

Original Research

Comparison of antimicrobial properties of thymoquinone and silver nanoparticles incorporated in denture base resin and their effect on mechanical properties - An in vitro study

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ABSTRACT:

Objectives: This study aimed : 1.To assess the antimicrobial properties of silver zinc zeolite nanoparticles and Thymoquinone embedded in acrylic denture base resin. 2. To assess the flexural strength of acrylic denture base resin incorporated with silver zinc zeolite nanoparticles and Thymoquinone. 3. To compare the antimicrobial and mechanical properties of acrylic denture base resin incorporated with silver zinc zeolite nanoparticles and Thymoquinone. 4. To assess the surface topography of denture base resin incorporated with silver zinc zeolite nanoparticles and thymoquinone. **Materials and methods:** Eighty-one acrylic specimens were fabricated and divided into three groups. GROUP A - Acrylic resin (Control group). GROUP B - Acrylic resin containing 1% Silver Zinc Zeolite nanoparticle. GROUP C - Acrylic resin containing 1% Thymoquinone. The acrylic samples were fabricated acrylic specimens, (65 x 10 x 2.5) according to the ADA Specification No.12 for all the three groups. The flexural strength was tested using three-point bending test in a universal testing machine. The test was carried out with a cross head speed of 1mm/min. The results obtained were compared by using One Way Anova Test. For antimicrobial test the acrylic samples were fabricated with dimensions 10x 10 x 3 mm for all the three groups. The specimens were placed in test tube with candida culture. The antimicrobial test was done by using serial dilution test and CFU/ml. Serial dilution was done from 10⁻² to 10⁻⁵. The culture was spread on petridish and after 24hrs colonies were counted and CFU/ml was determined. The results obtained were compared by using Kruskal Wallis Test and multiple comparison was done by using Mann Whitney Post hoc Test. The SEM analysis was done for all the three groups. The visual interpretation of the images obtained was done to determine the distribution of antimicrobial material within the polymer matrix. **Results:** Within the limitations of this study it can be concluded that 1) There was no statistical difference in the flexural strength of all the three. 2) The addition of thymoquinone and silver zinc zeolite nanoparticles significantly decreases the candida albicans count. 3) The addition of thymoquinone in denture base resins showed the least microbial count followed by silver zinc zeolite nanoparticles. 4) There was statistically significant difference of CFU/ml between the control group and other two groups. 5) There was no statistically significant difference of CFU/ml between the acrylic resin specimens incorporated with thymoquinone and acrylic resin specimens incorporated with silver zinc zeolite nanoparticles. 6) The SEM analysis of acrylic specimen incorporated with the thymoquinone showed fair dispersion of material within the polymer matrix and acrylic specimen incorporated with silver zinc zeolite nanoparticles showed little aggregation within the polymer matrix.

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INTRODUCTION

Poly methyl methacrylate (PMMA) resin was introduced in 1937 by Walter Wright.¹ It is the most widely used denture base material in the fabrication of removable dentures, implant-supported prostheses and maxillofacial prostheses.² PMMA has fulfilled almost

all the requirements of an ideal denture base material because of its favorable working characteristics, processing ease, accurate fit, adequate color stability, superior esthetics, and use with inexpensive equipment. However, the PMMA denture base is susceptible to microbial colonization in the oral

environment.³ Denture hygiene is recognized as an important part of oral hygiene in that dentures can harbor both bacterial and fungal micro-organisms such as streptococci, Candida and other organisms. The improvement of oral hygiene is generally achieved by the use of antimicrobial mouthwashes and appropriate tooth-brushing methods along with the use of denture cleansing tablets and prophylactic systemic antibiotics. However, all these methods have limited success in reducing the effectiveness of these pathogens⁴ due to surface roughness caused by denture cleansers and rapid microbial re-colonization, especially by Candida Species.⁵ Biofilm formation and adhesion reduce the cleansing efficacy against the biofilms and increase its resistance to antifungal therapy.⁶

