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Green Synthesis, Characterization of Zinc Oxide Nanoparticles and their Incorporation into Glass Ionomer Cement for Inhibition of *Streptococcus mutans*

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In present study, zinc oxide nanoparticles were synthesised using *Syzygium aromaticum* and characterized using UV visible spectroscopy, SEM, XRD and FTIR techniques. The characteristic hexagonal structure of the ZnO nanoparticles was confirmed through XRD analysis. The UV-Visible spectrum showed a strong absorbance at 366 nm confirming the presence of ZnO and the peak at 496 cm^{-1} in FTIR indicated the Zn-O stretch bond. Average size of the zinc oxide nanoparticles obtained from SEM analysis was found to be 86 nm. The zinc oxide nanoparticles exhibited better antibacterial activity than clove extract, when tested against clinical isolates of *Streptococcus mutans*. The nanoparticles incorporated into the dental restorative material, glass ionomer cement (GIC) were tested against *S. mutans* and exhibited better antibacterial activity than clove extract. Glass ionomer cement (GIC) did not exhibit antibacterial activity alone, but the incorporation of ZnO nanoparticles into GIC significantly improved antibacterial activity. Hence, ZnO embedded GIC is a promising material in restorative dentistry for preventing the recurrence of dental caries.

Keywords: Dental caries, *Streptococcus mutans*, Zinc Oxide nanoparticles, Glass ionomer cement.

INTRODUCTION

The emergence of nanotechnology as an interdisciplinary field has brought significant changes in dentistry. Although many diseases in dentistry can be treated by conventional methods, the new era of nanotechnology in dentistry will bring revolutionary approaches in diagnosis and treatment of dental diseases [1]. Dental caries commonly caused by *Streptococcus mutans,* is one of the most chronic diseases among adults and children [2]. Over the past few decades, there has been an increasing trend in the prevalence of dental caries, especially in the developing countries [3-6]. The treatment of dental caries involves the use of antibiotics and dental restorative materials such as glass ionomer cement (GIC), which acts as sealants in the pits and fissures in the tooth and release fluoride to prevent further enamel demineralisation. However, growing resistance to antibiotics makes the organism more persistent to survive and cause diseases [7]. These antibiotics also affect the oral and gut microflora, which may contribute to the development of drug resistance in them [8-11]. The antibiotics when administered alone may not inhibit the demineralisation completely and may result in secondary infection due to the prevalence of drug resistant microbes [12]. The microbial biofilm formation is also seen as a major cause for the failure of antibiotic therapy and development of drug resistance [13,14]. Studies reveal that fluoride-resistant microorganisms may be another key factor in the development of antibiotic resistance [15]. In addition the restorative materials such as GIC lack antibacterial properties and thus it cannot prevent secondary dental caries.

Nanoparticles exhibit antimicrobial properties and incorporating them into the dental restorative material can be explored for preventing the recurrence of secondary dental caries. In recent years, silver and zinc nanoparticles have gained more importance because of their significant antibacterial activity [16]. The antibacterial effect of nanoparticles can be attributed to the high surface area to volume ratio enabling maximum contact with microbial biofilms. Further, nanoparticles because of their smaller size can penetrate the cell membranes easily and affect the processes within the microbes, which results in significant antibacterial activity [17].

Green synthesis of nanoparticles like zinc oxide is more feasible than other chemical methods as it is simple, cost effec-

tive and gives more reproducible results [18]. In present study, clove extract was used to synthesize zinc oxide nanoparticles. The polyphenols present in plants *i.e*. the eugenol component of the clove oil [19], acts as a reducing agent for the synthesis of ZnO nanoparticles. The synthesized zinc oxide nanoparticles were characterized by X-ray diffraction for their crystal structure, by scanning electron microscopy (SEM) to define their morphology and also by FT-IR and UV-Vis spectroscopy. The synthesized zinc oxide nanoparticles were checked for their antibacterial activity against *S. mutans*. Followed by the confirmation of their antibacterial activity, they were then incorporated in the dental restorative material and again tested for their susceptibility against *S. mutans.*

