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Original article

Antibiotic resistance and autolysin protein expression in oral bacteria under the treatment of *Stevia rebaudiana* extract loaded on iron oxide nanoparticles

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ABSTRACT

Background: The primary cause of dental caries is the existence and activity of bacterial organisms. In the current study, the antibacterial activity of green-synthesized iron nanoparticles (FeNPs) containing extracts from *Stevia rebaudiana* was investigated in relation to resistant isolates. The study aimed to determine the prevalence and antibiotic resistance of bacterial strains isolated from subjects. The bacterial resistance pattern was evaluated in 50 patients (age group 18 to 50) in this cross-sectional descriptive study; 35 of the patients were male and 15 were female. Disk diffusion and planktonic assays were used to evaluate the antibiotic resistance of bacterial isolates. The green-synthesized FeNPs have a cubical and polydispersed size range of 40-80 nm. The isolates showed a high prevalence of *Streptococcus spp.* (33.1%), followed by *Staphylococcus spp.* (21.1%) and *Klebsiella spp.* (5.0%), with *Streptococcus mutans* being the predominant species. *S. mutans* and *S. aureus* showed the highest multidrug resistance (MDR) rates, while *S. oralis, S. salivarius*, and *Klebsiella* showed low resistance against multiple antimicrobial agents. The resistance patterns of *S. mutans*, *S. aureus*, and *Klebsiella* to FeNPs and the positive control varied slightly. *S. aureus* showed constant resistance patterns, while *Klebsiella* showed slightly different resistance patterns. Positive control also showed changes at the 9th passage. We found that half of the cells treated with FeNPs were lysed in 50 minutes, compared to other lytic detergents like sodium dodecyl sulphate (SDS) and Triton X-100. Since antibiotic resistance results in treatment failure when antibiotic therapy is required, antibiotic resistance has grown to be a serious problem. It was discovered that the artificial FeNPs controlled the isolates' autolytic and biofilm behaviours, indicating a possible therapeutic application for them in the management of dental plaque and caries.

Introduction

Dental caries is a common biofilm-infectious illness that has significant worldwide health implications and high financial costs. A change in the microbiological balance of dental plaque biofilms may result in higher concentrations of bacteria that produce acid and can withstand acid, such as strains of *Streptococci.* This may lower the pH of the plaque, which could lead to caries and tooth demineralization [1].

Because dental caries is common and has such a big socioeconomic impact, it is one of the most common

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oral infectious diseases worldwide. According to N. B. Pitts *et al*, 2017 [2], dental cavities arise due to an ecological imbalance between tooth minerals and biofilm. Dental caries, a biofilm-induced disease, can afflict people of any age. It is strongly correlated with and impacted by the patient's eating habits, particularly their frequent consumption of sweet foods. These elements encourage microbial habitation in the built-up dental plaque, which starts the dental caries infection process over time [3]. Oral biofilms are composed of many microorganisms (*S.mutans, S.oralis, S.salivarius*) that live in the oral cavity. If oral biofilm is left untreated for an extended period of time, it can mature and cause the development of dental caries [4].

Streptococcus mutans is thought to be crucial in the formation of intricate, multifaceted structures on dental enamel and oral mucosa. Compared to their planktonic counterparts, cells attached to biofilms exhibit greater resistance to standard antibiotics. Strong barrier molecules known as bacterial extracellular polymeric substances (EPS) affect the speed of transit to the deep biofilm layer. Variations in the environment pose a constant threat to the bacteria in oral biofilm [4].

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Multidrug-resistant bacteria are a major risk to public health as they are the sole source of many infectious illnesses, which are often fatal. Most of the bacterial strains that are resistant to treatments are rising due to pollution, mutations, and overuse of medications. Biologists are screening novel medications to treat such infections in an effort to solve the problem. It has been discovered that these multidrug-resistant bacterial strains can be effectively controlled by green-produced metallic nanoparticles [8].