Studies have shown that currently available acrylic denture base resins do not have any antimicrobial properties. With the addition of antimicrobial agents bacterial and fungal contamination can be reduced without impairing the mechanical properties of dental materials.⁷ Various antimicrobial materials have been used like nanosilver, nanotitanium dioxide or nanosilicon dioxide particles, chlorhexidine or organic compounds such as 2-tert butylaminoethyl methacrylate, ethylene glycol methacrylate phosphate, and quaternary ammonium salts such as methacryloyloxy undecyl pyridiniumbromide, quaternary ammonium polyethyleneimine nanoparticles, neem, heena and thymoquinone.^{8,9} Silver nanoparticles have been used for their antimicrobial effect in different biomedical applications¹⁰ and are reported to be nontoxic to humans and very effective against bacteria, viruses, and other eukaryotic micro-organisms at very low concentrations and without side effects.¹¹ Thymoquinone (TQ) is the bioactive phytochemical constituent of the seeds oil of *Nigella sativa*. *Nigella sativa* is an annually flowering medicinal plant native to South and Southwest Asia. *N.sativa* seed extract includes essential oil, alkaloids, fixed oil, proteins, and saponins. Its extract has been explored in the medical field and has been found to have antibacterial, anti-inflammatory, antioxidant, and anti-tumor properties.¹²

MATERIAL AND METHOD

Materials used the study

1. Heat polymerized denture base material,-polymer and monomer DPI-heat Cure (pink), Mumbai. Batch no. Polymer-762, monomer-766.
2. Silver Zinc Zeolite (20 nm, Degussa Company, Germany) particles and Thymoquinone ($\geq 98\%$; Sigma-Aldrich, Taufkirchen, Germany).

3. *Candida Albicans* cultures. (MTCC No. 183)

PREPARATION OF SPECIMEN

Eighty-one acrylic specimens were fabricated and divided into three groups. GROUP A - Acrylic resin (Control group). GROUP B - Acrylic resin containing 1% Silver Zinc Zeolite nanoparticle. GROUP C - Acrylic resin containing 1% Thymoquinone. For flexural strength test the acrylic resin specimens were fabricated with dimensions (65 x 10 x 2.5) according to the ADA Specification No.12 for all the three groups. The flexural strength was tested using three-point bending test with a universal testing machine. The test was carried out with a cross head speed of 1mm/min. The results obtained were compared by using One Way Anova Test.

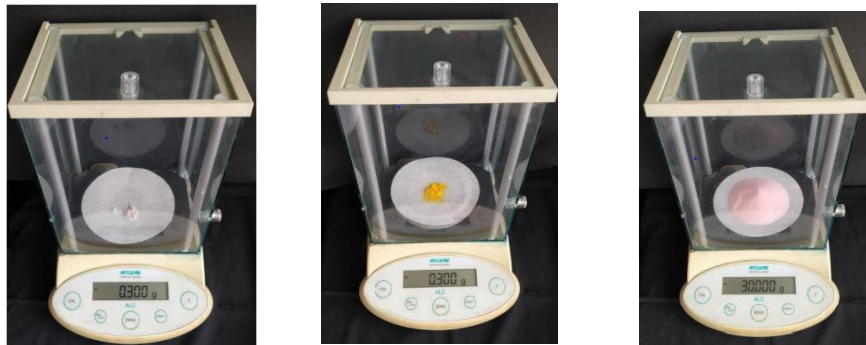
For antimicrobial test the acrylic resin specimens were fabricated with dimensions 10× 10 × 3 mm for all the three groups. The specimens were placed in test tube containing candida culture. The antimicrobial test was done by using serial dilution test and CFU/ml. Serial dilution was done from 10⁻² to 10⁻⁵. For SEM analysis acrylic resin specimens were fabricated with dimensions 5×5×2 mm for all the three groups. The visual interpretation of the images obtained was done to determine the distribution of antimicrobial material within the polymer matrix

These stainless-steel strips were used to form a standard mold for the fabrication of acrylic block specimens. A tab of modelling wax was attached at one end of the stainlesssteel strips to facilitate its removal. The metal strips were embedded up to the full thickness in dental stone in the base of the flask. The dental stone was allowed to set for half an hour and separating medium was applied. Second pour was made in dental stone, and the flask was held in compression till the final set of dental stone. After that, the flask was opened and metal strips were removed to obtain the mold.

The powder and liquid components of denture base resins were dispensed and mixed in the ratio of 3:1. For every 30gms of acrylic powder, 0.3 gm of silver zinc zeolite nanoparticles was added for group I, 0.3gm of thymoquinone was added to II group. Group III was the control without antimicrobial material.

