In-vivo antimicrobial potential of green mediated Zinc-oxide and Titanium oxide nanoparticles and nanocomposites coated orthodontic bands

By R. Mahesh

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In-vivo antimicrobial potential of green mediated Zinc-oxide and Titanium oxide nanoparticles and nanocomposites coated orthodontic bands

Mary Sheloni Missier[1], R. Mahesh[2], Saravana Dinesh[2], S.Rajeshkumar[3]

[1]Department Of Orthodontics, SIMATS, Saveetha University, Chennai, Tamilnadu, India

[2]Saveetha dental college and hospital, SIMATS, Saveetha University, Chennai, Tamilnadu, India

[3]Department of Pharmacology, Saveetha Dental College and Hospital, SIMATS, Saveetha University, Chennai, Tamilnadu, India

drkarthiks1981@gmail.com

ABSTRACT

This study delves into the *in-vivo* antimicrobial efficacy of orthodontic bands coated with environmentally-friendly, green-synthesized Zinc-oxide (ZnO) and Titanium oxide (TiO2) nanoparticles, as well as nanocomposites. The findings illuminate the remarkable ability of these coatings to hinder the proliferation of oral pathogens, particularly *Streptococcus mutans* and *Streptococcus mitis*, pivotal in preventing dental plaque formation. Utilizing green synthesis methods ensures not only the ecological soundness but also the biocompatibility of the nanoparticles, crucial factors for their application in orthodontic devices. Among the noteworthy outcomes, ZnO-coated bands demonstrated superior antimicrobial properties, especially against *S. mutans*, while nanocomposite coatings exhibited notable effectiveness as well. Importantly, these coatings maintained their antimicrobial effects consistently over a span of 30 days, with no fungal growth observed beyond the initial day. Significantly, the study

revealed that the coated bands pose no discernible toxicity to vital organs, affirming their clinical viability in orthodontics. This investigation not only paves the way for further research, encompassing long-term antimicrobial assessments, clinical trials, and molecular inquiries but also underscores the broader implications of eco-friendly nanoparticle synthesis in various fields.

Keywords: Antimicrobial efficacy, Biocompatibility, Eco-friendly, Green synthesis, Orthodontic bands

Introduction

Nanotechnology holds a wide spectrum of applications within the field of orthodontics, encompassing the augmentation of material properties and the fortification of antimicrobial characteristics [1,2]. It has been the subject of extensive investigation for its potential to reinforce orthodontic materials, regulate frictional forces, and mitigate enamel demineralization during orthodontic interventions [3]. The incorporation of nanoparticles into dental materials has facilitated notable enhancements in their physical and mechanical attributes, with several of these advancements having already transitioned into routine clinical use [4]. Furthermore, nanotechnology is actively contributing to the development of innovative orthodontic devices, such as elastomeric ligatures, power chains, and miniscrews, all of which are engineered with the specific goal of reducing frictional forces [5].

Zinc oxide nanoparticles (ZnO NPs) hold significant promise in the field of orthodontics, demonstrating versatile applications spanning restorative dentistry, endodontics, implantology, periodontal treatment, prosthodontics, and orthodontics [6]. Within the domain of orthodontics, ZnO NPs serve as a valuable tool with multifaceted utility. Their primary role lies in augmenting the antibacterial properties of established orthodontic materials, effectively bolstering protection against oral pathogens [7]. Additionally, ZnO NPs find application as a coating for orthodontic implants, a strategy that enhances implant stability and mitigates the associated infection risk [8]. Moreover, ZnO NPs exhibit potential for integration into orthodontic adhesives and cements, thereby elevating their antimicrobial efficacy and acting as a preventive measure against secondary caries development [9].

Titanium dioxide nanoparticles (TiO2 NPs) offer unique and promising applications in the field of orthodontics. These nanoparticles can be strategically integrated into orthodontic adhesives,

cements, or acrylic resins, serving as nanofillers. Their inclusion in these materials imparts valuable antimicrobial properties while effectively preventing enamel demineralization [10]. Furthermore, TiO2 NPs can play a pivotal role in enhancing the antimicrobial capabilities of orthodontic appliances, including brackets. This is achieved through the application of a specialized nitrogen-doped TiO2 thin film on their surfaces, thereby augmenting their capacity to resist microbial colonization and proliferation [11].

Overall, the distinctive attributes of ZnO NPs and TiO2 NPs position them as a promising material in orthodontics, offering the prospect of improved treatment outcomes and enhanced patient care.

Lemon juice has emerged as a valuable natural resource for the synthesis of diverse nanoparticles, encompassing nickel-cobalt bimetallic oxide (NiCo2O4) [12], bismuth (Bi) [13], and zinc oxide (ZnO) [14]. Its utilization as an eco-friendly alternative to traditional chemical reagents and solvents is underpinned by several advantages, including low toxicity, simplicity, and environmental benignity.

The efficacy of lemon juice in nanoparticle synthesis arises from its chemical composition, housing compounds that serve as stabilizing, capping, and chelating agents. These multifaceted agents exert influence over the surface morphology and crystal structure of the resultant nanoparticles.Remarkably, nanoparticles synthesized using lemon juice have demonstrated noteworthy catalytic attributes, exemplified by their activity in catalyzing the reduction of 4-nitrophenol to 4-aminophenol [15] and the reduction of various organic dyes [16]. Furthermore, the integration of lemon juice-derived nanoparticles into starch-based films has yielded enhancements in material properties. These improvements manifest as augmented mechanical robustness and heightened antimicrobial capabilities.Collectively, these findings underscore the versatile and sustainable nature of lemon juice as a resource for nanoparticle synthesis and its potential applications across various domains. This natural alternative holds promise in the pursuit of environmentally conscious and effective nanomaterial production [17].

In the current investigation, lemon juice served as the primary agent for the synthesis of zinc oxide nanoparticles (ZnO NPs), titanium dioxide nanoparticles (TiO2 NPs), and a Zn-Ti nanocomposite. These synthesized nanomaterials were subsequently applied as coatings on orthodontic bands, forming the basis for a comprehensive study evaluating their in vivo antimicrobial effectiveness, biocompatibility, nephrotoxicity, and histopathological impact.

The study encompasses a multifaceted examination, encompassing microbiological, biocompatibility, and safety aspects, contributing valuable insights to the field of orthodontics and nanomaterial applications in dentistry.

Materials and Methods

Preparation of lemon juice extract:

Fresh lemons were brought from a local supermarket near Poonamallee. The fresh lemons were cut into two pieces and squeezed to get up to 50 mL extract. The collected extract was subjected to filtration process using a muslin cloth to remove the lemon seeds and pulp. The freshly collected lemon juice extract was used as a reducing and capping agent to synthesize both zinc oxide and titanium di oxide nanoparticles.

Green synthesis of Titanium oxide nanoparticles (TiO2 NPs) and Zinc oxide nanoparticles (ZnO NPs):

For the preparation of titanium oxide nanoparticles, 0.395 g of titanium oxide was measured and added to 50 mL distilled water to attain a precursor solution for synthesizing titanium di oxide nanoparticles (TiO2 NPs). To the precursor solution, 50 mL of a filtered reducing agent (Lemon juice extract) was added. Similarly, 20mM of zinc sulphate was taken as precursor to synthesize zinc oxide nanoparticles. The measured zinc sulphate powder was added to 25 mL distilled water. To that, 25 mL of filtered lemon juice extract was added. Uniform dispersion is very essential in synthesizing nanoparticles, therefore both reaction mixture was kept on an individual magnetic stirrer at 700 rpm for upto 48 h. To preliminarily confirm the synthesis of TiO2 and ZnO nanoparticles, UV-visible spectroscopy analysis was performed.