EXPERIMENTAL

Preparation of clove extract: Fresh cloves (*Syzygium aromaticum*) was purchased from the local market of Bangalore, India. The cloves were washed thoroughly with distilled water, dried and then powdered. Clove powder (5 g) was added to a 10 mL of distilled water and heated for about 0.5 h to boil. This extract was filtered using Whatmann filter paper No. 1. The filtered extract was again mixed with 40 mL distilled water, making the volume up to 50 mL. This extract was used for the synthesis of zinc oxide nanoparticles.

Synthesis of zinc oxide nanoparticles: Zinc nitrate (0.1M) was added to 50 mL of the clove extract. This mixture was continuously stirred in a beaker for about 0.5 h to ensure homogeneity. Later, this solution mixture was kept in a preheated muffle furnace at 400 ºC for about 20 min. After cooling, the synthesized zinc oxide nanoparticles were used for further studies.

Characterization: The synthesized zinc oxide samples were characterized by X-ray Diffractometer (Bruker-D2-Phaser) to determine their phase purity and crystallinity. The UV absorbance of zinc oxide nanoparticles was recorded using UV-Vis spec instrument ELICO model. Infrared spectrum was obtained from Bruker-Alpha FT-IR spectrometer, while the morphology of the nanoparticles was determined by scanning electron microscope (model: ZEISS-GEMINI-ULTRA 55).

Isolation and identification of *Streptococcus mutans*: Forty plaque samples (labelled S1 to S40) were collected from the Faculty of Dental Sciences, Ramaiah University of Apllied Sciences, Bangalore. These samples were cultured on Mitis Salivarius agar plates containing bacitracin. The identification of *S. mutans* was done using gram staining, sorbitol and mannitol fermentation tests and blood agar haemolysis. *S. mutans* (MTCC 497) received from Microbial Type Culture Collection and Gene Bank, India was included as control.

Antibiotic sensitivity tests: The antibiotic sensitivity tests were conducted using the most frequently used antibiotics *viz*. penicillin G (10 mcg), erythromycin (15 mcg) and ciprofloxacin (5 mcg) for all the positive clinical isolates and MTCC 497 standard. Disc diffusion method was used to perform these tests on Mueller Hinton agar plates swabbed with the overnight cultures of clinical isolates of *S. mutans*. The zones of inhibition were measured in millimeters and the sensitivity of the clinical isolates as well as the standard towards these antibiotics was interpreted according to the CLSI guidelines.

Antibacterial activity: Based on the sensitivity of the clinical isolates to antibiotics, six clinical isolates (S1, S2, S4, S6, S22, S30) and the standard (MTCC 497) were chosen for the antibacterial activity of ZnO nanoparticles against *S. mutans*. Three concentrations of zinc oxide nanoparticles (0.5, 1.0 and 5 mg/mL) were chosen for the antibacterial susceptibility test. This test was done by agar well diffusion method on Mueller Hinton Agar plates [20]. The plates were incubated for 24 h at 37 ºC and then the zones of inhibition were measured in millimetres. The antibacterial activity of the clove extract alone and the synthesized zinc oxide nanoparticles was assessed.

Antibacterial activity of GIC incorporated ZnO nanoparticles against *S. mutans*: A mixture of 1 mg of zinc oxide nanoparticles and1 mg of dental restorative material *i.e*. glass ionomer cement (GIC-Fuji II) was suspended in 1 mL of DMSO. Similarly, 1 mg of zinc oxide nanoparticles in DMSO and 1 mg of prepared GIC in DMSO were also taken separately. The antibacterial susceptibility test was carried out with clinical isolates of *S. mutans* and the standard, on Mueller Hinton Agar plates by agar well diffusion method [20]. The zones of inhibition were measured after 24 h of incubation at 37 ºC and the results were recorded.