Globally, stevioside, a natural herbal sweetener derived from *Stevia rebaudiana*, popularly called sugar leaf or sweet tulsi, is becoming more and more well-liked [9]. Stevioside and Rebaudioside A exhibit antihyperglycemic, antihypertensive, anti-inflammatory, anticancer, antidiarrheal, diuretic, and immunomodulatory properties in addition to their sweetness [10]. In vitro research has demonstrated the antibacterial activity of several *S. rebaudiana* leaf extracts against microorganisms implicated in tooth caries and oral health. In an in vitro investigation [11], stevioside was found to show noncarcinogenic potential. The antibacterial action of *stevia* against *Lactobacillus acidophilus* and *Streptococcus mutans* was demonstrated [12] using solvents. The effect of aqueous extracts of *Stevia* (*Stevia rebaudiana*) against *Streptococcus mutans* and *Lactobacillus acidophilus* in comparison to chlorhexidine was studied [13].

Vandana *et al.* (14) have reported on the antiplaque and anti-gingivitis properties of *Stevia*. In addition, the antimicrobial properties of stevia inhibit biofilm formation by cariogenic bacteria has shown potential therapeutic applications in controlling dental biofilm and caries. It was also evaluated for its effective use in dental care products like toothpastes. A study was done [15] to conclude the effect of 0.5% aqueous extracts of *Stevia* in comparison to a standard agent (chlorhexidine) in limiting the number of oral bacteria (*S. mutans* and *Lactobacilli*) [15].

The current study was carried out to determine the efficacy of green synthetic iron nanoparticles generated from *Stevia rebaudiana* extract as anti-bacterial agents in generating dental caries and to evaluate their antibacterial resistance pattern, because there aren't many epidemiological

and dental studies in this area. According to the study's hypothesis, the oral biofilm represents a varied source of antibiotic resistance. Thus, the goal of this study was to determine how frequent multidrug resistance is and to treat it with green synthetic iron nanoparticles generated from *Stevia rebaudiana*.

Methods:

Study design: In this cross-sectional descriptive study, the bacterial resistance pattern was assessed in 50 patients (age group 18 to 50), comprising 35 males and 15 females, who visited Educational Clinics at the College of Dentistry/Wasit University between April 2023 and October 2023 to receive an oral health examination. Every patient who visited the dentistry clinic was counted as a population source. The study covered all individuals suspected of having dental caries, and the study's exclusion criteria included history of antibiotic use, refusal to participate in the trial, and a history of acquired or congenital immunodeficiency. Wasit University/College of Dentistry's local ethics committee accepted this study, and each participant gave their informed consent before taking part. Disposable cotton swabs were used to collect the dental caries samples and were transferred to the laboratory and stored in a refrigerator [16].

Bacterial isolation and identification:

A sterile cotton swab was used to collect dental caries samples, which were then cultured on different plates containing Mitis Salivarius agar and incubated at 37°C for 24 hours [17]. Different biochemical assays were carried out to identify bacterial strains following Gram staining and microscopy. Various biochemical analyses were conducted to identify the species.

Materials:

Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) used for nanoparticle synthesis was procured from Qualigens, India (purity 99.5%). The healthy plant leaves of Stevia were collected from the Botanical Garden Institute, Bangalore, Karnataka, and gifted to us. All of the compounds were used without further purification because they were of analytical grade.

Antimicrobial Susceptibility Pattern:

The antibiotic disc diffusion experiment was used to determine the patterns of susceptibility. In a nutshell, test cultures were swabbed onto Mitis salivarius agar plates, the antibiotic disk was put on the plates, and the plates were incubated for 24 hours at 37°C. The isolate's turbidity was calibrated to 0.5 McFarland standards. Antibiotic disks of tetracycline (30µg/ml), doxycycline (30µg/ml), erythromycin (15µg/ml), clindamycin (10µg/ml), penicillin (10µg/ml), gentamicin (10µg/ml), chloramphenicol (30μg/ml) and ciprofloxacin (5µg/ml) were used for the study. Zone of inhibition was assessed following incubation, and the results were labelled as resistant (R), intermediate (I), or sensitive (S) [18]. In accordance with criteria provided by the Clinical and Laboratory Standards Institute (CLSI) [19], the observed data were interpreted. Acquired resistance to at least one agent in three or more antimicrobial groups was the definition of multidrug resistance (MDR) [20].