Green synthesis of zinc oxide and Titanium di oxide nanocomposites

In a green synthesis approach, nanocomposites composed of zinc oxide (ZnO) and titanium dioxide (TiO2) were prepared. The synthesis involved the mixing of 50 mL of lemon juice-mediated ZnO nanoparticle solution with an equal volume of TiO2 nanoparticle solution.

The mixing of the ZnO and TiO2 nanoparticle solutions was conducted on a magnetic stirrer at a constant speed of 600 rpm for an uninterrupted duration of 24 hours. This extended period of agitation was essential to ensure the complete and homogeneous blending of the two nanoparticle types, thereby facilitating the formation of nanocomposites. To preliminarily confirm the synthesis of TiO2 and ZnO nanoparticles, UV-visible spectroscopy analysis was performed.

Coating of orthodontic bands with green synthesized nanoparticles and nanocomposites

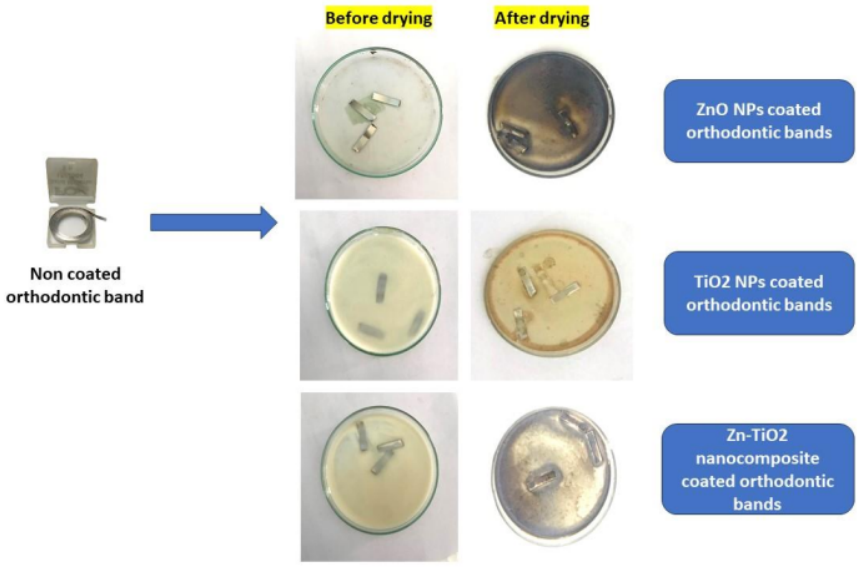

Figure 1: Lemon juice mediated Zinc oxide nanoparticles, Titanium di oxide nanoparticles and nanocomposite coated orthodontic bands

To extend the potential applications of these nanocomposites, orthodontic bands were introduced into the experimental setup. These bands were meticulously divided into nine segments, each measuring 2 cm in length. Subsequently, three separate conical flasks were prepared, each containing one of the following: the ZnO nanoparticle solution, the TiO2 nanoparticle solution, and the nanocomposite solution. Within each flask, three segments of orthodontic bands were carefully placed.

All three conical flasks, each containing orthodontic bands immersed in their respective nanoparticle solutions, were placed on a magnetic stirrer set at 410 rpm. This controlled agitation served the purpose of facilitating the impregnation of the orthodontic bands with the nanoparticle and nanocomposite solutions. After another 24-hour incubation period, the resulting nanoparticle solutions were carefully decanted into separate petri dishes, marking the conclusion of the synthesis phase. The next critical step involved the drying of the treated orthodontic bands. These bands, now potentially enriched with ZnO NPs, TiO2 NPs, and Zn-TiO2 NC, were individually placed in petri dishes and subjected to a hot air oven maintained at a temperature of 70°C for approximately 4 hours.

Characterization:

In this study, we examined three types of coated orthodontic bands: ZnO nanoparticles (ZnONPs) coated bands, TiO2 nanoparticles (TiO2NPs) coated bands, and Zn-TiO2 nanocomposite coated bands. SEM and EDAX was used to analyze their surface characteristics and elemental composition. These analyses provided insights into the structural and elemental properties of each coating, aiding in the evaluation of their suitability for orthodontic applications.

Cytotoxic effect – Brine shrimp lethality assay

The cytotoxic effect of green synthesized nanoparticles and nanocomposite coated orthodontic bands was performed by Brine Shrimp lethality assay. For the preparation of saline water 2 grams of iodine free salt was measured and dissolved in 200 mL of distilled water. 10 to 12 mL of saline water was added to six well ELISA plates. To that 10 nauplii were slowly added to each well. The coated orthodontic bands were added to the plates and incubated at room temperature for 24 hours. After 24 hours, the ELISA well plates were observed and counted for number of live nauplii present and calculated by using the following formula,

Number of dead nauplii/ number of Dead nauplii + number of live nauplii ×100

Isolation of pathogens from swabs of orthodontic patient bands

A swab culture procedure was conducted on a 37-size orthodontic band obtained from a patient undergoing orthodontic treatment. The orthodontic band was aseptically collected and placed in a sterile jar, followed by the addition of 20 mL of distilled water, which was gently stirred to create a suspension.Two conical flasks were prepared, with each containing 40 mL of distilled water. In one flask, 1.12 mg of nutrient agar was meticulously weighed and dissolved in the distilled water. In the other flask, 3.932 mg of Mutans-Sanguis agar was similarly weighed and dissolved in the distilled water. Additionally, 0.40 mg of Agar-Agar Type I was accurately measured and added to both flasks.To ensure sterility, the conical flasks were subjected to moist heat sterilization at 121°C for 15 minutes under a pressure of 15 lbs. Subsequently, the agar-containing solutions were poured onto separate petri dishes and allowed to solidify at a controlled temperature of 45°C.Following the solidification, a swab culture of the patient's orthodontic band was meticulously performed, and the petri dishes were placed in a dark room at room temperature for an incubation period of 24 hours.After the 24-hour

incubation period, the petri dishes were examined to assess the growth and enumeration of microbial colonies. This procedure provided valuable insights into the microbial load and composition associated with the patient's orthodontic band, facilitating a better understanding of potential oral health implications and the need for appropriate interventions.

In the laboratory setting, for the isolation and cultivation of potential pathogens from orthodontic patient bands sterile nutrient agar and Sanguis mutans agar were prepared and subjected to sterilization procedures to ensure an aseptic environment.

Patient swabs, collected from the orthodontic bands, were gently mixed with 10 mL of sterile distilled water, creating a suspension of potential microbial content. Subsequently, this suspension was evenly swabbed onto the sterile surfaces of both the nutrient agar and Sanguis mutans agar plates.The agar plates, now inoculated with the patient swabs, were placed into a controlled incubation environment set at 37 degrees Celsius. Over the course of 24 hours, the plates were allowed to incubate, facilitating the growth and proliferation of any potential pathogens present in the patient's oral microbiota.

Isolation of S.mutans and S.mitis from the swab culture using Mutans-Sanguis agar:

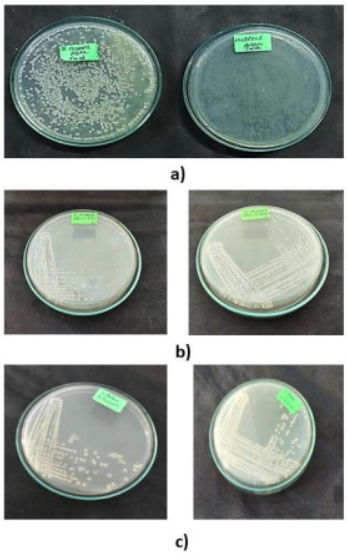

Figure 2: Isolation of pathogens from patient orthodontic bands a) Pathogens isolated from orthodontic band swabs b) Isolation of pathogens using Mutans-Sanguis Agar using Streak technique c) Subculture of *S.mutans* and *S.mitis* to observe morphological features

In preparation of the culture medium, 3.92 grams of Mutan Sanguis Agar were dissolved in 40 milliliters of distilled water, and an additional 0.40 milligrams of Agar Agar Type I were meticulously added to the mixture. Subsequently, the culture medium was subjected to sterilization using moist heat at 121°C for 15 minutes, maintaining a pressure of 15 lbs. After sterilization, the selective media was poured on the surface of sterile Petriplates and allowed for solidification process.

