ORIGINAL ARTICLE

Effect of Adding 3% Chitosan, 2.5% Neem, and 0.5% Sodium Metabisulphite on Anti-microbial and Mechanical Properties of Heat Activated Denture Base Resin- An In Vitro Study.

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ABSTRACT

Background

Denture stomatitis, commonly linked to microbial colonization on denture surfaces, remains a persistent challenge in prosthodontics. Incorporating antimicrobial agents into denture base resin represents a promising strategy to combat this condition while maintaining essential mechanical properties. The study evaluated the antimicrobial efficacy, flexural strength, and impact resistance of heat-activated denture base resin modified with chitosan, neem, and sodium metabisulphite.

Methodology

A total of 160 samples were fabricated and allocated to four groups: Group 1 (control), Group 2 (3% chitosan), Group 3 (2.5% neem), and Group 4 (0.5% sodium metabisulphite). Each group was categorized into four subgroups, with 10 samples in each subset. Antimicrobial activity was assessed by counting colony-forming units (CFUs). Flexural strength (MPa) was determined with a universal testing machine, while impact strength (kJ/m²) was assessed using a Charpy impact tester. Data was analysed using the Kruskal-Wallis test with post-hoc comparisons, and results were considered significant at $p < 0.05$.

Results

All experimental groups exhibited reduced CFU counts. Chitosan and neem groups demonstrated statistically significant antimicrobial activity ($p < 0.001$), whereas sodium metabisulphite exhibited a limited effect. Flexural strength was reduced in the chitosan and neem groups, but it was not altered in the sodium metabisulphite group. No significant differences in impact strength were observed ($p = 0.224$).

Conclusion

Natural antimicrobials like chitosan and neem effectively enhance the antimicrobial performance of denture base resins, though with some compromise in flexural strength. Sodium metabisulphite, while mechanically stable, lacks strong antimicrobial potency. These findings highlight the potential of bioactive additives for functional improvement of denture materials, with implications for the prevention of denture-related infections.

Keywords

denture base resin, chitosan, neem, Sodium metabisulphite, antimicrobial, flexural strength, impact strength.

INTRODUCTION

Polymethyl methacrylate (PMMA) remains the material of choice for denture base fabrication because of its excellent balance of processability, affordability, lightweight nature, biocompatibility, aesthetic appeal, and satisfactory mechanical porperties. Despite these advantages, conventional PMMA exhibits notable shortcomings, particularly its suboptimal mechanical strength, rendering it susceptible to impact fractures and fatigue failure under repetitive masticatory stresses. Additionally, PMMA surfaces provide a favourable environment for microbial colonization and biofilm development by opportunistic oral pathogens such as *Candida albicans* and *Streptococcus mutans*. Microbial adherence plays a crucial role in the pathogenesis of denture stomatitis, a common inflammatory condition characterized by mucosal erythema, hyperplasia, and discomfort, which affects an estimated 20–67% of denture wearers.[1]

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In response to these challenges, extensive research has focused on incorporating antimicrobial agents into PMMA to inhibit microbial adhesion and biofilm formation without compromising its critical mechanical properties. Various modification strategies have been evaluated, including the integration of antimicrobial copolymers, surface coatings, bioactive nanoparticles[2] (e.g., silver, titanium dioxide, zinc oxide), natural organic extracts (e.g., neem, thymoquinone, tea tree oil), and food-grade preservatives (e.g., potassium sorbate, sodium metabisulphite).[3] While these modifications show potential for enhancing the biological performance of denture base resins, concerns remain regarding their influence on essential mechanical properties such as flexural strength, impact resistance, and long-term durability factors integral to the clinical success and patient satisfaction associated with removable prostheses. Among natural biopolymers, chitosan, a deacetylated derivative of chitin obtained from crustaceans, fungi, and insects, has gained attention due to its broad-spectrum antimicrobial, antifungal, anti-inflammatory, mucoadhesive, and osteoconductive properties.[4] Its cationic nature in acidic environments facilitates electrostatic interactions with negatively charged microbial membranes, leading to membrane disruption and cell death.[5] Similarly, *Azadirachta indica* (neem), widely recognized in traditional medicine exhibits antimicrobial, anti-inflammatory, and antioxidant activities primarily attributed to compounds such as azadirachtin. Neem's incorporation into dental materials has demonstrated promise in mitigating microbial growth[6] , yet its effect on the mechanical properties of denture base resins remains underexplored. Sodium metabisulphite ($Na_2S_2O_5$), a commonly used preservative and antioxidant in the food, pharmaceutical, and cosmetic industries, has demonstrated antimicrobial efficacy with minimal cytotoxicity, as evidenced by studies showing no significant adverse effects on mammalian fibroblasts.[7]