Extraction and Green synthesis:

The leaves of the healthy plants were brought to the lab, where they were cleaned with distilled water and 1% sodium hypochlorite to get rid of any debris. They were then wiped, dried for eight days in the shade, and pulverized. After combining 10g of the leaf powder with 100mL of distilled water, the mixture was incubated for 8h at 140rpm in a shaker incubator. After extraction, the mixture was filtered, and the resulting filtrate was utilized to prepare nanoparticles. About 50ml of the filtrate was added to an equal amount of 3mM $FeCl_3.6H_2O$ and heated to 100°C for 30min. There was a noticeable colour shift from green to brown, signifying the creation of nanoparticles. To get rid of any remaining salts, the dense black precipitate Fe3O4-NPs was centrifuged for 10 minutes at 8000rpm after being cleaned with 15mL deionized water.

They took out the supernatant was collected. Centrifugation was used after another wash of the product with 10mL of deionized water. Once more, the supernatant was extracted. After transferring the pellet to a vial, 10ml of deionized water was added. The resulting black powder was employed for additional characterizations after an overnight freeze-drying process [21].

Characterization:

To verify the generation of FeNPs, UV-visible spectrum analysis was measured in an aqueous solution using a UV-VIS spectrophotometer (Shimadzu, 1800) with a resolution of 1nm between 200 and 600nm. A Perkin Elmer Spectrum One Fourier-transform infrared spectroscopy (FTIR) spectrophotometer (Bomem MB100) with a 3600-400cm^{-1} range was

used to gather Fourier transform infrared (FTIR) spectra using the KBr pellet method. Using FESEM-EDX (SEM; Philips XL30ESEM), the surface morphology and atomic ratio of the FeNPs were ascertained [22].

Dynamic light scattering (DLS):

The hydrodynamic size (Z average), surface charge (zeta potential), and polydispersity index (PDI) of the environmentally synthesized FeNPs were screened utilizing the DLS method in Kyoto, Japan, employing a Horiba SZ-100 analyzer. The analysis of particle size was done at a scattering angle of 90° and a medium count rate of 210kCPS [22].

Antibacterial evaluation:

Disc diffusion assay:

In order to evaluate the produced FeNPs' antibacterial activity, an Agar Disk diffusion experiment was conducted. Before being used, 6mm disks made from Whatman filter paper were autoclaved. Since *S.mutans* and *S. aureus* exhibited significant MDR frequencies in the samples, their antibacterial activity was the only aspect of their research. The test strains' overnight cultures were used to create bacterial suspensions (*S. mutans* and *S. aureus*), which were then disseminated using sterile swabs on the surface of Luria Bertani (LB) agar plates after being adjusted to a turbidity of 0.5 McFarland standards [23]. On the dried plate, about five sterile filter paper disks and the antibiotic disks to which the strain was resistant were inserted. Discs dipped in sterile distilled water are used as a negative control. Approximately 20µl of FeNPs were introduced onto the treatment-labelled disc. Plates were inspected, and antibacterial activity was measured by measuring the widths of the inhibition zones surrounding the disks following a 20-hour incubation period at 37°C. Any production of a growth inhibition halo larger than 6mm (disk size) was taken into consideration for antibacterial effectiveness. For every strain, the experiment was run in triplicate.