For subculturing purposes, two distinct colonies were identified in the previous culture, with one being S. mutans and the other S. mitis. Subculturing was undertaken to maintain the purity of these cultures. The selected colonies were aseptically transferred using an inoculating loop.The subcultured colonies were streaked onto the surface of Mutan Sanguis Agar within the Petri dishes. This streaking method was employed to isolate individual colonies and facilitate their subsequent growth.Subsequently, the Petri dishes, now containing the subcultured colonies, were placed within a dark room at room temperature. The incubation period spanned 24 hours, allowing ample time for the colonies to proliferate and develop.Upon completion of the 24-hour incubation, the colonies that had grown were subjected to identification procedures, confirming their identities as S. mutans and S. mitis. The primary goal of these preparations was to observe the morphological features of the bacteria. morphology in microbiology refers to the physical characteristics and shapes of microorganisms. By studying these features, researchers can gain insights into the nature of the microbes, which can be valuable for various purposes, including diagnosis, treatment, or understanding their role in oral health.

In vivo antimicrobial activity of coated orthodontic bands:

A total of 24 male Wistar rats, each weighing approximately 200 grams, were selected for this study. These rats were divided into four groups, with six rats in each group.Coated orthodontic bands were fabricated to match the shape of the incisor teeth of Wistar rats. These bands were then securely cemented onto the lower incisor teeth using dental luting cement. This orthodontic banding procedure was performed on all 24 rats.

Group	Orthodontic Band Type
Group I	Control (Non-coated orthodontic bands)

Group II	ZnO Coated Orthodontic Bands
Group III	TiO2 Coated Orthodontic Bands
Group IV	Nanocomposite Coated Orthodontic Bands

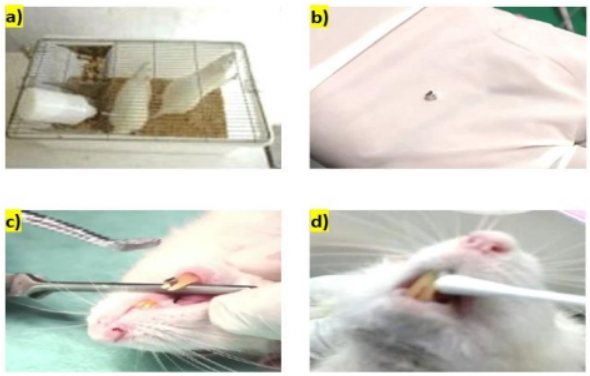

Figure 3: In vivo antimicrobial activity of coated orthodontic bands a) male wistar rats b) Coated orthodontic bands were fabricated to match the shape of the incisor teeth c) Insertion of bands onto the lower incisor teeth d) Swab collection from 0-30th day

Swabs were collected from both the labial and lingual surfaces of the orthodontic banded teeth at defined time points, including 0, 3, 6, 9, 15, and 30 days following the band application. These collected swabs were subsequently plated onto Mutans-Sanguis agar plates using the spread plate technique.

For the identification of fungal growth, Rose Bengal agar plates were employed as the culture medium. The swabbed plates were then incubated at a controlled temperature of 37 degrees Celsius for a duration of 24 hours.This approach enabled the comprehensive analysis of microbial colonization on the orthodontic bands, facilitating the detection and differentiation of both bacterial and fungal growth patterns over the specified time intervals.

On the 30th day of the experiment, blood samples were collected from the rats for further analysis. At the end of the 30-day experimental period, the rats were humanely euthanized

following established ethical guidelines. Following euthanasia, the liver, kidney, and spleen were carefully collected for histopathological examination.

Nephrotoxicity Study

At the end of the experimental period of 30 days, blood samples were collected by puncturing retro-orbital venous plexus and the serum biochemistry were analyzed for toxicity. The rats were anaesthetized by ether (Anaesthetic Grade) and the blood samples were collected in glass test tubes. The exuded serum was decanted and centrifuged at 2500 rpm for 20 minutes. The clear supernatant serum which was obtained was subjected to liver and renal function tests, such as Bilirubin, Albumin, Total protein, Aspartate amino S transferase (AST), Alanine transaminase (ALT) Alkaline phosphatase (ALP), Urea and Creatinine.

HISTOPATHOLOGICAL STUDY

Euthanasia and Tissue Harvesting

Animals were euthanized at the end of the intended experimental period (end of 30th day) by administering over dose of anesthesia (Sodium Pentothal - i.p). After the respiration ceases out the animals were transcardially perfused using normal saline and then the tissues were fixed with formal saline. Tissues such as liver spleen and kidney were dissected out and post-fixed in freshly prepared 10% formalin and processed for histopathological investigation. The tissue sections were taken at 5μm thickness and stained with routine Haematoxylin and Eosin staining and permanently mounted in DPX, then analyzed for histopathology.

Light Microscopy

Histopathological examinations of Liver, Spleen and Kidney were done using H&E stain for hepatotoxicity and nephrotoxicity.

Haematoxylin and Eosin Stain

For light microscopic study and for analyzing the histopathology of liver, spleen and kidney, the fixed tissues were processed for routine paraffin sectioning and stained with Haematoxylin and Eosin (Bancroft and Gamble, 2008). For paraffin sectioning the tissues were hydrated, then dehydrated in graded alcohol series, cleared in chloroform and xylene and then embedded in paraffin wax. For H&E staining the paraffin embedded tissues were sectioned at 5µm thickness using Rotary microtome (Leica Microsystems, Germany) they were incubated overnight at room temperature, then the sections were deparaffinized& rehydrated through descending alcohol series (100% alcohol, 90% alcohol, 70% alcohol and 50% alcohol)

followed by distilled water. Now these sections were stained with Haematoxylin and Eosin and then rapidly carried through ascending alcohol series (50% alcohol, 70% alcohol, 90% alcohol and three changes of 100% alcohol) & then the sections were cleared in three changes of xylene and they were mounted with DPX.

This comprehensive experimental setup allowed us to investigate the effects of the coated orthodontic bands on the oral health of the Wistar rats over a specified time frame. It involved the collection of swabs, blood samples, and histopathological examination to assess potential systemic and local impacts. This study contributes to our understanding of the biocompatibility and safety profile of orthodontic bands in a preclinical animal model.

Statistical analysis:

In this study, all experimental procedures were performed in triplicates to ensure the reliability and reproducibility of the results. To assess a comprehensive range of in vivo antimicrobial, biochemical, oxidative stress, and antioxidant parameters, a statistical analysis was conducted using a two-way analysis of variance (ANOVA). Subsequently, a Dunnett post hoc test was employed for pairwise comparisons. This rigorous statistical approach was chosen to rigorously evaluate the experimental data and to draw meaningful conclusions from the observed outcomes.