Although sodium metabisulphite has been utilized in dental formulations, its role in enhancing the antimicrobial potential of denture base resins, in addition to its effects on mechanical performance, requires further investigation.

Therefore, the present study aims to evaluate the effect of 3% chitosan, 2.5% neem, and 0.5% sodium metabisulphite on antimicrobial efficacy against *Candida albicans* and *Streptococcus mutans* and mechanical properties, specifically flexural strength and impact strength, to determine whether these natural additives compromise or sustain the material's functional integrity. The null hypothesis of this study was that incorporating chitosan, neem powder, and sodium metabisulphite into PMMA would not significantly affect its antimicrobial activity or mechanical properties.

MATERIALS AND METHODS

The present study was conducted after obtaining ethical approval from the Institutional Ethics Committee, Government Dental College, Thiruvananthapuram, Kerala. (IEC NO: IEC/T/17/23 & 25-05-2023).

PROCEDURE

Fabrication of specimens

Heat-activated acrylic resin specimens modified with 3% chitosan, 2.5% neem, and 0.5% sodium metabisulphite were prepared using a stainless-steel mold and conventional heat polymerization. Wax patterns were created using the mold, followed by flasking and polymerization at 74°C for two hours and an additional hour at 100°C. The specimens were then finished and polished using acrylic stones, sandpaper, polishing tips, rouge, and pumice.

Additive Incorporation into PMMA

For the chitosan group, 4.2 grams of low molecular weight chitosan was dissolved in 100 ml of 1% acetic acid using a magnetic stirrer and incorporated into a PMMA mix consisting of 100 g polymer and 40 g monomer, ensuring a 3% concentration relative to total mass (3/100)×(100+40)=4.2 g. For the neem group, 3.5 grams of neem leaf powder was added to the PMMA to achieve a 2.5% concentration using geometric dilution. Similarly, 0.7 grams of sodium metabisulphite were added to the PMMA mix to obtain a 0.5% concentration.

Methods for evaluating anti-microbial property

Eighty-disc specimens, having a thickness of 2 mm and a diameter of 8 mm, were fabricated for all groups. After that, the samples were washed using 70% ethanol and sterilized at 121°C in an autoclave for 15 minutes. For this study, two oral microbial species, *Candida albicans* and *Streptococcus mutans*, were selected as strains of interest. A bacterial suspension of *Streptococcus mutans* was prepared in brain–heart infusion broth, and sterile saline was used for preparing a suspension of *Candida albicans.* These suspensions were kept at 37°C for one

hour in an incubator to enable the microorganisms to reach the logarithmic phase of growth. Ten samples were placed in separate sterile microtubes in each group. The microbial suspension was adjusted to achieve a turbidity of 0.5 McFarland standard (approximately 2.5×10^5 to 1.0×10^6 cells/mL), followed by the addition of 400 microliters of suspension into each of the microtubes. The specimens with microbial suspension are incubated at 35±1°C for 24 hours. Post-incubation, each microtube's 0.01 ml of suspension was swirled onto a blood agar plate for *Streptococcus mutans*, while a plate of Sabouraud dextrose agar was used for *Candida albicans* using a loop inoculator. After that, these agar plates were incubated for a further 48 hours at about 37°C. The number of colonies was assessed using a colony counter and expressed in colony-forming units (CFU) (Figures 1 and 2).