RESULTS AND DISCUSSION

The ZnO nanoparticles were synthesized using clove extract. The clove extract was used as fuel and zinc nitrate served as an oxidizer for the synthesis of nanoparticles.

X-Ray diffraction studies: The X-ray diffraction pattern of the synthesized zinc oxide nanoparticle using clove extract was obtained. The observed peaks at different value of 2θ are in completion accordance with the JCPDS (File No. 5-664) standard diffraction pattern for ZnO nanoparticles (Fig. 1), which confirms that the nanoparticles are of hexagonal phase having a wurtzite structure. The X-ray diffraction pattern reported in this study was in agreement with the earlier studies [21,22]. Average particle size of ZnO nanoparticles was found to be 80 nm using Scherrer's equation ($D = 0.9\lambda/\beta\cos\theta$).

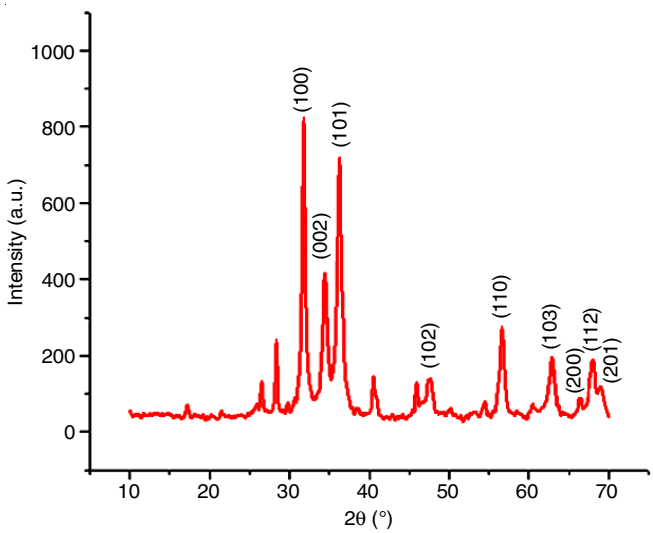

Fig. 1. XRD pattern of zinc oxide nanoparticles synthesized using clove extract

UV-visible studies: The absorbance peak of synthesized zinc oxide nanoparticles was observed at 366 nm (Fig. 2). This is within the band gap range for zinc oxide nanoparticles [23]. The absence of any peaks or drops before and after the absorbance peak of ZnO nanoparticle confirms that other dopants are not present in the sample.

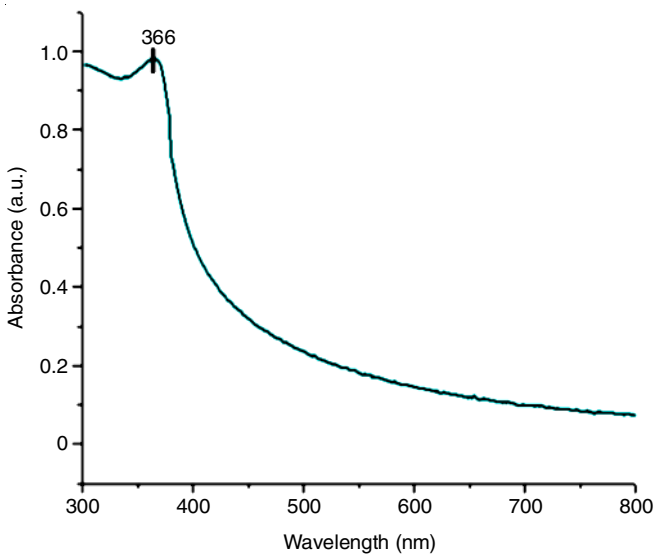

Fig. 2. UV-visible spectra of zinc oxide nanoparticles

FTIR analysis: The FTIR pattern of the synthesized ZnO nanoparticles by combustion method using clove extract was examined in the range of 4000-400 cm^{-1}. A peak at 520-480 cm^{-1} is attributed due to metal oxide bond. In the FTIR pattern, a peak at 496 cm^{-1} indicates the presence of ZnO nanoparticles, the sharp peak around 1800 cm^{-1} indicates the presence of C=O group. A small peak around 2900 cm^{-1} is due to C-H stretch and a band at 3400 cm^{-1} shows O-H stretch (Fig. 3).

