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ORIGINAL ARTICLE

IN VITRO EVALUATION OF ANTIMICROBIAL EFFICACY OF SILVER ZEOLITE AGAINST COMMON ORAL PATHOGENS

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ABSTRACT

Objectives. Antimicrobial efficacy and toxicity analysis of 5 different concentrations of silver zeolite (SZ) compound against 5 common oral pathogens *Streptococcus mutans, Streptococcus pyogenes, Lactobacillus sp., Staphylococcus aureus, Candida albicans*.

Material and methods. The antimicrobial efficacy of 5 different concentrations of SZ was tested against 5 common oral pathogens using the agar well diffusion method and the MIC and MBC values were determined using the micro broth dilution method. The toxicity of all 5 different concentrations was evaluated using brine shrimp assay and lethal concentration (Lc50) value was determined.

Results. At 10 µg/mL the antimicrobial activity of SZ was almost negligible. The antimicrobial activity was observed in an increasing trend against all the test microorganisms as the concentration increased. At 75 & 100 µg/mL the zone of inhibitions was more than the control. Furthermore, MIC and MBC values of SZ with concentrations 25, 50, 75, and 100 µg/mL were determined and recorded. SZ was equally effective against all the test organisms. The LC25 (lethal concentration25) value was 1.6 µg/mL, whereas the LC50 value was 1.77 µg/mL and the LC75 value was 1.90 µg/mL, calculated from the probit computational method.

Conclusion. SZ has the potential to change the ongoing system and bring about a revolution as an antimicrobial drug. However, the dose must be regulated as it can be toxic in higher concentrations. SZ compounds with the correct study of physicochemical properties and toxicity analysis can increase their pharmacological use and market value.

Key words: *silver zeolite, oral pathogens, antimicrobial efficacy, toxicity analysis; pharmaceutical applications*

INTRODUCTION

The oral cavity is an ambient location for the growth of microorganisms. Several pathogenic or nonpathogenic microbes find an excellent incubation area inside the mouth [10]. This growth of microbes exponentially increases in the presence of a prosthesis or other dental apertures [22, 23]. The use of a partial or complete removable prosthesis imposes a large problem as it provides a greater surface area for microbial adhesion to the oral tissues [18]. In the same way, the use of partial and complete fixed prosthesis poses a challenge and difficulty in maintaining proper oral hygiene and following the mouth cleaning protocol [18, 22]. This, in turn, results in increased chances of microbial infection in the oral tissues and the remaining teeth. Various common pathogenic bacteria and fungi like *Streptococcus mutans*, *Streptococcus pyogenes*, *Lactobacillus sp.*, *Staphylococcus aureus*, *Candida albicans* are responsible for causing hard tissue as well as soft tissue infections in the oral cavity [4]. Dental caries and oral candidiasis are the most frequently occurring microbial infections in the oral cavity [27] These clinical consternations resulted in the need to have a new and effective antimicrobial agent that is effective against all these common pathogens, thereby protecting the oral cavity of patients who are partially or completely edentulous.

The noble metals bioactivity as well as their uses are in the focus of interest amongst the researchers

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due to multiple factors such as high reactivity, superior compatibly and the non-toxic nature of the eukaryotic cells [2, 8]. Out of the wide range of noble metals, silver seems to be quite popular in the usage of the biomedical field because of its remarkable physicochemical properties and its cost-effectiveness [3, 6, 13]. Silver is generally non-toxic and has been used in several different forms in the field of medicine and pharmacology for treating various ailments and infections. Silver has been described to be "oligodynamic" because of its potential bactericidal activity even at minimal concentrations [3, 6, 13]. Several inorganic materials act as a carrier for silver particles such as phosphate, zeolite, titanium dioxide, activated carbon that increases its pharmaceutical properties and shelf life, without changing its basic physiochemical properties [3, 6, 13].

The molecule Silver-zeolite (SZ) has a wide range of antimicrobial properties and is rarely used in the field of dentistry. In recent times there has been an increased interest in the usage of these silver nanoparticles (AgNPs) for their properties [25]. The concept is based on integrating AgNPs in between the minute voids present in the crystalline structure of the zeolites and the carrier that is inorganic such as zirconium phosphate silicate and zirconium phosphate [25]. It has been hypothesized that the release AgNPs and the silver ions tend to restrict the growth of nearby microorganisms. Several antimicrobial action mechanisms have been hypothesized for AgNPs like inactivation of vital microbial enzymes, RNA replication interruption and alterations in cell wall permeability [7, 14, 15, 25]. SZ has been introduced as a crystalline aluminosilicate material with silver ions. SZ is known as a pharmacological product effective against various microbes but has not been used extensively in the dental sciences, instead, products like zinc-undecylenate have been used whose antimicrobial efficiency and coverage is far lesser than that of SZ [7, 14, 15, 25]. Therefore, this study aimed to evaluate the antimicrobial efficacy of 5 different concentrations of SZ against five common oral pathogens, *S. mutans, S. pyogenes, Lactobacillus sp., S. aureus, C. albicans* in both *in vitro* along with its toxicity analysis.