Methods for testing flexural strength

The flexural strength of the denture base added with 3% chitosan, 2.5% neem, and 0.5% sodium metabisulphite was investigated using three-point flexural tests following ISO 20795-1:2013. In each group, specimens were prepared in accordance with ISO 20795- 1:2013 standards (64 x 10 x 3.3 mm in thickness). The prepared acrylic specimens were immersed in distilled water at 37˚C for 48 hours after being ground with 15 mm of silicon carbide impervious abrasive sheets under a constant stream of water. Ten specimens of each material were prepared and evaluated utilizing a Universal Testing Machine from Instron. A load was applied centrally to each specimen at 5 mm per minute for the crosshead speed. The maximum force sustained before fracture was documented and reported in terms of megapascals (MPa).

Methods for testing Impact strength

The impact strength of heat-activated denture base resins incorporated with 3% chitosan, 2.5% neem, and 0.5% sodium metabisulphite was investigated using a Charpy impact strength analyzer (TINIUS OLSEN MODEL IMPACT 503). Ten samples from every subgroup were fabricated with dimensions of 55×10×10 mm, following ASTM D-256 standards. Each specimen was positioned on the impact testing apparatus with the smooth, unnotched surface facing away from the pendulum. The test was carried out with a pendulum capable of delivering a maximum of 2 joules of energy at an impact speed of 3.46 m/s. The energy absorbed by each specimen upon fracture was recorded using a calibrated scale and reported in kilojoules per square meter (kJ/m^2).

STATISTICAL ANALYSIS

The observed data were coded, tabulated, and assessed using IBM SPSS Version 20. The mean ± standard deviation, median, and interquartile range (IQR) were used to express descriptive statistics. As the number of samples was below 30 in each group, non-parametric tests were used for analysis. Comparison of CFUs (Colony Forming Units) of Candida albicans, Streptococcus mutans, Flexural strength, and Impact strength was done using Kruskal-Wallis ANOVA, followed by a post-hoc analysis. A p-value below 0.05 was considered statistically significant.

RESULTS

Table 1 summarizes the antimicrobial and mechanical performance of heat-activated denture base resin modified with 3% Chitosan, 2.5% Neem, and 0.5% Sodium Metabisulphite ($Na_2S_2O_5$) compared to the Control. For *Candida albicans*, the Control group showed the highest mean CFU count (503 ± 18), while all modified groups demonstrated reduced values, with 3% Chitosan exhibiting the strongest antifungal effect (Figure 3). Similarly, for *Streptococcus mutans*, the Control group recorded the highest mean CFU (599 ± 18), and the greatest antibacterial reduction was again observed in the Chitosan group (Figure 4). Regarding flexural strength, the Control group had the highest mean value (63.80 ± 9.80 MPa); both Chitosan and Neem significantly reduced this property, while $Na_2S_2O_5$ maintained values similar to the Control, indicating minimal adverse impact (Figure 5). In terms of impact strength, the Control group exhibited highest mean (2.63 ± 0.41 kJ/m^2), and although the modified groups had slightly lower values, the differences were not significant, with $Na_2S_2O_5$ showing the lowest impact strength among them (Figure 6).