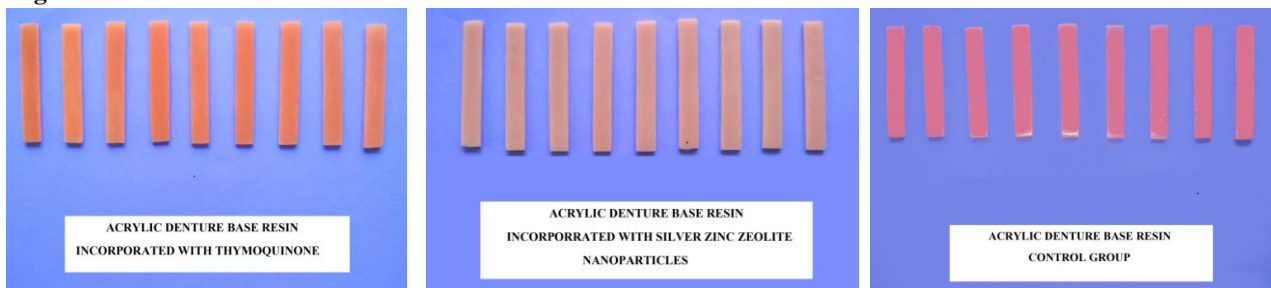
The polymer for control group was mixed with monomer in a ratio of 3:1 by volume in a mixing jar with a tight-fitting lid followed by packing. The modified resin polymer for specimens of group 2 and 3 was then mixed with monomer in a ratio 3:1 by volume in a mixing jar with a tight-fitting lid followed by packing. (Fig No.1)

Fig 1:



Bench curing was done for 1 hour. The samples were cured following the short cycle. The samples were cured by heating the flasks at 65°C for 90 minutes, followed by heating at 100°C for 1 hour. Bench cooling was done for 30 minutes followed by de-flasking. Finishing and polishing of samples. Samples were trimmed by using acrylic trimmer bur. Finishing was done by using sand paper (Emery 120 grit and 240 grit). Polishing was done using pumice and polishing cake. (Fig No.2)

Fig 2:



Specimens were stored in water for 24 hours. Specimens were autoclaved.

Testing the flexural strength

Total no. of 27 acrylic specimens were taken and were divided into 3 groups; 1) Control(C) 2) Nanoparticles(N) 3) Thymoquinone (T).

The flexural strength was tested using three-point bending test with a universal testing machine (Fig No.3). The test was carried out with a cross head speed of 1mm/min. The results obtained were compared by using One Way Anova Test.

Transverse strength was calculated using following equation

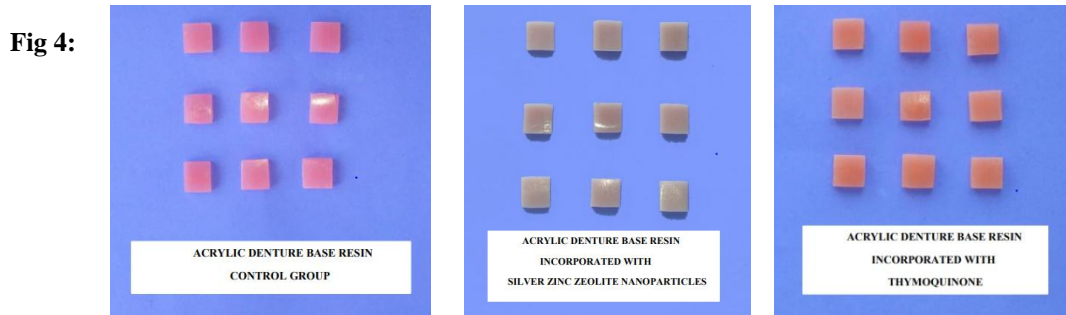
$$T_s = 3PL / 2WT^2$$

Fig 3:



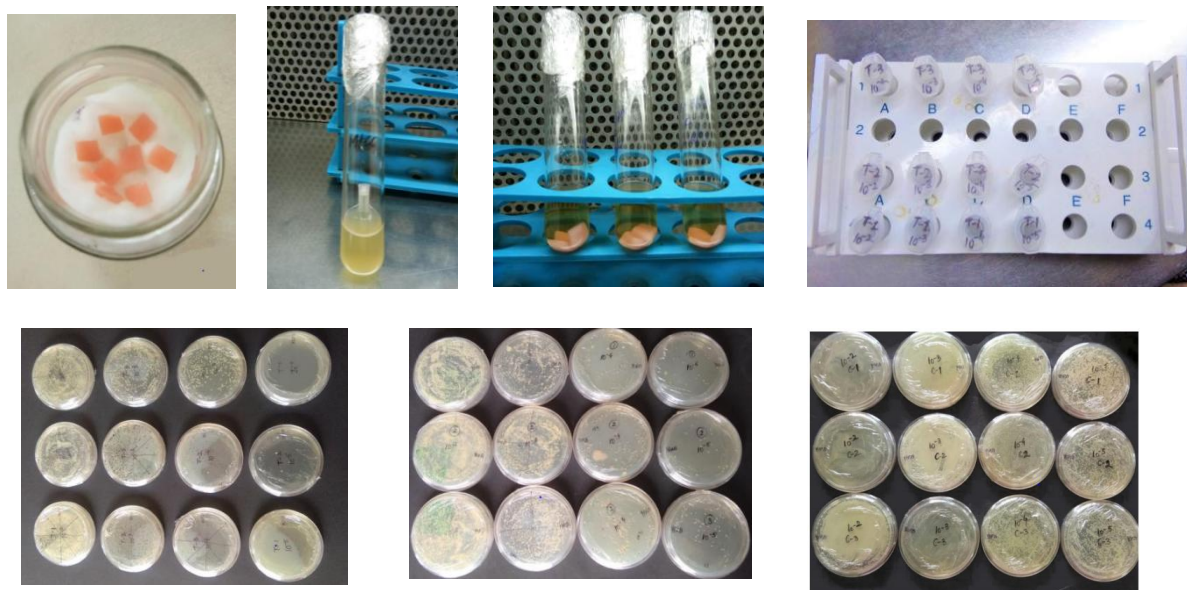
ANTI-MICROBIAL TEST

Total no. of 27 acrylic specimens were taken and were divided into 3 groups; 1) Control(C) 2) Nanoparticles(N) 3) Thymoquinone (T). These groups were further divided into subgroups; C1, C2, C3; N1, N2, N3; T1, T2, T3. Each sub-group consisted of 3 specimens which were kept in 3 test tubes with candida culture. These test tubes were incubated and vibrated for 24 hours at 30°C (Fig 4).



Serial Dilution Test A 20µl of culture was taken from each test tube with the help of micro pipette and then it was diluted serially and spread on a petri dish containing YEPD medium and incubated for 48h at 37°C. A marker pen counter was used to count the number of *Candida albicans* colonies in each quadrant where acceptable growth was noted and the final number was corrected for the dilution factor (Fig No.5).

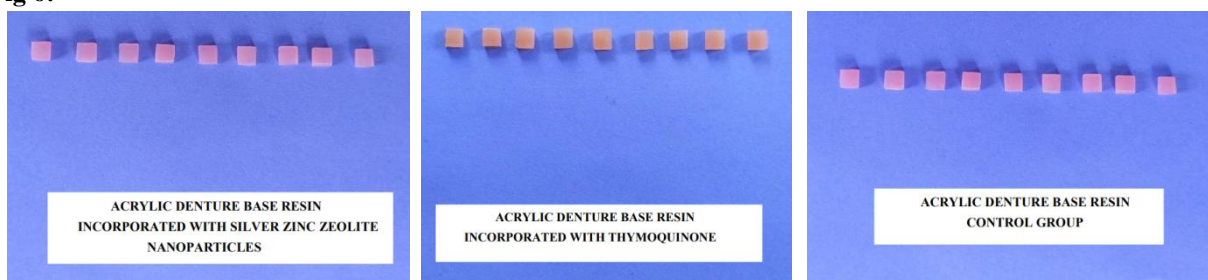
Fig 5:



SCANNING ELECTRON MICROSCOPY

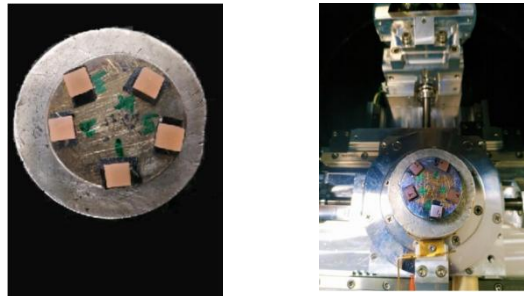
Total no. of 27 acrylic specimens were taken and were divided into 3 groups; 1) Control(C) 2) Nanoparticles(N) 3) Thymoquinone (T).