MATERIAL AND METHODS

Collections of microorganisms

The test organisms were isolated from patient samples attending the Department of Oral Pathology and Microbiology of our institution and were identified in the Central Research Laboratory of our Institute using standard microbiological methods and safety guidelines.

Agar well diffusion method

The agar-well diffusion procedure was followed to evaluate the antibacterial activity. Blood agar plates were used for *S. mutans* and *S. pyogenes*, Muller Hinton agar was used for *S. aureus* and *Lactobacillus* and Sabouraud dextrose agar was used for *C. albicans*. The bacterial strains were spread with an L- shape spreader with a fully grown broth culture of respective bacteria. Further, 0.5mm wells were bored on each plate with the help of a sterile cork borer. Six wells per plate in equidistant were made. 50 µl of 5 different concentrations: 10, 25, 50, 75 and 100 µg/ml was added to the wells. In the 6th well, amoxiclav (against all bacteria) and amphotericin B (against *Candida*) was used as a positive control. Then the plates were incubated at 37°C for 18-24 hours. The diameter of the zone of inhibitions (ZI, in mm) was measured after the incubation period [16, 30].

Micro dilution method

Use of standardized bacterial colony numbers: Comparison of different microorganisms was difficult since they had different optical densities, growth timing, and requirements. Also, at a time, the number of bacteria of different bacterial species in their respective medium is different. Hence, a concentration of 5×10^5 CFU/ml of each test bacteria were used [16, 30].

*Preparation of bacterial culture***:** Under aseptic conditions, a single colony was inoculated into a 5 ml test tube containing its respective broth, and incubated at 37°C. After incubation, 100µl of that culture was transferred into the 10 ml test tube containing its broth and further incubator until its OD_{600} was obtained at 0.5. When the absorbance was 0.5, serial dilution was done. Here 10^{-5} dilutions were taken. In this way, all the strains were prepared for this assay [16, 30].

P*reparation of plates:* Sterile 96 well microtiter plate was coated with resazurin, media, drug, and bacteria under aseptic conditions inside the laminar airflow. A stock solution of 100 µg/ml was prepared. After that, 4 different lower concentrations were prepared i.e. 10, 25, 50 and 75 µg/ml. Then the wells were filled up with 50 µl of respective broth media, different concentrations of extracts, 10 µl resazurin indicator solution and 10µl of bacterial suspension (5×10^6 CFU/ml) was added to each well to attain 5×10^5 CFU/ml concentration. Two blank wells were made in which the first one no drug was there and in the second well there was no bacterial culture. Each plate was wrapped with aluminium foil and they were incubated overnight at 37°C. The colour change from purple to pink or colourless was recorded as the growth of bacteria. The lowest concentration at which colour does not change to pink colour and remain blue is considered as the MIC value of an extract. Amoxiclav,

a broad-spectrum antibiotic was taken as the standard for the bacteria and whereas amphoteric b was the control used against *C. albicans*. The Minimum Inhibitory concentration (MIC) calculation of the control for the 5 microbial strains was done separately [16, 30].

Brine shrimp toxicity assay

The 5 different concentrations of SZ compounds were routinely evaluated in a test for lethality to brine shrimp larvae as described by *Waghulde* et al [29]. Toxicities of compounds were tested at 10, 24, 50, 75 and 100 µg/mL in 10 mL sea-water solutions with 1% DMSO (v/v). Ten, nauplii were utilized in each test and survivors checked after 24 hours. Distilled water was used as a negative control. The observed percentage of the lethality of the different concentrations SZ was determined based on probit analysis. The $\log_{10}$ concentration values were taken on X-axis and probits were taken Y-axis. The probit values were calculated from the probit computational table [21].