Pairwise comparisons revealed that the 3% Chitosan group exhibited a significant reduction in CFU counts of both Candida albicans and Streptococcus mutans when compared to the 0.5% sodium metabisulphite ($Na_2S_2O_5$) group (Adj. $p = 0.002$ and 0.001, respectively) and the Control group (Adj. $p < 0.001$ for both), indicating its superior antimicrobial efficacy. The 2.5% Neem group also showed a statistically significant reduction in CFU counts of both organisms compared to the Control group (Adj. $p < 0.001$ for Candida albicans and Adj. p

= 0.001 for S. mutans), but did not differ significantly from the 0.5% $Na_2S_2O_5$ or the 3% Chitosan groups. In contrast, no significant difference was noticed between the 0.5% $Na_2S_2O_5$ group and the Control group for either microorganism (Adj. $p > 0.05$). These findings suggest that incorporating 3% Chitosan and 2.5% Neem into the denture base resin significantly reduced microbial growth, with Chitosan demonstrating the greatest antifungal and antibacterial efficacy, while $Na_2S_2O_5$ exhibited limited antimicrobial activity. This trend was clearly illustrated in Figures 7 and 8, where greater average rank separations between groups indicated more pronounced and statistically significant differences.

In terms of mechanical properties, both 3% Chitosan and 2.5% Neem groups exhibited a marked reduction in flexural strength compared to the 0.5% $Na_2S_2O_5$ group (Adj. $p = 0.001$ and 0.010, respectively) and the Control group (Adj. $p = 0.001$ and 0.009, respectively). No significant difference was observed between the Chitosan and Neem groups (Adj. $p = 1.000$), indicating a similar weakening effect. The 0.5% $Na_2S_2O_5$ group showed no significant difference from the Control (Adj. $p = 1.000$), suggesting no adverse effect on flexural strength (Figure 9). Thus, while Chitosan and Neem offered superior antimicrobial benefits, they compromised the mechanical strength of the denture base material, whereas $Na_2S_2O_5$ preserved flexural integrity but was less effective antimicrobially. Regarding impact strength, no statistically significant difference was found among the groups ($p = 0.224$), with median values ranging from 2.06 to 2.60 kJ/m^2, indicating that the additives had no significantly influence on impact resistance of the denture base resin.

DISCUSSION

Denture stomatitis is a prevalent inflammatory condition among complete denture wearers, commonly linked to poor oral hygiene, continuous denture wear, systemic conditions, and reduced salivary flow. *Candida albicans* is widely recognized as the primary causative organism due to its strong adherence to denture surfaces and ability to form persistent biofilms.[8] However, increasing evidence highlights the synergistic role of *Streptococcus mutans*, which is present in approximately 74% of denture-wearing individuals.[9] *S. mutans* not only enhances biofilm formation but also promotes the adhesion and proliferation of *C. albicans*, contributing to complex polymicrobial infections that are more resistant to treatment.[10]

In this context, the incorporation of antimicrobial agents into denture base resins (DBRs) has emerged as a promising strategy to reduce microbial colonization and biofilm development. While various agents ranging from organic compounds to quaternary ammonium derivatives have been explored,[11] most studies have focused on antifungal activity alone, often neglecting concurrent presence of bacterial involvement and the mechanical implications that arise after material modification. Given the essential role of physical durability in clinical performance, especially in resisting occlusal and masticatory stresses, this study employed a dual approach to evaluate both antimicrobial and mechanical properties of modified DBRs.

The findings demonstrated that incorporation of 3% chitosan and 2.5% neem into heat-activated PMMA resin significantly reduced CFU counts of both *C. albicans* and *S. mutans*, confirming their potent antimicrobial efficacy. Specifically, chitosan showed the greatest reduction (*C. albicans*: 94 CFUs; *S. mutans*: 298 CFUs), consistent with previous studies. Rajabnia et al.[12] demonstrated effective inhibition of *S. mutans* with 2–5% chitosan in resin sealants, while Gondim et al.[13] found chitosan nanoparticles to significantly reduce *C. albicans* with minimal impact on resin surface properties. Similarly, Ardestani et al.[14] showed dose-dependent biofilm reduction of *Candida* species with nano chitosan. Neem, known for its bioactive compounds like azadirachtin and flavonoids, also showed significant microbial reduction (*C. albicans*: 116 ± 14; *S. mutans*: 371 ± 27), aligning with the results of Hamid et al.[6] and Saeed et al.,[15] who reported prolonged antifungal effects with 2.5% neem in denture resins and tissue conditioners. In contrast, sodium metabisulphite at 0.5% concentration exhibited relatively limited antimicrobial action (*C. albicans*: 403 ± 34; *S. mutans*: 498 ± 21), though its mechanism, based on sulfur dioxide release disrupting microbial proteins, has shown modest efficacy in previous studies. Ratanajanchai et al.[16] reported that 0.5% sodium metabisulphite significantly reduced *C. albicans* viability without compromising biocompatibility or mechanical integrity. The mechanical testing revealed a significant decrease in flexural strength for the chitosan (42.91 ± 9.88 MPa) and neem (48.32 ± 6.67 MPa) groups compared to the control (63.80 ± 9.80 MPa).