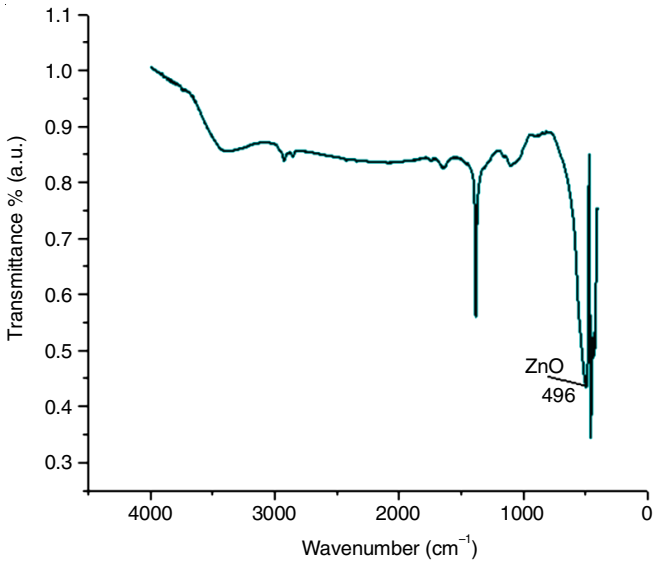

Fig. 3. FTIR pattern of zinc oxide nanoparticles

In the case of nanoparticles, dipolar interactions and high internal stress play an important role and variation in this can affect the observed vibrational frequencies. According to earlier studies, the Zn-O stretch bond was observed at around 500 cm^{-1}. The slight variation in the band position of zinc oxide may be due to the variation in zinc and oxygen bond length because of the different particle size [24].

SEM studies: The SEM morphology of the synthesized ZnO particles by combustion method shows clearly the presence of ZnO nanoparticles within the nanometer range in an agglomerated form. The ZnO nanoparticles synthesized using clove extract had an average particle size of 86 nm and were irregular shaped (Fig. 4). If the size of nanoparticle is lesser than 100 nm, it has more applications and lesser the size of the nanoparticles better is their antibacterial activity. This is because smaller particles have larger surface areas that promote more interaction between the cell and particles as well as promote a faster rate of release of zinc ions [25].

Isolation and identification of *S. mutans*: From the collected 40 dental plaque samples, 23 clinical isolates grown on mitis salivarius agar plates were Gram-positive and showed positive for both sorbitol and mannitol fermentation studies and hence were identified as *S. mutans.* The confirmation of these isolates as *S. mutans*, was done by blood agar haemolysis, where α-haemolysis (greenish haemolysis) on blood agar plates was observed.

Antibiotic sensitivity tests: The antibiotic sensitivity tests were conducted against *S. mutans* clinical isolates and standard using the most frequently used antibiotics. The antibiotics penicillin G, erythromycin and ciprofloxacin, belongs to the class of β-lactam, macrolides and flouroquinolones, respectively. The results were interpreted according to the CLSI guidelines. Out of 23 clinical isolates, 11 isolates showed sensitivity (28-40 mm), 9 isolates showed intermediate sensitivity (19-28 mm) and 3 isolates (0-10 mm) showed resistance to Penicillin G. For ciprofloxacin, 18 isolates showed sensitivity (23-31 mm), 2 isolates showed intermediate sensitivity (15-19 mm) and 3 isolates (0-14 mm) were resistant. For erythromycin, eight isolates were sensitive (21-26 mm), nine isolates showed intermediate sensitivity (15-19 mm), and six isolates exhibited resistance (0-13 mm). Penicillin G and ciprofloxacin proved to be the most effective antibiotics against *S. mutans*. The standard MTCC497 was sensitive to all the antibiotics tested. Based on the sensitivity of clinical isolates to antibiotics, six clinical isolates (S1, S2, S4, S6, S22 and S30) were chosen along with standard for further studies. Among the clinical isolates chosen, S1 and S2 were susceptible to all the three antibiotics whereas S4 and S22 were resistant to all the three antibiotics. The isolate S6 was resistant to erythromycin but susceptible to penicillin and ciprofloxacin and S30 was resistant to erythromycin and ciprofloxacin but sensitive to penicillin.