RESULTS

The antimicrobial activity was observed in an increasing trend against all the microorganisms as the concentration increased. At 10 µg/ml the antimicrobial activity of SZ was almost negligible. However, at 25 µg/ml there was a significant increase in the antibacterial activity where *S. aureus* was most sensitive to SZ with a ZI of 19 and against *C. albicans* the ZI was 12 mm. Similarly, the ZI was recorded against all the concentrations (Figure 1, Table 1). At 75 & 100 µg/ml the ZI was more than the control. Furthermore, MIC and MBC values of silver zeolite with concentrations 25, 50, 75, and 100 were determined and recorded. The minimum inhibitory concentration (MIC) was found at 10 µg/mL. Higher ZI or MIC levels do not generally mean, that those concentrations can be directly used in manufacturing drugs or incorporated into any other antimicrobial drug to enhance its activity. Therefore, the host toxicity of each concentration exhibiting antimicrobial activity should be done.

From the graph plotted (Figure 2), the $LC_{25,}$ LC_{50}, and LC_{75} values were determined along with probit values and their corresponding $\log_{10}$ concentration values, respectively (Table 2). Antilog values of these $\log_{10}$ concentration values are 39.81 (LC_{25}), 58.88 (LC_{50}) and 79.43 (LC_{75}), which are the computed LC values. The individual MIC and LC_{100} values are directly interpreted from the experiments. The LC_{25} value was 1.6 µg/mL, whereas the LC_{50} value was 1.77 µg/mL and the LC_{75} value was 1.90 µg/mL, calculated from the probit computational method (Table 2).

Table 1. Antimicrobial assay by agar well diffusion method and determination of MIC and MBC values of silver zeolite in 5 different concentrations against 5 common oral pathogens (zone of inhibition in mm)

Strain	10 µg/ml			25 µg/ml			50 µg/ml			75 g/ml			100 µg/ml			Control		
	ZI	MIC	MBC	ZI	MIC	MBC	ZI	MIC	MBC	ZI	MIC	MBC	ZI	MIC	MBC	ZI	MIC	MBC
S. mutans	05	-	-	18	15	24	23	15	24	26	12	21	28	9	18	24	10	19
S. pyogenes	-	-	-	15	18	25	22	18	25	26	10	18	27	6	12	23	8	13
S. aureus	04	-	-	19	15	22	23	15	22	23	10	21	25	6	15	25	8	17
Lactobacillus sp	07	-	-	18	21	25	20	21	25	21	12	18	22	9	21	22	10	19
C. albicans	-	-	-	12	18	22	22	18	22	24	12	20	24	12	23	24	11	20

ZI, Zone of inhibition; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

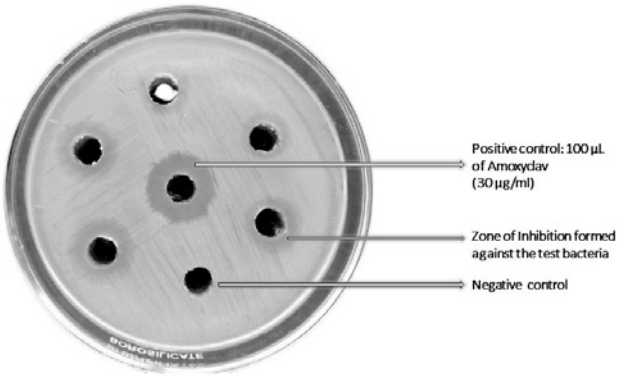

Figure 1. Determination of the antimicrobial activity of silver zeolite against different test organisms by Agar well diffusion method

Table 2. Lethality values during silver zeolite (SZ) toxicity to shrimp nauplii by probit computational method

SZ extract concentration	Log_{10} Concentrations	Percentage lethality	Probit values
0	-	0	-
10	1	6.67	3.5
25	1.4	16.67	4.0
50	1.7	43.34	4.8
75	1.9	70.0	5.5
100	2.0	99.94	8.1