Result

Scanning Electron Microscopy (SEM)

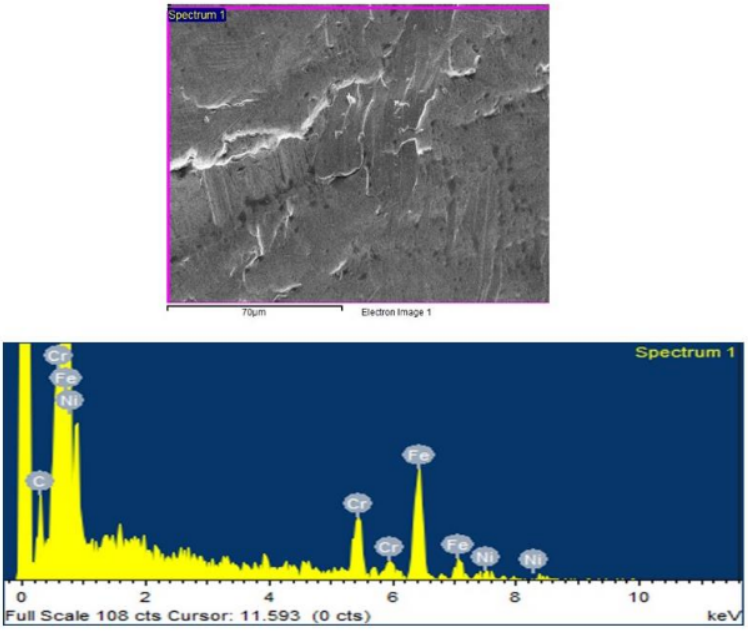

Figure 4: SEM image & EDX spectra of non coated control orthodontic bands

The scanning electron microscopy (SEM) analysis of the non-coated control orthodontic bands revealed distinctive surface characteristics. The SEM images exhibited both rough and smooth regions on the orthodontic bands, highlighting the heterogeneity of the surface topography.

The energy-dispersive X-ray spectroscopy (EDX) spectra provided valuable insights into the elemental composition of the control orthodontic bands. The EDX analysis detected the presence of several key elements, with their respective weight percentages and atomic percentages as follows:

- Carbon (C): Found at 2.93% by weight and 12.19% by atomic composition.

- Chromium (Cr): Present at 22.25% by weight and 21.36% by atomic composition.

- Iron (Fe): Predominantly identified at 64.92% by weight and 58.03% by atomic composition.

- Nickel (Ni): Detected at 9.89% by weight and 8.41% by atomic composition.

These findings highlight the multi-elemental composition of the non-coated control orthodontic bands, with iron (Fe) being the most abundant element. The presence of these elements contributes to the overall material properties and may have implications for the bands'

mechanical and biocompatible characteristics. Further investigations and analyses may be warranted to fully understand the significance of these elements in relation to the performance of orthodontic bands.

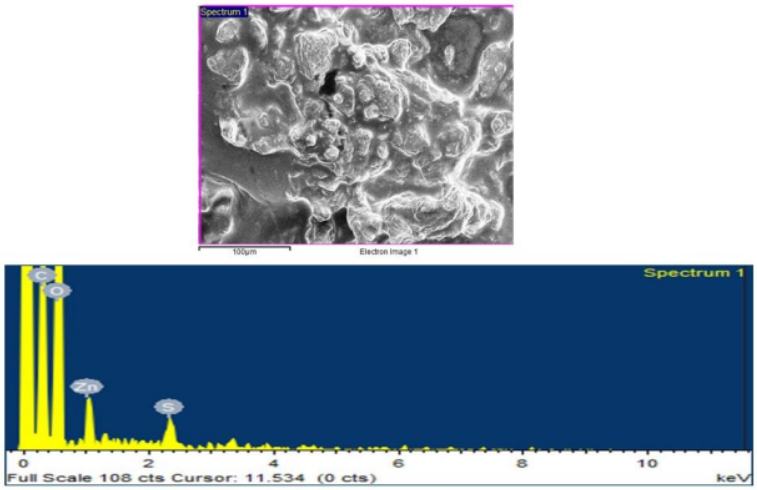

Figure 5: SEM image & EDX spectra of ZnONPs coated orthodontic bands

The examination of ZnO nanoparticles (ZnONPs) coated orthodontic bands through scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) yielded significant insights into the surface characteristics and elemental composition.

The SEM images revealed distinctive features on the surface of the orthodontic bands. The ZnO nanoparticles, used for coating, were observed to be present as spherical-shaped agglomerates. These agglomerates were distributed across the orthodontic band's surface, indicating successful coating with ZnO nanoparticles. The spherical morphology of the nanoparticles suggested effective adhesion and distribution on the band's surface.

The EDX spectra provided valuable information regarding the elemental composition of the ZnONPs coated orthodontic bands. The major elements detected, along with their respective weight percentages and atomic percentages, are as follows:

- Carbon (C): Identified at 35.26% by weight and 44.24% by atomic composition.

- Oxygen (O): Predominantly found at 56.67% by weight and 53.37% by atomic composition.

- Sulfur (S): Detected at 2.19% by weight and 1.03% by atomic composition.

- Zinc (Zn): Present at 5.88% by weight and 1.35% by atomic composition.

These results confirm the successful coating of ZnO nanoparticles on the orthodontic bands, as evidenced by the presence of zinc (Zn) in the EDX spectra. The agglomerated, spherical shape of the nanoparticles contributes to their adhesion and distribution, potentially offering unique properties and applications to the coated orthodontic bands.

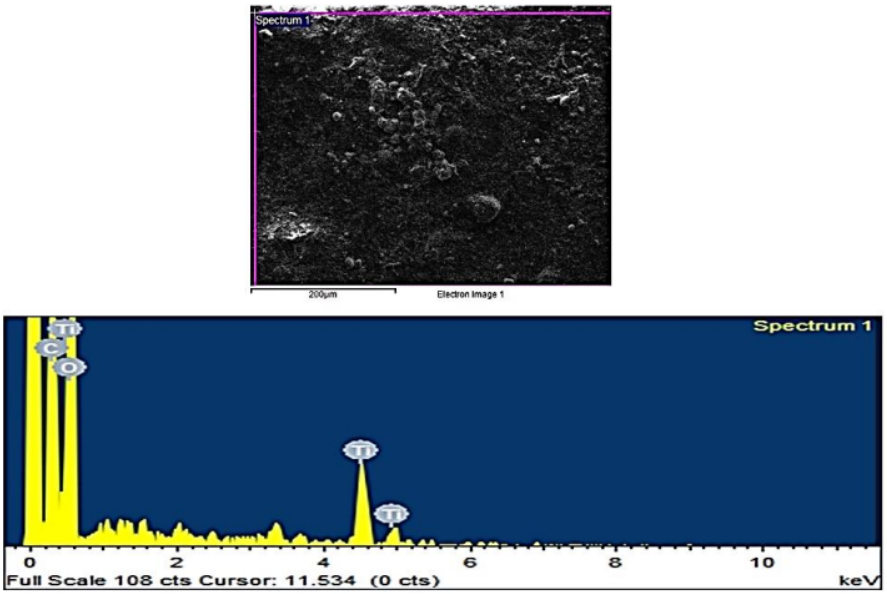

Figure 6: SEM image & EDX spectra of TiO2 NPs coated orthodontic bands

The investigation of orthodontic bands coated with TiO2 nanoparticles (TiO2 NPs) through scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) provided valuable insights into the surface characteristics and elemental composition of the coated bands.

The SEM images of the TiO2 NPs coated orthodontic bands revealed intriguing surface features. Small spherical-shaped nanoparticles were observed to be evenly distributed across the orthodontic band's surface. These nanoparticles displayed a smooth appearance, indicating effective coating with TiO2 NPs. The spherical morphology of the nanoparticles suggests uniform dispersion and adherence to the band's surface.

The EDX spectra yielded crucial information about the elemental composition of the TiO2 NPs coated orthodontic bands. The primary elements detected, along with their respective weight percentages and atomic percentages, are as follows:

- Carbon (C): Identified at 32.62% by weight and 43.75% by atomic composition.

- Oxygen (O): Predominantly found at 50.09% by weight and 50.44% by atomic composition.