This reduction may be attributed to poor interfacial bonding or non-uniform dispersion within the resin matrix, challenges commonly associated with blending natural polymers like chitosan into synthetic PMMA, as Li et al.[17] suggested, poor solubility and hydrogen bonding of chitosan hinder homogeneous integration, although solvent-mediated approaches using acetic acid have shown improvement. Supporting this, Bayiumy and Elsokkary[18] observed flexural strength reduction with higher concentrations of chitosan nanoparticles, while Hamid et al.[7] found that neem powder above 0.5% adversely affected PMMA strength. Interestingly, the 0.5% $Na_2S_2O_5$ group maintained flexural strength (61.22 ± 3.57 MPa) comparable to the control, suggesting that it did not compromise the structural performance of the denture base material. This reinforces its potential role where mechanical durability is prioritized, though with limited antimicrobial protection.

Impact strength, critical for withstanding accidental drops or masticatory forces, did not show statistically significant variation across groups. The control (2.63 ± 0.41 kJ/m²), chitosan (2.59 ± 1.18 kJ/m²), neem (2.87 ± 1.42 kJ/m²), and $Na_2S_2O_5$ (2.23 ± 0.63 kJ/m²) groups maintained comparable values. These findings agree with those of Chander et al.,[19] Hamid et al.,[7] and Ismiyati et al.,[20] who reported that moderate incorporation of bioactive powders did not significantly alter the impact resistance of heat-cured PMMA.

Overall, the study confirms that chitosan and neem are effective natural antimicrobial agents for use in denture base materials, offering considerable reductions in microbial load. However, the drawback lies in decreased flexural strength, necessitating optimization in concentration and dispersion to achieve clinical viability. Sodium metabisulphite, while less effective antimicrobially, preserved mechanical strength and may serve as a viable additive when maintaining structural integrity is a priority. As this study was conducted under in vitro conditions, further in vivo research is essential to validate the findings within the dynamic oral environment and assess long-term clinical performance.

The study does have certain limitations, including its in vitro design, which does not fully mimic the complex oral environment. Antimicrobial evaluation was limited to *Candida albicans* and *Streptococcus mutans*, excluding the broader microbial spectrum found in denture biofilms. Only single concentrations of each additive were tested, and assessments were short-term, without exploring long-term effects or sustained antimicrobial release. Additionally, physical properties like surface roughness and hardness, which may influence microbial adhesion and patient comfort, were not evaluated.

CONCLUSION

- Incorporation of 3% chitosan and 2.5% neem into heat-activated denture base resin led to a significant reduction in colony-forming units (CFUs) of Candida albicans and Streptococcus mutans, indicating notable antimicrobial efficacy.
- The group containing 0.5% sodium metabisulphite demonstrated comparatively limited antimicrobial activity, with microbial counts remaining greater than those of the chitosan and neem groups, yet still lesser than the unmodified heat-activated denture base resin.
- A statistically significant decrease in flexural strength was observed in the chitosan and neem groups when compared to the control, which may be attributed to factors such as poor interfacial bonding and inadequate dispersion of the additives throughout the resin matrix.
- The sodium metabisulphite group exhibited flexural strength values comparable to the unmodified control, suggesting favourable mechanical compatibility with the heat activated denture base resin.
- No significant differences in impact strength were noted between any of the test groups, indicating that the addition of these agents did not negatively influence the material's resistance to impact forces.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