Fig 6:



Specimens were kept in dessicator for 3 days to avoid any moisture contamination. After 3 days these specimens were subjected to gold sputtering. Specimens were coated with a layer of 12nm layer of gold sputter and were placed on stub/holder and subjected to scanning electron microscopy under 25000 magnification power.(Fig No.7)

Fig 7:

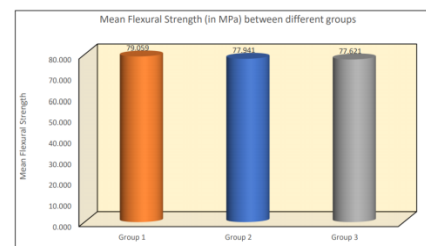


RESULT

Comparison of mean flexural strength (in Mpa) between different groups was done by using Oneway ANOVA Test. (Table 1 and graph 1) Comparison of mean candida Albicans CFUs/ml at serial dilutions from 10^{-2} to 10^{-5} between different groups using Kruskal Wallis Test. (Table 2,4,6,8) Multiple comparison of mean difference in Candida Albicans CFU/ml (10^7) at serial dilutions from 10^{-2} to 10^{-5} between different groups using Mann Whitney post hoc test. (Table 3,5,7,9). Mean candida Albicans CFUs/ml (10^7) at serial dilutions from 10^{-2} to 10^{-5} between different groups was also given (Graph 2,3,4,5). Three groups which were tested were: Group 1 - Conventional Denture Base Resin [Control Group], Group 2 - Denture Base Resin incorporated with Zinc Zeolite Nano Particles, Group 3 - Denture Base Resin incorporated with Thymoquinone.

The test results demonstrate the comparison of mean Flexural Strength (in MPa) between different groups. The mean Flexural Strength values for Group 1 was 79.059 ± 6.289 , for Group 2 was 77.941 ± 4.297 and Group 3 was 77.621 ± 4.932 . This difference in the mean flexural strength (in MPa) between 03 groups was not statistically significant [P=0.83].(table 1& graph1)

Comparison of mean Flexural Strength (in Mpa) between different groups using One-way ANOVA Test						
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	9	79.059	6.289	71.70	88.42	0.83
Group 2	9	77.941	4.297	74.88	88.32	
Group 3	9	77.621	4.932	71.62	86.88	

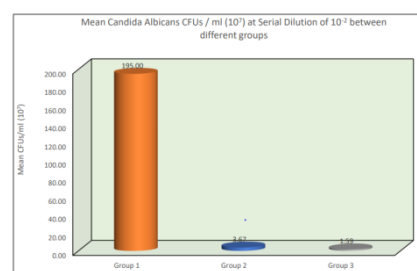


The test results demonstrate the comparison of CFUs / ml (10^7) at Serial Dilution of 10^{-2} between different groups. The mean CFUs (per ml) for Group 1 was 195.00 ± 26.46 , for Group 2 was 3.67 ± 1.51 and Group 3 was 1.59 ± 0.65 . This difference in the mean CFUs (per ml) between 03 groups was statistically significant at $P<0.001$ (Table 2)

Comparison of mean Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-2} between different groups using Kruskal Wallis Test						
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	3	195.00	26.46	175	225	<0.001*
Group 2	3	3.67	1.51	2.64	5.40	
Group 3	3	1.59	0.65	0.9	2.2	

Multiple comparison of mean difference in the mean CFUs / ml (10^7) at Serial Dilution of 10^{-2} between groups revealed that Group 1 showed significantly highest mean CFUs (per ml) as compared to Group 2 & Group 3, both at $P<0.001$ (Table 3& Graph 2)