- Titanium (Ti): Detected at 17.29% by weight and 5.81% by atomic composition.

These results confirm the successful coating of TiO2 nanoparticles on the orthodontic bands, as evidenced by the presence of titanium (Ti) in the EDX spectra. The smooth and spherical shape of the nanoparticles indicates uniform dispersion and adhesion on the band's surface, which may have implications for the bands' properties and applications.

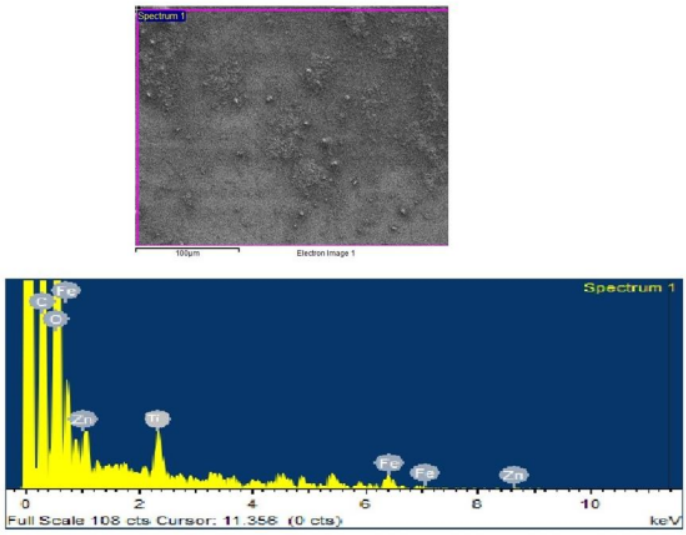

Figure 7: SEM image and EDX spectra of Zn-Ti nanocomposite coated orthodontic bands

The SEM image of the Zn-Ti nanocomposite-coated orthodontic bands revealed a smooth surface adorned with small spherical-shaped structures. These structures are indicative of the presence of the nanocomposite coating.The EDX (Energy-Dispersive X-ray) spectra further confirmed the composition of the coating. The results showed the following elemental composition:

- Carbon (C): 31.29% (Weight%), 46.84% (Atomic%)

- Oxygen (O): 38.33% (Weight%), 43.07% (Atomic%)

- Titanium (Ti): 1.88% (Weight%), 1.06% (Atomic%)

- Iron (Fe): 25.48% (Weight%), 8.20% (Atomic%)

- Zinc (Zn): 3.01% (Weight%), 0.83% (Atomic%)

These findings establish the presence of carbon, oxygen, titanium, iron, and zinc within the nanocomposite coating. This comprehensive analysis provides valuable insights into the elemental composition of the Zn-Ti nanocomposite, which is integral to understanding its potential properties and applications in orthodontic treatments.

Cytotoxic effect-Brine shrimp lethality assay

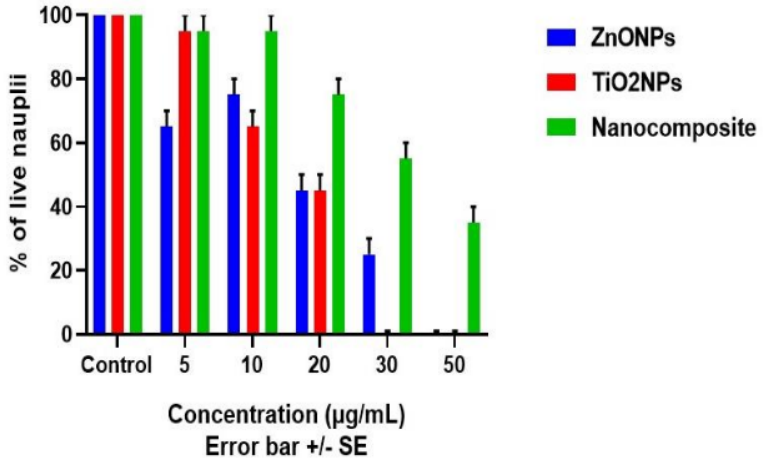

Figure 8: Brine shrimp lethality assay of green synthesized nanoparticles and nanocomposite

In the brine shrimp lethality assay, we evaluated the biocompatibility and potential cytotoxicity of different concentrations of nanoparticles, specifically ZnONPs, TiO2NPs, and a Nanocomposite, over 48h. The percentage of live nauplii was assessed as an indicator of the samples impact on the survival of the brine shrimp.

At the control concentration (100 μg/mL), all three tested materials, ZnONPs, TiO2NPs, and Nanocomposite, exhibited no significant lethality to the brine shrimp, with a survival rate of 100%. As the concentration of ZnONPs increased to 5 μg/mL, the survival rate of the brine shrimp decreased to 60%. TiO2NPs at 5 μg/mL also showed a slight reduction in survival to 90%, while Nanocomposite at this concentration maintained 100% survival.At 10 μg/mL concentration, ZnONPs and TiO2NPs caused reduced survival rates of 70% and 60%, respectively, whereas the Nanocomposite still showed no lethality (100% survival).

Further increasing the concentration to 20 μg/mL, ZnONPs and TiO2NPs continued to exhibit cytotoxic effects with survival rates of 40% and 40%, respectively. The Nanocomposite showed a less pronounced effect with a survival rate of 70%.At higher concentrations of 30 μg/mL and 50 μg/mL, ZnONPs caused a significant decrease in survival, reducing it to 20% and 0%, respectively. TiO2NPs and Nanocomposite at these concentrations were even more cytotoxic, with survival rates dropping to 0% for both materials.

These results suggest that ZnONPs, TiO2NPs, and the Nanocomposite exhibit concentration-dependent cytotoxicity in the brine shrimp lethality assay. Higher concentrations and longer exposure periods led to increased lethality, particularly for TiO2NPs and Nanocomposite, while ZnONPs displayed intermediate toxicity. These findings highlight the importance of considering nanoparticle concentration and exposure duration when assessing their potential impact on aquatic organisms like brine shrimp.

In vivo antimicrobial activity:

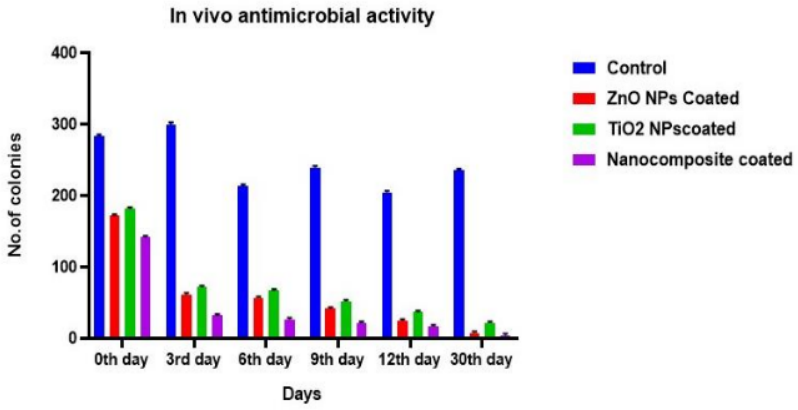

Figure 9: Evaluation of pathogens from swabs collected after treatment with coated orthodontic bands

The interaction term had an F-statistic of 12930 and a highly significant p-value (<0.0001).Treated days showed an F-statistic of 537.3 and a highly significant p-value (<0.0001).Groups had an extremely high F-statistic of 1303900 and a highly significant p-value (<0.0001).

Dunnett's Multiple Comparisons Test: This test compared the control group with the treated groups (ZnO NPs Coated, TiO2 NPs Coated, and Nanocomposite Coated). It provided mean differences and confidence intervals:

Control vs. ZnO NPs Coated: The mean difference was 185.5, with a 95% confidence interval from 185.2 to 185.8. This difference was highly significant (****) with an adjusted p-value <0.0001.