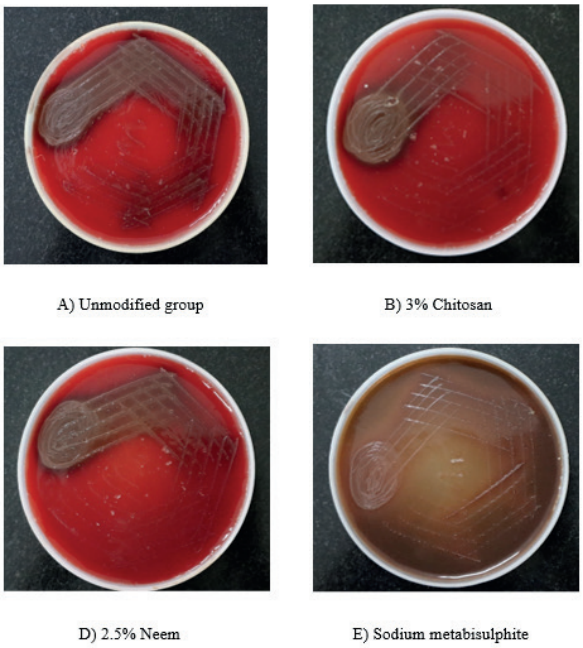


Figure 1: *Streptococcus mutans* colonies cultured on a blood agar plate.

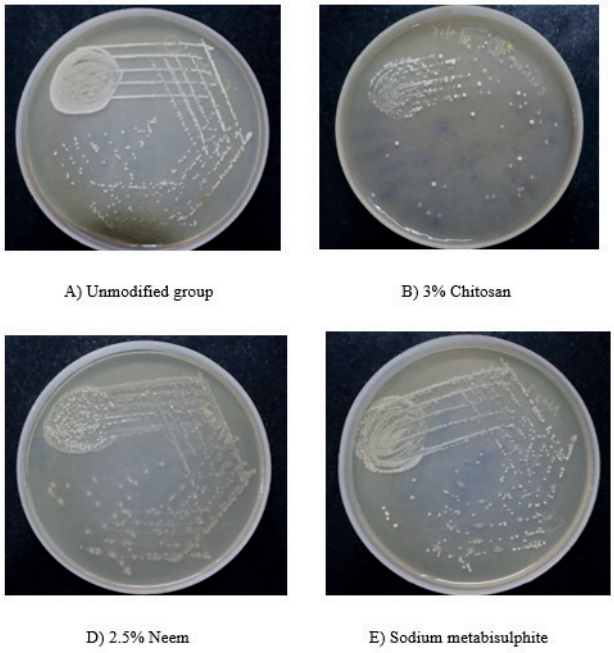


Figure 2: *Candida albicans* colonies cultured on a Sabouraud dextrose agar plate.

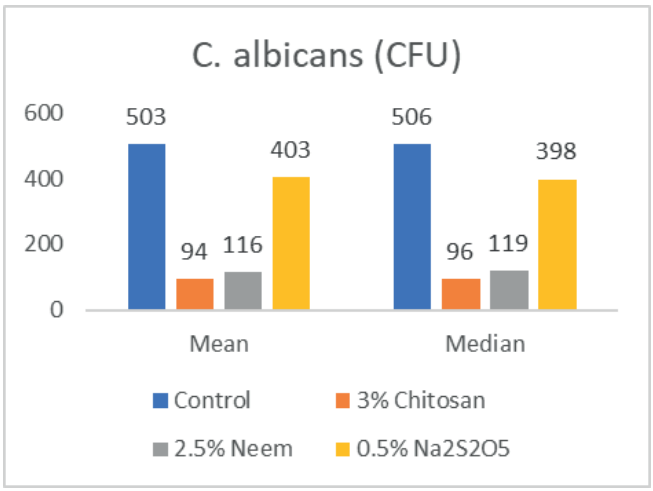


Figure 3: Comparison of colony-forming units of *Candida albicans* of study groups