Antibacterial activity of ZnO nanoparticles against *S. mutans*: Three concentrations of ZnO nanoparticles (0.5, 1.0 and 5 mg/mL) were chosen for the antibacterial susceptibility test. The antibacterial activity of the clove extract and the green synthesized zinc oxide nanoparticles were tested against *S. mutans* and the results were compared. Zone of Inhibition were observed for all the clinical isolates (S1, S2, S4, S6, S22, S30) and the standard (MTCC 497) (Fig. 5). For the clove

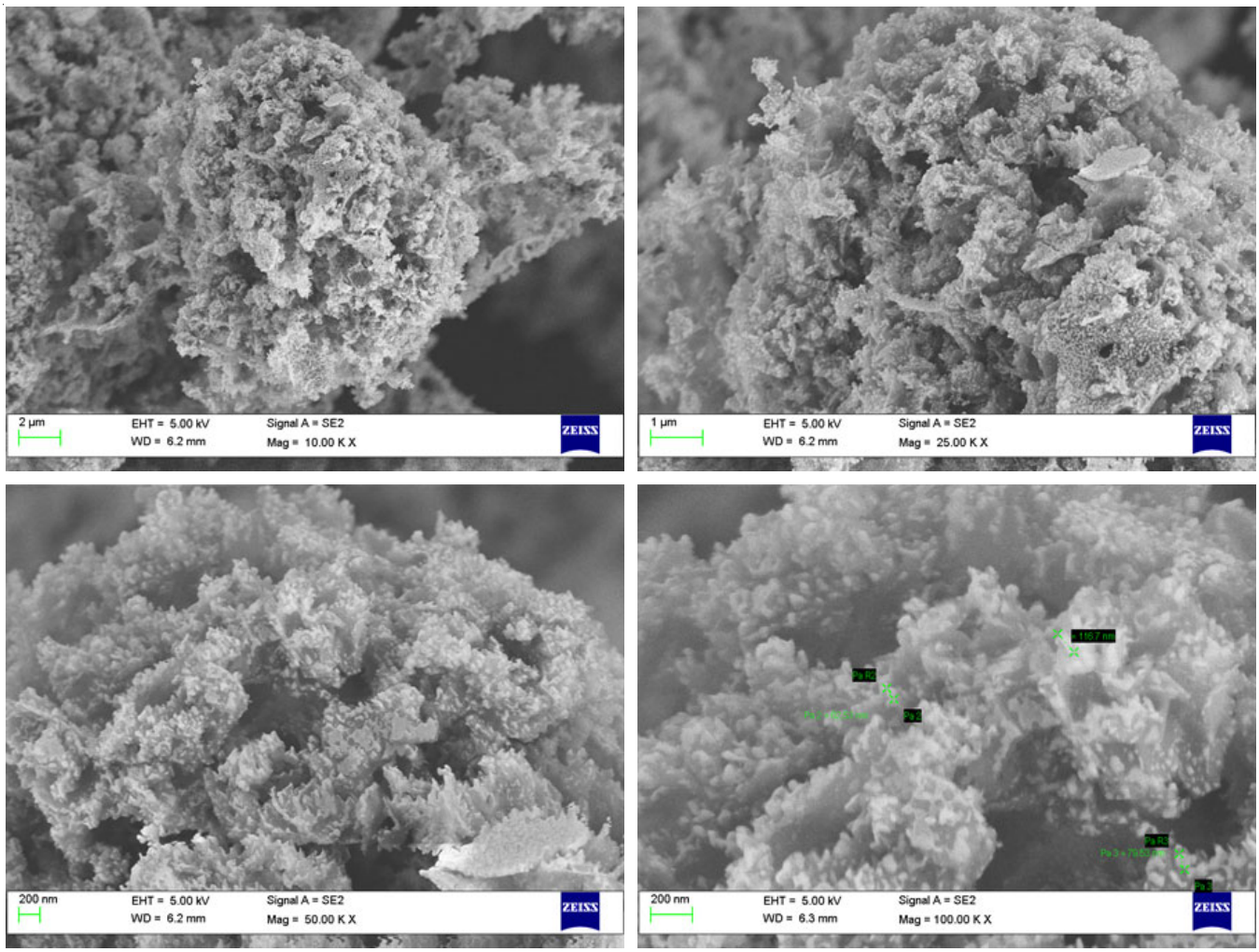

Fig. 4. SEM images of zinc oxide nanoparticles

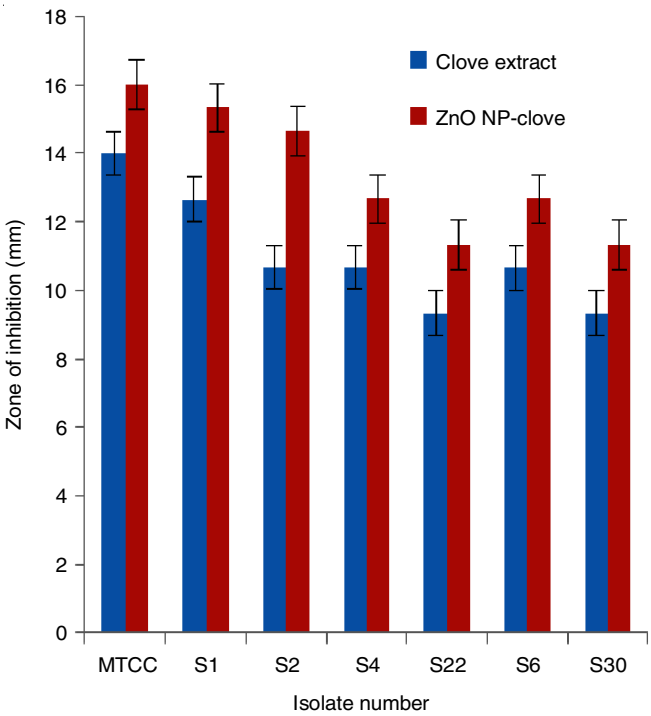

Fig. 5. Zone of inhibition for clove extract and ZnO nanoparticles

extract, the zone of inhibition ranging from 9 mm to 13 mm was observed against the clinical isolates. Whereas, ZnO nanoparticles synthesized using clove extract had a higher antibacterial activity with the zone of inhibition ranging from 11 mm to 16 mm. It was observed that for all six isolates and the standard *S. mutans*, the antibacterial activity exhibited by the synthesized ZnO nanoparticles was higher when compared to the clove extract alone.

The zinc oxide nanoparticles kill the bacteria by disrupting the cell membrane of the bacterial species. There is a high rate of generation of reactive oxygen species, which disrupt and destabilize the membrane [26,27]. Another possibility of bactericidal activity could be the induction of intercellular reactive oxygen species like hydrogen peroxide. Under UV or visible light, ZnO get activated to generate OH^-, O_2^-, H_2O_2. These reactive oxygen species could penetrate the cell membrane of the bacteria, cause oxidative stress and kill them. In the present study, the antibacterial activity of clove extract can be attributed to the presence of alkaloid and phenolic compounds like eugenol.