Control vs. TiO2 NPs Coated: The mean difference was 174.5, with a 95% confidence interval from 174.2 to 174.8. This difference was highly significant (****) with an adjusted p-value <0.0001.

Control vs. Nanocomposite Coated: The mean difference was 205.7, with a 95% confidence interval from 205.4 to 206.0. This difference was highly significant (****) with an adjusted p-value <0.0001.

The statistical analysis revealed that the choice of treatment group and treatment duration significantly influenced the antimicrobial outcomes. All three treatment groups (ZnO NPs Coated, TiO2 NPs Coated, and Nanocomposite Coated) showed significantly reduced microbial growth compared to the control group. This indicates the potential antimicrobial efficacy of these coated orthodontic bands over a 30-day period.

Nephrotoxicity study:

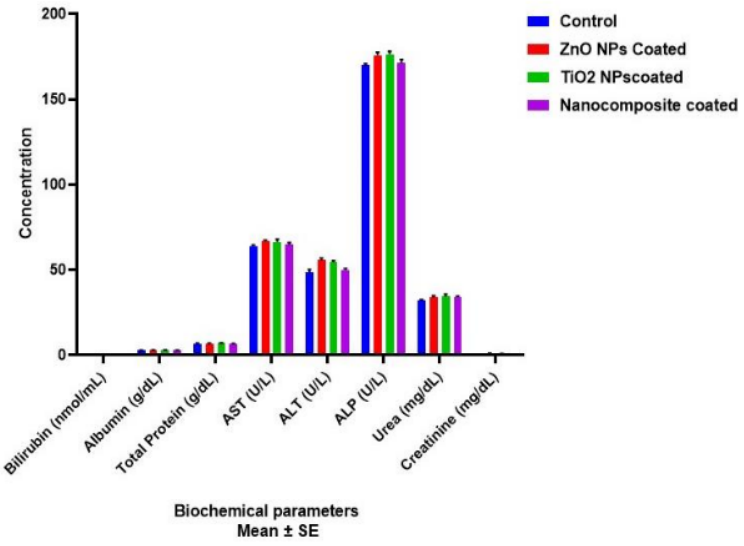

Figure 10: Biochemical parameters of the coated orthodontic bands

Two-way repeated measures ANOVA and Dunnett's multiple comparisons test was conducted to assess the significance of differences in various biochemical parameters between different groups. The main effect of the parameters was highly significant ($F(1.657, 4.972) = 21628$, $p < 0.0001$), indicating that the biochemical parameters varied significantly across the groups.The main effect of Control, ZnO NPs, TiO2 NPs, nanocomposite coated groups was significant ($F(1.882, 5.647) = 13.78$, $p = 0.0070$), suggesting that there were differences between the control and treated groups in terms of the biochemical parameters.The interaction between parameters and control-treated groups ($F(21, 63) = 2.589$) was not significant, indicating that the impact of parameters did not differ significantly between control and treated groups.

Post hoc analysis using Dunnett's multiple comparisons test was conducted to compare each treatment group with the control group for individual biochemical parameters. For all biochemical parameters (Bilirubin, Albumin, Total Protein, AST, ALT, Urea, and Creatinine), there were no significant differences between the control group and the treated groups. The mean differences and confidence intervals for all comparisons included zero, indicating that the treatments did not significantly impact these parameters.However, for ALP (Alkaline Phosphatase), a significant difference was observed between the control group and ZnO NPs Coated group ($p < 0.05$).Overall, the results suggest that the treatments did not have a significant impact on most of the biochemical parameters, except for ALP.

Evaluating oxidative stress and antioxidant activity within biological samples

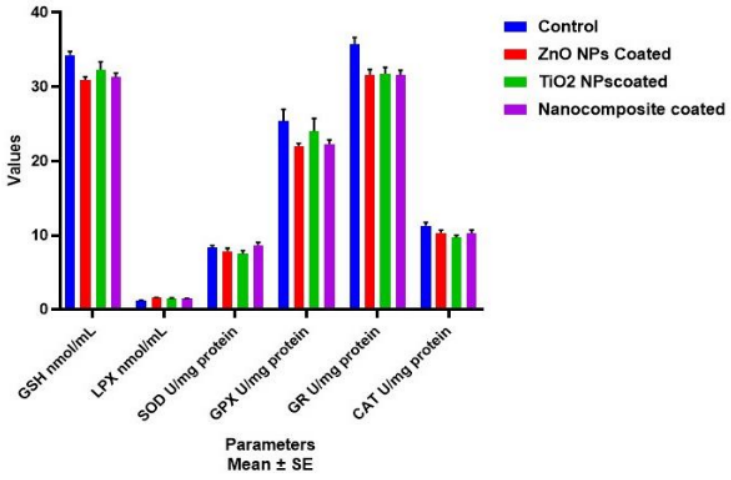

Figure 11: Evaluation of oxidative stress and antioxidant parameters for coated and non-coated orthodontic bands

The results of the antioxidant parameters compared to the control group indicate significant differences in certain parameters, suggesting variations in antioxidant activity between the control group and the groups treated with different coatings.

GSH (Glutathione):

Control vs. ZnO NPs Coated: There is a significant increase in GSH levels in the ZnO-coated group compared to the control group. This suggests that the ZnO-coated material may have a positive impact on the antioxidant defense system by increasing GSH levels.

Control vs. TiO2 NPs Coated: There is no significant difference in GSH levels between the TiO2-coated group and the control group. This indicates that TiO2 coating does not significantly affect GSH levels.

Control vs. Nanocomposite Coated: Similar to ZnO, there is a significant increase in GSH levels in the nanocomposite-coated group compared to the control group. This suggests that the nanocomposite coating also enhances the antioxidant defense system by increasing GSH levels.

LPX (Lipid Peroxides), SOD (Superoxide Dismutase), GPX (Glutathione Peroxidase), and CAT (Catalase):

For these parameters, there are no significant differences between the control group and the coated groups. This indicates that the coatings with ZnO, TiO2, or nanocomposites do not have a substantial impact on these specific antioxidant parameters in comparison to the control group.

GR (Glutathione Reductase):

Control vs. ZnO NPs Coated: There is a significant increase in GR levels in the ZnO-coated group compared to the control group. This suggests that the ZnO-coated material may enhance the activity of GR, which plays a role in the regeneration of reduced glutathione (GSH).

Control vs. TiO2 NPs Coated: Similar to ZnO, there is a significant increase in GR levels in the TiO2-coated group compared to the control group. This indicates that TiO2 coating also enhances GR activity.

Control vs. Nanocomposite Coated: Similar to ZnO and TiO2, there is a significant increase in GR levels in the nanocomposite-coated group compared to the control group. This suggests that the nanocomposite coating enhances GR activity as well.

In summary, the results show that ZnO, TiO2, and nanocomposite coatings have varying effects on antioxidant parameters. They significantly increase GSH and GR levels, indicating potential enhancement of the antioxidant defense system. However, other parameters like LPX, SOD, GPX, and CAT do not show significant differences, suggesting that these coatings may not have a substantial impact on these specific aspects of antioxidant activity compared to the control group.