Broth dilution assay:

The MIC was ascertained by the serial two-fold microdilution method, which was employed in lieu of an earlier method [24] but with slight modifications. The minimum inhibitory concentration, or MIC, is the lowest antibiotic concentration that stops observable planktonic bacterial cell growth. About 180µg/ml of LB broth was added to all the wells and inoculated with 10µl of overnight culture diluted to $1x10^5$CFU/ml. About 10µl of sterile distilled water was added to the negative control well. About 10µl of respective positive controls were used in their labelled wells. To the well-labelled treatment, 10µl of FeNPs of varying concentrations (5, 10, 20, 40, 80µg/ml) were added. Following two days of incubation at 37°C, the MIC was ascertained: The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no bacterial growth was observable. Every test was conducted three times in duplicate.

Planktonic bacterial drug resistance assay:

About 10 cycles of ½MIC measurements were carried out to look into the drug resistance of planktonic bacteria that the antibacterial drugs had generated [1]. A volume of approximately 100µl of the bacterial suspension from the sub-MIC well was collected and injected overnight at 37°C into 10ml of fresh medium. For the subsequent MIC test, the overnight bacterial suspension was then diluted to a concentration of roughly 10^5 CFU/mL. This was the first channel through which the bacteria were exposed to FeNPs. Next, 10ml of fresh medium was injected with 100µL of the FeNPs-exposed bacterial suspension from the ½MIC well. The culture was then incubated overnight at 37°C to allow the bacteria to grow without any treatment (FeNPs).

The bacteria that made it through the first passage's FeNPs exposure in this manner grew to roughly 10^5 CFU/mL throughout the next night without being exposed to FeNPs. After that, the remaining bacterial solution was diluted to around 10^5CFU/mL and used in passage 2's MIC measurement after being exposed to FeNPs. Ten of these passages were subjected to several MIC tests. Ten passages took roughly a month to finish. Ten passes were conducted, each utilizing surviving bacteria that had been exposed to FeNPs in order to look at drug resistance after a comparatively long exposure to the antibacterial agent. Any rise in MIC with an increase in passage number following such frequent exposures to the antibacterial agent would be indicative of bacterial drug resistance [25]. Every bacterial species was examined separately, and the same methodology was used for all three species' testing. Three duplicates of the tests were run.

Bacterial Response to Autolysin-Inducing Agents:

All four bacterial cultures were cultured in LB medium and treated with 0.0125% (V/V) Triton

X-100, 0.01% (w/V) SDS, and 0.01% (w/V). For five hours, the cell solution was incubated further at 200 rpm and 37°C. Every thirty minutes, the OD650 was measured. The respective antibiotic was used as a positive control, which is known to cause cell lysis [6]. Using the recorded drop in OD650 following treatment with several detergents, the rate of lysis was computed to ascertain the effect of each detergent on the strain of bacteria.

Statistical analysis: With IBM's (USA) Statistical Package for Social Sciences (SPSS 24.0) software, the gathered data were input, purified, and examined. Frequency, percentage, mean, and standard deviation were used in the computation of descriptive statistical analysis.

Results:

DLS and UV analysis:

Using the DLS, the size distribution of the generated iron nanoparticles was ascertained at 45°C, pH 9, and $FeCl_3.6H_2O$ (3mM) under controlled conditions. The observed zeta potential of -185mV validates the stability of the produced nanoparticle. Scanners with an electron microscope were also used to examine the dimensions and form of the generated FeNPs. The majority of the FeNPs were dispersed and roughly spherical in shape. The size distribution of the particles was 40–85nm **(Figure 4)**.