Antibacterial activity of GIC incorporated with ZnO nanoparticles against *S. mutans*: The glass ionomer cement (GIC), the ZnO nanoparticles and the ZnO incorporated GIC

were tested for their antibacterial activity against the clinical isolates of *S. mutans* and the standard. The GIC alone did not exhibit any antibacterial activity, which was evident with no zone of inhibition. The dental restorative material glass ionomer cement (GIC) constitutes fluoro-alumino-silicate glass and does not exhibit any antibacterial activity. Whereas, the ZnO nanoparticles synthesized using clove extract had an antibacterial activity with the zone of inhibition (11-16 mm) against the *S. mutans* isolates and standard. Zinc oxide nanoparticles embedded in GIC also showed a good antimicrobial activity against the clinical isolates of *S. mutans* with the zone size ranging from 10 mm to 15 mm. The zone of inhibition was observed for all the six isolates (S1, S2, S4, S6, S22 & S30) and standard (Fig. 6). The diameter of the zone of inhibition of GIC incorporated with ZnO nanoparticles was lesser when compared to the ZnO nanoparticles alone, as expected. This is mainly because the dental cement prevents the complete dispersion of the nanoparticles into the medium. In the present study, zinc oxide nanoparticles embedded in GIC showed good antimicrobial activity against *S. mutans*. This is in agreement with the earlier studies where silver and titanium dioxide nanoparticles incorporated into restorative materials exhibited improved antibacterial activity [28,29]. Such improvised restorative materials with antibacterial activity will reduce the proliferation of cariogenic bacteria and decrease the recurrence of dental caries.

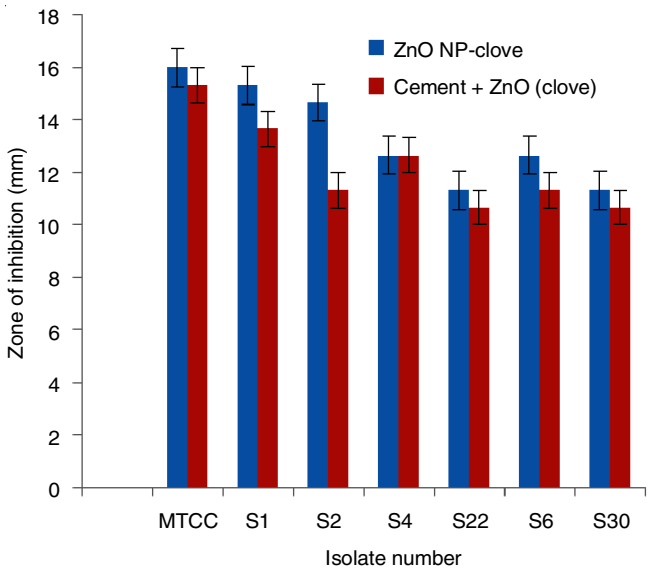

Fig. 6. Zone of inhibition for ZnO nanoparticles and GIC incorporated with ZnO nanoparticles

Conclusion

In current study, zinc oxide nanoparticles were synthesized using clove (*Syzygium aromaticum*) extract. The synthesized nanoparticles were characterized using UV-vis, FTIR, XRD and SEM, and the average size of the nanoparticles was found to be 86 nm. Drug resistance was observed among the clinical isolates of *S. mutans*, when tested against the standard antibiotics *viz*. Penicillin G, erythromycin and ciprofloxacin. The synthesized ZnO nanoparticles, glass ionomer cement (GIC) and ZnO nanoparticle incorporated GIC were tested for their antibacterial activity against *S. mutans*. The incorporation of ZnO nanoparticles into dental restorative material GIC significantly improved antibacterial activity against *S. mutans*. From the present study, it can be concluded that ZnO embedded GIC is a promising material for restorative dentistry because of its improved antibacterial properties and therefore, are efficient in preventing the recurrence of dental caries.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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