Histopathological studies:

Histopathology Evaluation of Liver

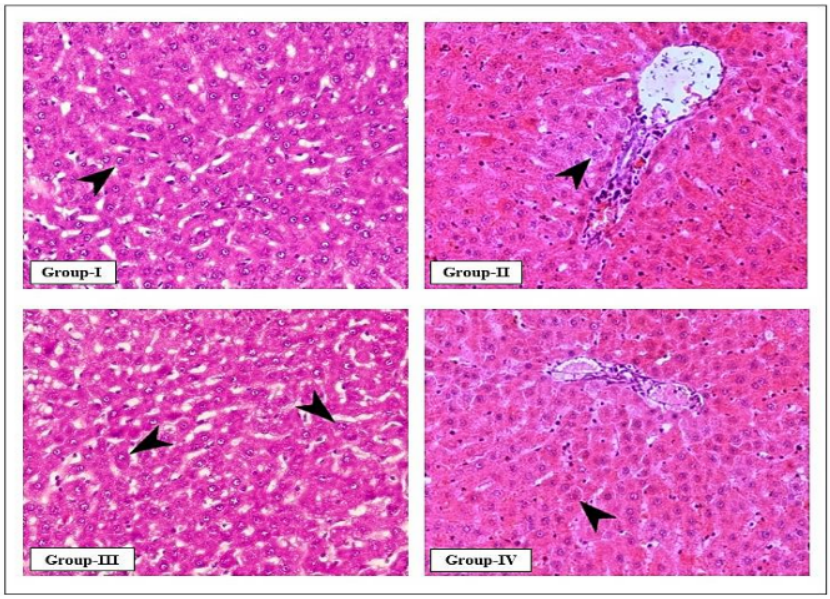

Figure 12: Photomicrographs showing the histopathology of liver stained with Haematoxylin & Eosin at 40X Magnification.

The histopathological assessment of liver tissues from all experimental groups, including Group-I (Control – Non-coated orthodontic bands), Group-II (ZnO coated orthodontic bands), Group-III (TiO_2 coated orthodontic bands), and Group-IV (Nanocomposite coated orthodontic bands), was conducted using Haematoxylin & Eosin staining at 40X magnification.

In all experimental groups, the liver parenchyma exhibited an intact appearance, characterized by hepatocytes with vesicular nuclei. Furthermore, a normal hepatic architecture was observed in all groups, featuring a central vein and radiating cords of hepatocytes, along with an intact portal triad and normal sinusoids. Notably, the photomicrographs (Figure 1) illustrate these observations, with arrows indicating normal hepatocytes.Crucially, no significant gross cellular damage or alterations in hepatic architecture were detected in any of the experimental groups. This absence of evident tissue-level toxic manifestations suggests that the application of coated orthodontic bands, including ZnO, TiO_2, and Nanocomposite coatings, did not induce adverse effects on liver histology during the course of this study.These findings indicate that the evaluated orthodontic band coatings did not produce discernible hepatic histopathological abnormalities, supporting their potential safety for oral applications.

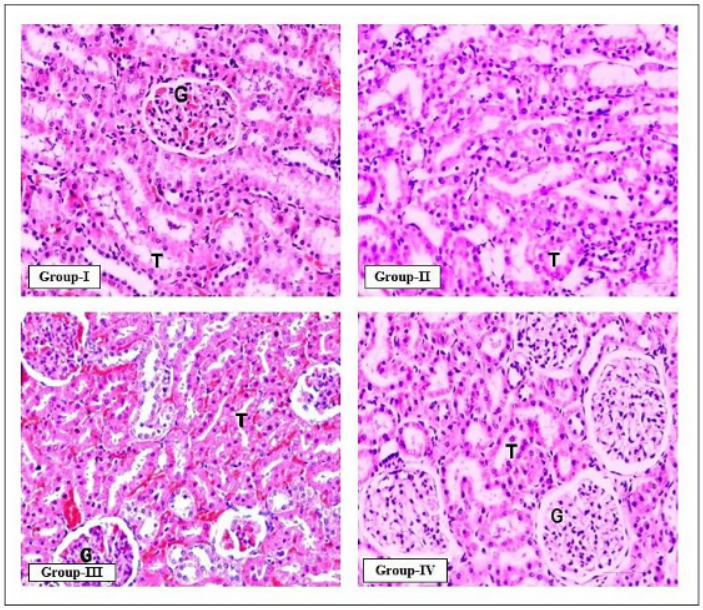

Figure 13: Photomicrographs showing the histopathology of kidney stained with Haematoxylin & Eosin at 40X Magnification.

Histopathological examination of kidney tissues from all experimental groups, including Group-I (Control – Non-coated orthodontic bands), Group-II (ZnO coated orthodontic bands), Group-III (TiO2 coated orthodontic bands), and Group-IV (Nanocomposite coated orthodontic bands), was carried out using Haematoxylin & Eosin staining at 40X magnification.In all experimental groups, the kidney histology revealed an intact and normal glomerular structure (G), as well as proximal and distal convoluted tubules (T) lined by simple cuboidal epithelium. Importantly, these findings indicated the absence of gross nephrotoxic manifestations in all groups.

Furthermore, there were no observed instances of gross cellular damage or alterations in renal architecture in any of the experimental groups. These histopathological results collectively demonstrate the absence of tissue-level toxic manifestations in the kidney.Taken together, these findings suggest that the application of various orthodontic band coatings, including ZnO, TiO2, and Nanocomposite coatings, did not induce evident renal histopathological abnormalities. This supports the potential safety of these coated orthodontic bands for oral applications and indicates no significant nephrotoxic effects during the study period.

Histopathological Evaluation of Spleen:

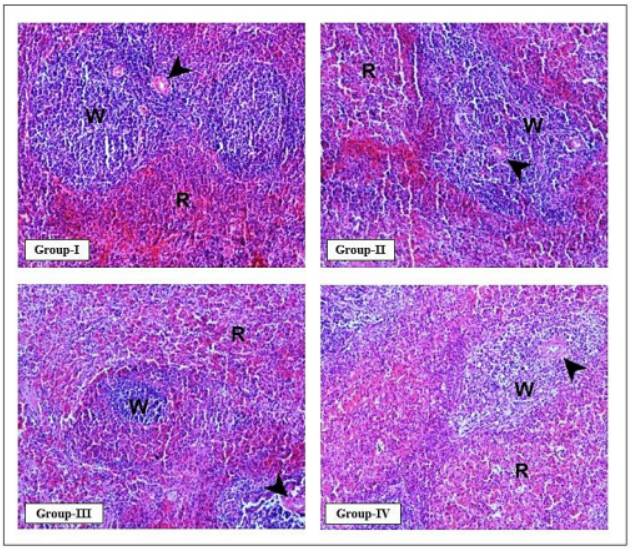

Figure 14: Photomicrographs showing the histopathology of spleen stained with Haematoxylin & Eosin at 40X Magnification.

Histopathological examination of spleen tissues from all experimental groups, including Group-I (Control – Non-coated orthodontic bands), Group-II (ZnO coated orthodontic bands), Group-III (TiO2 coated orthodontic bands), and Group-IV (Nanocomposite coated orthodontic bands), was conducted using Haematoxylin & Eosin staining at 40X magnification.

In Group-I (Control – Non-coated orthodontic bands), the spleen displayed characteristic features, including the central arteriole (arrow-heads), white pulp (W), and red pulp (R). Interestingly, Group-II (ZnO coated orthodontic bands) exhibited mild damage, as indicated by leucocyte infiltration in the arteriole (arrow), suggesting a minimal level of histological alteration.

Remarkably, all other experimental groups, including Group-III (TiO2 coated orthodontic bands) and Group-IV (Nanocomposite coated orthodontic bands), displayed spleen histopathology that closely resembled the control group (Group-I). These observations collectively indicated no obvious histological alterations or signs of tissue-level toxicity in the spleen among any of the experimental groups.These findings strongly support the biocompatibility of the materials used in the coated orthodontic bands and suggest that the majority of the bands, including TiO2 and Nanocomposite coatings, did not induce significant histopathological changes in the spleen during the course of the study.