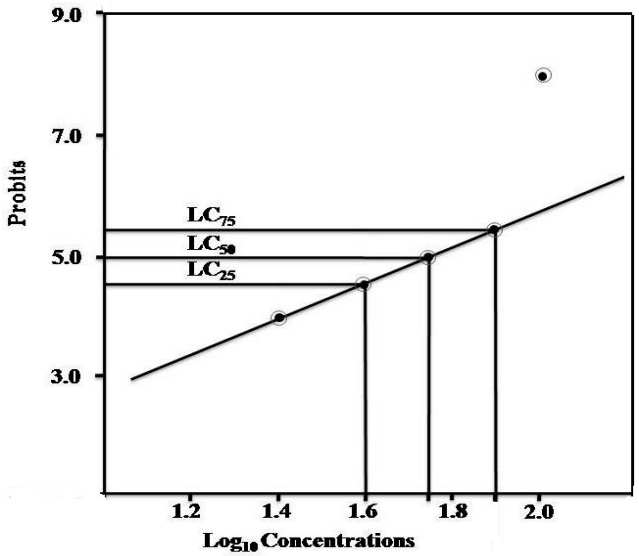

Figure 2. Graphical determination of LC_{50} value

DISCUSSION

The continuous increase in oral infections has led to the search for noble antimicrobial agents which integrated into various daily use oral/dental products. The noble metals are well sought-after agents because of their therapeutic non-toxic nature towards eukaryotic cells. Out of the wide range of noble metals, silver is being extensively used in the biomedical field because of its amazing physiochemical properties and its cost-effectiveness. The molecule SZ has a wide range of antimicrobial properties and is rarely used in the field of dentistry. The present *in vitro* study validated that SZ can inhibit the growth of 5 common oral pathogens. However, the increase in the toxicity level to nauplii cells, with an increase in concentration was a point of concern.

The antibacterial efficacy of SZ is similar to that of silver nitrate, where the inorganic salts and ion chelators generate reactive oxygen species, when they come in contact with the bacterial cell, resulting in cell wall damage [15]. In another study, the antibacterial activity of silver-loaded zeolite X was found effective in controlling pathogenic bacteria, such as *Escherichia coli*, *S. aureus*, and *Pseudomonas aeruginosa*, where zeolite X was synthesized and loaded with silver by ion-exchange method [12]. A study from the United Kingdom reported that silicone elastomers complex containing silver (14 wt%) & zeolite (2 wt%) was effective in limiting the growth of *E. coli* and *Staphylococcus epidermidis* [21]. Further, the study reported, the amalgamation of SZ with organo-silicanes, not only increased the mechanical strength of the elastomers, but they were also restricting the colonization of *C. albicans*, gram-positive and gram-negative bacteria, beyond a period of 24 hours incubation [5]. Similarly, a comparative study with polyvinyl chloride (PVC) tube containing SZ was effective in preventing the colonization of *S. epidermis* and *E. coli* for 5 and 20 days, respectively. In comparison, a simple plasticized PVC tube exhibited no antibacterial property [32]. Antimicrobial tests of silver in zeolite-loaded dental acrylic were active against *C. albicans*, *S. mutans* and *Fusobacterium nucleatum* [14]. An Indian study compared the antimicrobial effectiveness of SZ incorporated into two different types of softliners when used *in vivo* for 28 days in complete denture patients, where SZ showed a significant reduction in colony-forming units in both the soft liners [28]. Furthermore, the use of SZ was endorsed by many studies as an antifungal and antibacterial agent [11, 20]. Studies also confirmed, SZ acts as an ion pump, providing the controlled timely release of silver ions, and thus, the controlled release provides continuous antimicrobial protection [17]. The results obtained in our study also reported good antimicrobial activity.

A study with tissue conditioners reported conditioners incorporated with SZ reported slight toxicity to the dermal cells [1]. A cytotoxicity assay of SZ against lung carcinoma, human hepatocellular, colon carcinoma cell-line and breast adenocarcinoma revealed that SZ was nontoxic and silver substituted micronized zeolite-can be used as an antitumor

drug [31]. Further, Zeolite-silver-zinc nanoparticles biocompatibility was evaluated against pulmonary adenocarcinoma cells using MTT assay, where no cytotoxicity was reported [26]. However, a comet assay of SZ with silver nanoparticles against the MRC-5 cell line indicated DNA damage in results [19]. SZ has been incorporated into polymers, textiles, metal coatings, dental/medical materials, environmental/ consumer products because of its high antimicrobial and less host toxicity [8, 9]. Our study also reported higher toxicity to the nauplii, with an increase in SZ concentrations. The limitations of this study was the exact physiochemical properties were not analyzed. Moreover, the toxicity study was done using an *in vitro* model, which cannot be accurate. Hence, additional efficient *in vivo* studies must be done using cell lines to confirm the toxicity levels of SZ.

CONCLUSIONS

With the opportunities for new therapeutic options, it is evident that one must opt for new trials and implications of unexplored zones of medicine. SZ has the potential to change the ongoing system and bring about a revolution. It has been proving to show its beneficial effects on its application in the field of dentistry. Various studies have shown that even in low concentrations of AgNPs it is effective on opportunistic microorganisms. However, the dose must be regulated as it can be toxic in higher concentrations. In the coming years, SZ compounds with the correct study of physicochemical properties and toxicity analysis can increase their pharmacological use and market value.

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Conflict of interest
The authors declare they have no conflicts of interest.

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