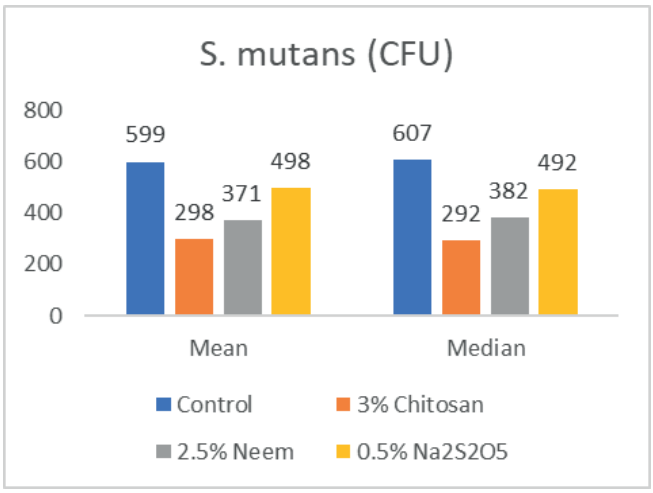


Figure 4: Comparison of Colony-forming units of *Streptococcus mutans* of study groups

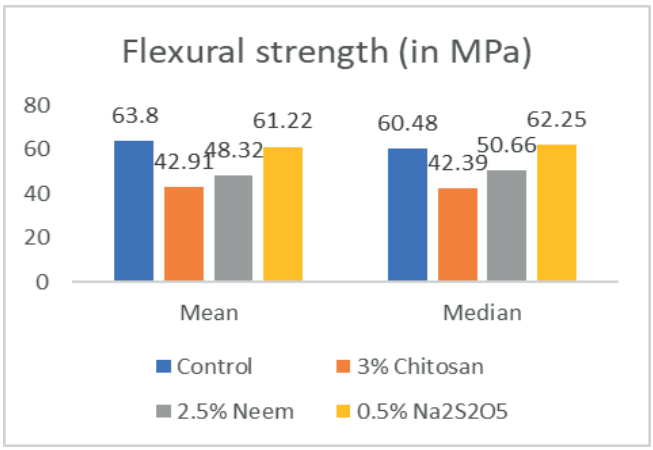


Figure 5: Comparison of the Flexural strength of the study groups

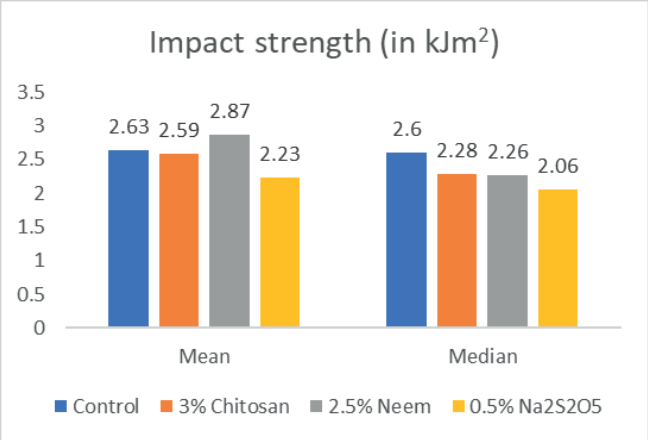
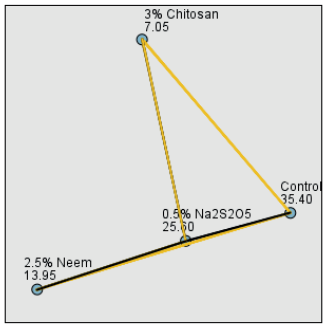