Discussion

The study investigated the antimicrobial potential of orthodontic bands coated with green-synthesized Zinc-oxide (ZnO) and Titanium oxide (TiO_2) nanoparticles, as well as nanocomposites (Zn-TiO_2 NC). These coatings are of significant interest in orthodontics due to their potential to inhibit the growth of oral pathogens, such as Streptococcus mutans and Streptococcus mitis. Controlling microbial colonization on orthodontic appliances is crucial for preventing dental plaque formation, which can lead to various oral health issues during orthodontic treatment.

Orthodontic bands are frequently associated with challenges related to the adherence of Streptococcus mutans (S. mutans), a bacterium linked to dental caries. Numerous research studies have explored S. mutans adherence to various orthodontic band materials and cements. For instance, one study discovered that Transbond Plus Band Cement and Ketac Cem exhibited reduced S. mutans adherence compared to other band cement materials [18]. Another investigation found that S. mutans had a lower initial affinity for metal brackets when compared to plastic and porcelain brackets, whether or not saliva coating was present [19]. Furthermore, research revealed that the prevalence of S. mutans in plaque was higher among patients wearing orthodontic bands compared to those without bands. Among caries-free patients, those with orthodontic bands had more S. mutans-infected sample sites [20].

Moreover, the microbial composition of plaque was found to vary depending on the bracket materials and dental cements used, with a higher proportion of S. mutans observed in plaque from plastic brackets and bands that were cemented with zinc phosphate cement [21]. Finally, the effectiveness of orthodontic cements containing antibacterial nanoparticles was assessed, and certain cements demonstrated sustained antibacterial properties against S. mutans [22]. These findings collectively highlight the significance of material selection in orthodontic treatment and its potential impact on oral health.

An important aspect of this study is the green synthesis of nanoparticles. The utilization of environmentally friendly methods for nanoparticle synthesis is commendable, as it reduces the environmental impact associated with traditional chemical methods. The integration of green-synthesized nanoparticle coatings into orthodontic applications has emerged as an innovative and environmentally conscious approach. These coatings offer a sustainable means to enhance the properties of orthodontic bands while aligning with the principles of green chemistry, which prioritize safer and less toxic routes for nanoparticle synthesis [23]. Green synthesis methods

entail the judicious use of non-toxic capping and reducing agents, the selection of harmless solvents, and the development of energy-efficient synthetic techniques [24]. Through the incorporation of green-synthesized nanoparticles into orthodontic band coatings, the mechanical and antimicrobial characteristics can be improved [25]. This enhancement can lead to superior functionality, biocompatibility, and tissue response of orthodontic bands, ensuring compliance with regulatory standards for oral cavity use [26]. The adoption of green-synthesized nanoparticle coatings in orthodontics holds significant promise for the advancement of orthodontic materials, offering enhanced performance while minimizing environmental impact.

The SEM images of non-coated control orthodontic bands reveal a surface with a combination of rough and smooth areas. This variation in surface texture is likely due to the manufacturing process and materials used. The EDX spectra of the control bands confirm the presence of elements commonly found in stainless steel orthodontic appliances, including Carbon (C), Chromium (Cr), Iron (Fe), and Nickel (Ni). These elements are consistent with the composition of stainless steel and are not unexpected in orthodontic devices.

In contrast, the SEM images of ZnO NPs coated orthodontic bands show spherical-shaped nanoparticles agglomerated on the band's surface. This morphology is consistent with previous reports of ZnO NPs. The EDX spectra of the ZnO NPs coated bands reveal the presence of Carbon (C), Oxygen (O), Sulfur (S), and Zinc (Zn). The presence of Zinc (Zn) is particularly noteworthy, as it indicates the successful deposition of ZnO NPs on the band's surface. ZnO NPs are known for their antimicrobial properties, making them a potential candidate for improving the biocompatibility of orthodontic bands.

Similarly, the SEM images of TiO2 NPs coated orthodontic bands display small, spherical-shaped nanoparticles distributed evenly on the band's surface, giving it a smoother appearance. The EDX spectra of the TiO2 NPs coated bands confirm the presence of Carbon (C), Oxygen (O), and Titanium (Ti). The presence of Titanium (Ti) indicates the successful deposition of TiO2 NPs on the band. TiO2 NPs are known for their photocatalytic properties, which can induce antimicrobial effects under specific conditions. The SEM image of the Zn-Ti nanocomposite-coated orthodontic bands reveals a smooth surface with small spherical structures, indicating the presence of the nanocomposite coating. The EDX spectra confirm the composition, with elements like carbon, oxygen, titanium, iron, and zinc detected.

This coating's composition suggests its potential in orthodontics and other applications, given its unique properties.

These SEM and EDX analyses provide strong evidence that the intended nanoparticles (ZnO and TiO2) have been successfully deposited onto the orthodontic bands. These findings suggest that these coated bands have the potential for antimicrobial applications due to the presence of Zn and TiO2, which are known for their antimicrobial properties.

The cytotoxic effect was initially tested by using Brine shrimp lethality assay which revealed nanocomposite to be less toxic among zinc oxide and titanium di oxide nanoparticles. Furthermore, the biocompatibility of these green-synthesized nanoparticles is crucial when considering their application in orthodontic devices. The study's results indicating no signs of tissue-level toxicity in vital organs like the liver, kidney, and spleen are reassuring.

The findings demonstrated differential antimicrobial efficacy among the coated orthodontic bands. Notably, ZnO-coated bands showed higher antimicrobial activity against S. mutans compared to TiO2-coated bands. This discrepancy may be attributed to variations in the size, surface charge, or release kinetics of nanoparticles between ZnO and TiO2. The nanocomposite-coated bands also exhibited promising antimicrobial potential, indicating the synergistic effects of multiple materials. The study assessed the antimicrobial efficacy of the coated bands over a 30-day period. Interestingly, fungal growth was observed only at day 0, indicating the immediate impact of the coatings on inhibiting microbial colonization. This result aligns with the clinical need for orthodontic appliances to maintain antimicrobial properties throughout treatment.

One of the most important aspects of orthodontic materials is their biocompatibility. The study results revealed no significant signs of toxicity in major organs affirm the potential clinical relevance of these coated bands. Patients can benefit from orthodontic appliances that not only correct dental misalignments but also contribute to oral health by reducing the risk of plaque-related issues [27].

This study opens avenues for future research. Further investigations into the long-term antimicrobial properties of these coatings and their clinical performance in patients are needed. Additionally, exploring the molecular mechanisms underlying the antimicrobial activity of these nanoparticles and nanocomposites can provide deeper insights into their mode of action.Beyond orthodontics, the environmentally friendly synthesis of nanoparticles holds promise for various other applications.

Conclusion

This study investigated the in-vivo antimicrobial effectiveness of orthodontic bands coated with green-synthesized Zinc-oxide (ZnO) and Titanium oxide (TiO2) nanoparticles, along with nanocomposites. Results demonstrated that these coatings effectively inhibit the growth of oral pathogens like Streptococcus mutans and Streptococcus mitis, crucial in preventing dental plaque formation. Green synthesis methods ensure eco-friendliness and biocompatibility of the nanoparticles, essential for their use in orthodontic devices. ZnO coated bands exhibited superior antimicrobial properties against S. mutans, while nanocomposite coatings also displayed higher activity. The study found that these coatings maintained their antimicrobial effects over 30 days, with no fungal growth observed after the initial day. Importantly, the coated bands showed no signs of toxicity in vital organs. This suggests their clinical relevance in orthodontics as they improve oral health and it also opens doors for future studies, including long-term antimicrobial assessments, clinical trials, and molecular investigations. Furthermore, the environmentally friendly synthesis of nanoparticles has broader implications beyond orthodontics. Overall, these coatings offer significant potential for enhancing orthodontic treatment outcomes and promoting sustainable nanomaterial applications.

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