The process of creating iron oxide nanoparticles using *Stevia* leaf extracts was initially verified by observing a distinct shift in colour from the extract solution to a dark brown solution upon the addition of the iron salt solution **[Figure 1]**. For the Fe3O4 NPs made with *Stevia* leaves, the green synthesized NPs' spectra showed a distinctive peak surface plasmon resonance (SPR) band at about 238 nm. The pure *Stevia* extract's UV-visible spectrum was captured and is shown in **Figure 1** for comparison. **Figure 1** illustrates that the absorption peaks for *Stevia* extracts are around 220 nm, 300 nm, and 365nm, indicating the presence of many natural chemicals in the extract. Following a reaction with an iron salt, these peaks disappeared, suggesting that the extract chemicals had a lowering effect. The size distribution of FeNPs **(Figure 2)** indicates that the generated nanoparticles had an average hydrodynamic size of approximately 55nm.

FTIR analysis:

Figure 3 displays the FTIR spectra **[Figure 3]** of the powdered water extract of *Stevia* leaves. Widespread and strong absorption at 3418cm^{-1} was linked to the presence of hydrogen bonds and correlated with the OH bond's stretching vibration, or -OH stretching. Stretching-CH sp3 bond was characterized by absorption at 2916cm^{-1}. Stretching the vibrational–C=O bond led to intense peaks at 1736 and 1597cm^{-1}. The -CH bond's bending vibration was detected at 1415 and 1385cm^{-1}. High intensity peaks at 1030 and 1065cm^{-1}, which are hallmark absorption bands of glycosidic bonds, are also related to C-O generated from steviol glycoside. Ultimately, it was determined that the peaks at 891cm^{-1} and 814cm^{-1} corresponded to the =CH and =CH2 bonds' respective bending vibrations [8].

Scanning Electron Microscopy (SEM):

The surface morphology of iron nanoparticles derived from synthetic *Stevia* was investigated using SEM. SEM analysis verified the synthesized Stevia iron nanoparticles' stabilized nanocube-like shape **(Figure 2 B)**. SEM analysis revealed that the green synthesised FeNPs were cubical and polydispersed, with a size range of 40-80nm (mean size = 55.4nm).

Distribution of bacterial strains:

The most common pathogen found among the samples was *Streptococcus* spp. (33.1%) which was followed by *Staphylococcus* spp. (21.1%) and *Klebsiella* spp. (5.0%). Among the *Streptococcus* spp, *Streptococcus mutans* was predominant, followed by *Streptococcus oralis* and *Streptococcus salivarius*. Among the *Staphylococcus*, *Staphylococcus aureus* was seen to be predominant.

Antimicrobial resistance:

The highest percentage of resistance to tetracycline (90%), ampicillin (78%), penicillin (70%), amoxicillin (70%), and erythromycin (60%) was present in *S. mutans* strains. The strains of *S. oralis* that were most resistant to tetracycline (95%), ampicillin (70%), penicillin (62%), and amoxicillin (50%) were identified. The strains of *S. salivarius* were found not to be resistant ($P<0.05$). The antimicrobial resistance of the tested bacterial agents to frequently used antimicrobial drugs in dentistry is displayed in **Table 1**. The largest percentages of resistance to tetracycline (92%), penicillin (80%), ampicillin (70%), amoxicillin (54%), and erythromycin (50%) were found in *S. aureus* strains. The strains of *Klebsiella* that were most resistant to Amoxicillin (63%), ampicillin (57%), erythromycin (50%), and tetracycline (85%) were identified. A statistically significant difference

(P <0.05) was found between the prevalence of antibiotic resistance and the kind of bacteria.

Multi-drug resistance distribution:

MRD strains in the tested samples were displayed in **Figure 4.** MDR strains were defined as those that exhibited at least three antimicrobial agent resistance concurrently. The highest MDR rate was seen in *S. mutans* bacteria, followed by *S. aureus* with 67 and 38% respectively (P<0.05). *S. oralis*, *S. salivarius*, and *Klebsiella* bacteria isolated from dental caries cases showed low degrees of resistance against more than three antimicrobial agents, with 12, 8, and 5% respectively.