Multiple comparison of mean difference in Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-2} between different groups using Mann Whitney Post hoc Test					
(I) Group	(J) Group	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	191.33	152.99	229.67	<0.001*
	Group 3	193.41	155.06	231.75	<0.001*
Group 2	Group 3	2.08	-36.27	40.42	0.99

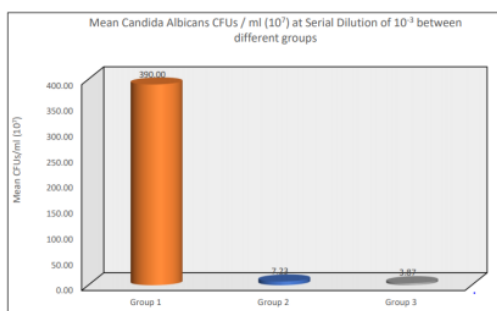


The test results demonstrate the comparison of CFUs / ml (10^7) at Serial Dilution of 10^{-3} between different groups. The mean CFUs (per ml) for Group 1 was 390.00 ± 52.92 , for Group 2 was 7.23 ± 1.37 and Group 3 was 3.87 ± 1.03 . This difference in the mean CFUs (per ml) between 03 groups was statistically significant at $P < 0.001$ (Table 4).

Comparison of mean Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-3} between different groups using Kruskal Wallis Test						
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	3	390.00	52.92	350	450	<0.001*
Group 2	3	7.23	1.37	6	8.7	
Group 3	3	3.87	1.03	3	5	

Multiple comparison of mean difference in the mean CFUs / ml (10^7) at Serial Dilution of 10^{-3} between groups revealed that Group 1 showed significantly highest mean CFUs (per ml) as compared to Group 2 & Group 3, both at P .Group 2 relatively showed higher mean CFUs (per ml) as compared to Group 3. However, there was no significant difference in the mean CFUs (per ml) between Group 2 & Group 3 [$P=0.99$]. This result infers that Group 1 had significantly highest mean CFUs / ml (10^7) at Serial Dilution of 10^{-3} as compared to Group 2 and Group 3.(Table 5& graph 3)

Multiple comparison of mean difference in Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-3} between different groups using Mann Whitney Post hoc Test					
(I) Group	(J) Group	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	382.77	306.19	459.34	<0.001*
	Group 3	386.13	309.56	462.71	<0.001*
Group 2	Group 3	3.37	-73.21	79.94	0.99

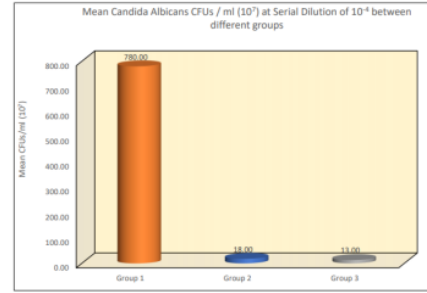


The test results demonstrate the comparison of CFUs / ml (10^7) at Serial Dilution of 10^{-4} between different groups. The mean CFUs (per ml) for Group 1 was 780.00 ± 105.83 , for Group 2 was 18.00 ± 5.20 and Group 3 was 13.00 ± 2.65 . This difference in the mean CFUs (per ml) between 03 groups was statistically significant at $P < 0.001$.(Table 6)

Comparison of mean Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-4} between different groups using Kruskal Wallis Test						
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	3	780.00	105.83	700	900	<0.001*
Group 2	3	18.00	5.20	15	24	
Group 3	3	13.00	2.65	10	15	

Multiple comparison of mean difference in the mean CFUs / ml (10^7) at Serial Dilution of 10^{-4} between groups revealed that Group 1 showed significantly highest mean CFUs (per ml) as compared to Group 2 & Group 3, both at P Group 2 relatively showed higher mean CFUs (per ml) as compared to Group 3. However, there was no significant difference in the mean CFUs (per ml) between Group 2 & Group 3 [$P=0.99$]. This result infers that Group 1 had significantly highest mean CFUs / ml (10^7) at Serial Dilution of 10^{-4} as compared to Group 2 and Group 3. (Table 7 & graph 4)

Multiple comparison of mean difference in Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-4} between different groups using Mann Whitney Post hoc Test					
(I) Group	(J) Group	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	762.00	608.70	915.30	<0.001*
	Group 3	767.00	613.70	920.30	<0.001*
Group 2	Group 3	5.00	-148.30	158.30	0.99