Figure 6: Comparison of the Impact strength of the study groups

Pairwise Comparisons of Group



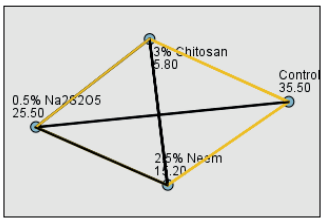
Each node shows the sample average rank of Group.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3% Chitosan-2.5% Neem	-6.900	5.227	-1.320	.187	1.000
3% Chitosan-0.5% Na2S2O5	-18.550	5.227	-3.549	.000	.002
3% Chitosan-Control	28.350	5.227	5.424	.000	.000
2.5% Neem-0.5% Na2S2O5	-11.650	5.227	-2.229	.026	.155
2.5% Neem-Control	21.450	5.227	4.104	.000	.000
0.5% Na2S2O5-Control	9.800	5.227	1.875	.061	.365

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 7: Pairwise comparisons of Colony Forming Unit of the *Candida albicans* group

Pairwise Comparisons of Group



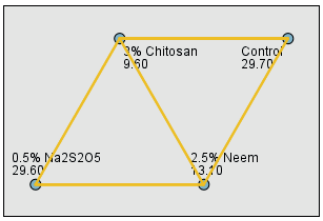
Each node shows the sample average rank of Group.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3% Chitosan-2.5% Neem	-9.400	5.228	-1.798	.072	.433
3% Chitosan-0.5% Na2S2O5	-19.700	5.228	-3.768	.000	.001
3% Chitosan-Control	29.700	5.228	5.681	.000	.000
2.5% Neem-0.5% Na2S2O5	-10.300	5.228	-1.970	.049	.293
2.5% Neem-Control	20.300	5.228	3.883	.000	.001
0.5% Na2S2O5-Control	10.000	5.228	1.913	.056	.335

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 8: Pairwise comparison of Colony Forming Unit of the Streptococcus mutans group

Pairwise Comparisons of Group



Each node shows the sample average rank of Group.

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
3% Chitosan-2.5% Neem	-3.500	5.228	-.669	.503	1.000
3% Chitosan-0.5% Na2S2O5	-20.000	5.228	-3.825	.000	.001
3% Chitosan-Control	20.100	5.228	3.845	.000	.001
2.5% Neem-0.5% Na2S2O5	-16.500	5.228	-3.156	.002	.010
2.5% Neem-Control	16.600	5.228	3.175	.001	.009
0.5% Na2S2O5-Control	.100	5.228	.019	.985	1.000

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.

Figure 9: Pairwise comparisons of flexural strength

Table 1: Descriptive Statistics of Candida albicans CFU, Streptococcus mutans CFU, Flexural Strength (MPa), and Impact Strength (kJ/m²) of Study Groups

Group	Candida albicans (CFU) Mean ± SD	Median (IQR)	Streptococcus mutans (CFU) Mean ± SD	Median (IQR)	Flexural Strength (MPa) Mean ± SD	Median (IQR)	Impact Strength (kJ/m²) Mean ± SD	Median (IQR)
Control	503 ± 18	506 (493, 517)	599 ± 18	607 (586, 612)	63.80 ± 9.80	60.48 (58.46, 75.71)	2.63 ± 0.41	2.60 (2.24, 2.90)
3% Chitosan	94 ± 15	96 (84, 107)	298 ± 25	292 (279, 321)	42.91 ± 9.88	42.39 (34.69, 52.09)	2.59 ± 1.18	2.28 (1.88, 2.83)
2.5% Neem	116 ± 14	119 (106, 127)	371 ± 27	382 (349, 391)	48.32 ± 6.67	50.66 (40.14, 53.44)	2.87 ± 1.42	2.26 (2.01, 2.66)
0.5% $Na_2S_2O_5$	403 ± 34	398 (372, 419)	498 ± 21	492 (485, 516)	61.22 ± 3.57	62.25 (58.25, 64.07)	2.23 ± 0.63	2.06 (1.98, 2.24)

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