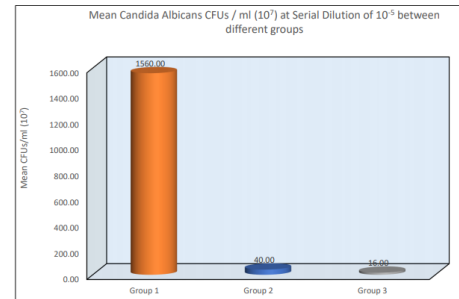


The test results demonstrate the comparison of CFUs / ml (10^7) at Serial Dilution of 10^{-5} between different groups. The mean CFUs (per ml) for Group 1 was 1560.00 ± 211.66 , for Group 2 was 40.00 ± 17.32 and Group 3 was 16.00 ± 15.10 . This difference in the mean CFUs (per ml) between 03 groups was statistically significant at $P < 0.001$. (Table 8)

Comparison of mean Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-5} between different groups using Kruskal Wallis Test						
Groups	N	Mean	SD	Min	Max	P-Value
Group 1	3	1560.00	211.66	1400	1800	<0.001*
Group 2	3	40.00	17.32	30	60	
Group 3	3	16.00	15.10	0	30	

Multiple comparison of mean difference in the mean CFUs / ml (10^7) at Serial Dilution of 10^{-5} between groups revealed that Group 1 showed significantly highest mean CFUs (per ml) as compared to Group 2 & Group 3, both at P Group 2 relatively showed higher mean CFUs (per ml) as compared to Group 3. However, there was no significant difference in the mean CFUs (per ml) between Group 2 & Group 3 [$P=0.97$]. This result infers that Group 1 had significantly highest mean CFUs / ml (10^7) at Serial Dilution of 10^{-5} as compared to Group 2 and Group 3. (Table 9 & graph 5).

Multiple comparison of mean difference in Candida Albicans CFUs / ml (10^7) at Serial Dilution of 10^{-5} between different groups using Mann Whitney Post hoc Test					
(I) Group	(J) Group	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
Group 1	Group 2	1520.00	1212.06	1827.94	<0.001*
	Group 3	1544.00	1236.06	1851.94	<0.001*
Group 2	Group 3	24.00	-283.94	331.94	0.97



The SEM analysis of the acrylic resin incorporated with silver zinc zeolite nanoparticles showed a fairly good dispersion of silver zinc zeolite nanoparticles in the polymer matrix with little aggregation. (fig 9). The SEM analysis of the acrylic resin incorporated with thymoquinone particles showed a fairly good dispersion of thymoquinone particles in the polymer matrix without aggregation. (fig 10). The SEM analysis of the acrylic resin (control group), (fig 11)

Fig 9:

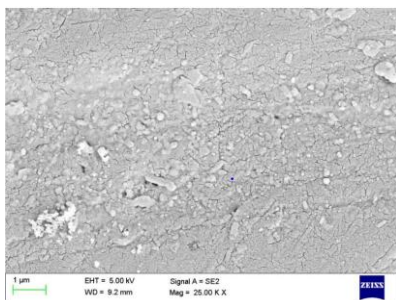


Fig 10:

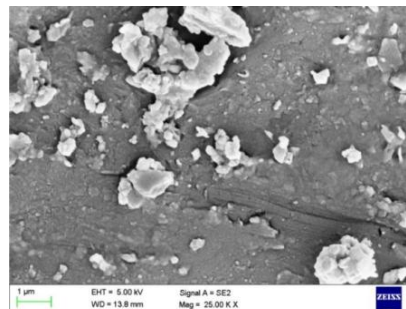
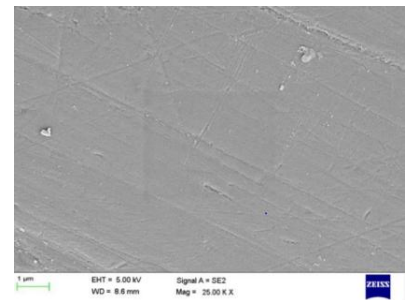


Fig 11:



DISCUSSION

Removable complete dentures have been widely used as the main rehabilitative modality for edentulous patients. The demand for complete dentures is still high due to the rise in life expectancy and is likely to be same over the next decade. Denture wearing is associated with certain adverse effects, such as denture stomatitis, on the denture-bearing areas. Denture stomatitis is common among denture wearers and its main cause is infection by *Candida* species.¹³ Denture stomatitis is characterized by inflamed mucosa, particularly under the upper denture, and patients may complain of a burning sensation, discomfort, or bad taste.¹⁴ *Candida* adheres to denture acrylic through plaque-forming bacteria. Despite antifungal therapy to treat denture stomatitis, infection recurs soon after the treatment stops. Chronic usage of these drugs often results in their resistance. So, if the antimicrobial material is incorporated within the denture base material the adherence of the *Candida albicans* can be reduced which in turn can reduce the occurrence of denture stomatitis.¹⁵ In this present study comparison of two different antimicrobial materials against *Candida albicans* which is a common causative agent of denture stomatitis was done after incorporating these materials into heat cure denture base resin. The antimicrobial materials chosen has been already used in several studies but their comparison was yet to be done. Two materials used were thymoquinone and silver zinc zeolite nanoparticles. *Nigella sativa* is an annual flowering plant and its seeds have wide therapeutic effects. It is also known as Black cumin. Its seeds are used as traditional medicine for the treatment of microbial diseases without any reported side effects. Therefore, this plant can provide a valuable agent for microbial diseases. Thymoquinone is an active compound of the *nigella sativa* (black cumin), which has a property of anti-bacterial, anti-tumor, anti-fungal. Thymoquinone acts by mechanism of producing reactive oxygen species (ROS) and has been used in several studies.⁵¹ Likewise silver zinc zeolite nanoparticles has also been used in many antimicrobial studies and has potential antimicrobial property. Silver has long been extensively used in medical fields due to its oligodynamic nature to prevent and treat a variety of diseases. It exhibits bactericidal activity even at minute concentrations. Silver has broad-spectrum antimicrobial effects and causes low toxicity to humans. Inorganic carriers such as zeolite, phosphate, titanium dioxide, activated carbon, montmorillonite, water soluble glass and mesoporous silica are developed to extend lifetime of silver.¹⁶

The higher clinical efficacy of silver is due to the prolonged constant dissociation of silver ions into the surrounding environment. The antimicrobial activities of silver depend on the silver cation Ag^+ , which binds strongly to electron donor groups in biological molecules containing sulphur, oxygen or nitrogen.

These silver-based materials have to release Ag^+ to a pathogenic environment to be effective. In this present study 1% of antimicrobial material was added to the polymer and specimens were fabricated by conventional method. Antimicrobial property of both materials was tested and compared with control group that is acrylic specimen without any antimicrobial material. Effect of these antimicrobial material on the flexural strength of the acrylic specimen was also evaluated and comparison was done. And SEM study was done to visualize the distribution of particles within the polymer matrix.

Thymoquinone showed the highest antimicrobial property followed by silver zinc zeolite nanoparticles. The effect of addition of antimicrobial material on flexural strength was not clinically significant. Both the materials were fairly dispersed within the polymer matrix.

CONCLUSION

Within the limitations of this study it can be concluded that

- 1) There was no statistical difference in the flexural strength of all the three groups that is: control group, acrylic specimens incorporated with thymoquinone and acrylic specimens incorporated with silver zinc zeolite nanoparticles.
- 2) The addition of thymoquinone and silver zinc zeolite nanoparticles significantly decreases the *Candida albicans* count.
- 3) The addition of thymoquinone in denture base resins showed the least microbial count followed by silver zinc zeolite nanoparticles.
- 4) There was statistically significant difference of CFU/ml between the control group and other two groups.
- 5) There was no statistically significant difference of CFU/ml between the acrylic resin specimens incorporated with thymoquinone and acrylic resin specimens incorporated with silver zinc zeolite nanoparticles.
- 6) The SEM analysis of acrylic specimen incorporated with the thymoquinone showed fair dispersion of material within the polymer matrix and acrylic specimen incorporated with silver zinc zeolite nanoparticles showed little aggregation within the polymer matrix.

COMPETING INTEREST

No conflict (s) of interest.

ETHICS APPROVAL

Not applicable

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