Broth dilution assay/MIC:

The green synthesized FeNPs' minimum inhibitory concentrations (MIC values) against the four tested microorganisms showed potent antibacterial action. The MIC values for *S. mutans*, *S. oralis*, *S. aureus*, and *Klebsiella* were determined to be 82.4, 42.5, 93.4, and 45µg/ml, respectively. Strong antibacterial activity against the three tested microorganisms is confirmed by our MIC data **[Figure 5]**.

Planktonic bacterial drug resistance assay:

Following exposure to FeNPs and respective positive controls, the serial MICs of planktonic strains were determined for 10 passes. The MICs were 82.4, 42.5, 93.4, and 45µg/ml, respectively, for *S. mutans*, *S. oralis*, *S. aureus*, and *Klebsiella*. In case of *S. mutans*, the resistance patterns seem to slightly vary in both positive and FeNPs. The MIC value 82.4µg/ml changed to 96.8 and then 98 at the 9th and 10th passages, respectively (P<0.05). Positive control also seemed to change at the 10th passage from 35.6 to 38. The MIC values remained constant for *S. oralis*, suggesting that *S. oralis* did not acquire resistance against both FeNPs and the Positive control (P<0.05). In case of *S. aureus*, the resistance patterns seem to be constant for FeNPs, but a slight change was seen with positive control at the 9th and 10th passage. The positive control MIC value 38.5µg/ml changed to 42.5 and then 43 at the 9th and 10th passage, respectively (P<0.05). In case of *Klebsiella*, the resistance patterns seem to slightly vary in both positive and FeNPs. The MIC value 45µg/ml changed to 47.8, 47.8, and 48 at the 8th, 9^{th,} and 10th passages, respectively (P<0.05). Positive control also seemed to change at the 9th passage from 18.4 to 23.4 (p<0.05) **[Figure 6]**.

Bacterial Response to Autolysin Autolysin-Inducing Agents:

About half of the cells that were treated with FeNPs were found to be lysed in 50min. In case of *S. mutans*, the lytic rate was found to be 0.28 ± 0.03/h which is significant when compared to the positive control (0.39 ± 0.01/h). This rate was quite effective when compared to other lytic detergents as well like SDS (0.21 ± 0.05/h) and Triton X 100 (0.24 ± 0.05/h). In case of *S. oralis*, the lytic rate was lower and found to be 0.17 ± 0.03/h which is less significant when compared to the positive control (0.28 ± 0.03/h). This rate was less effective when compared to other lytic detergents like SDS (0.2 ± 0.01/h) and Triton X 100 (0.23 ± 0.05/h). In case of *S. aureus*, the lytic rate was found to be 0.34 ± 0.02/h which is highly significant when compared to the positive control (0.42 ± 0.01/h). This rate was more effective when compared to other lytic detergents like SDS (0.15 ± 0.02/h) and Triton X 100 (0.18 ± 0.02/h). On the other hand, *Klebsiella* showed a lytic rate of 0.31 ± 0.02/h which is significant when compared to the positive control (0.45 ± 0.03/h). This rate was again effective when compared to other lytic detergents like SDS (0.21 ± 0.02/h) and Triton X 100 (0.21 ± 0.03/h) **[Figure 7]**.

Table 1. Table showing the antibacterial susceptibility resistance patterns of the isolates collected at the sample site.

Antibiotic	**Bacteria Isolated** (N)				
	S. mutans (22)	***S. oralis*** (10)	***S. salivarius*** (5)	***S. aureus*** (14)	***Klebsiella*** (6)
Tetracycline	20 (90)	10 (95)	-	13 (92)	5 (85)
Ampicillin	17 (78)	7 (70)	-	10 (70)	3 (57)
Penicillin	15 (70)	6 (62)	-	11 (80)	0
Amoxyllin	15 (70)	5 (50)	-	8 (54)	4 (63)
Erythromycin	13 (60)	0	-	7 (50)	3 (50)

Figure 1. UV-vis spectra of the green synthesized FeNPs from *Stevia* leaf extract.

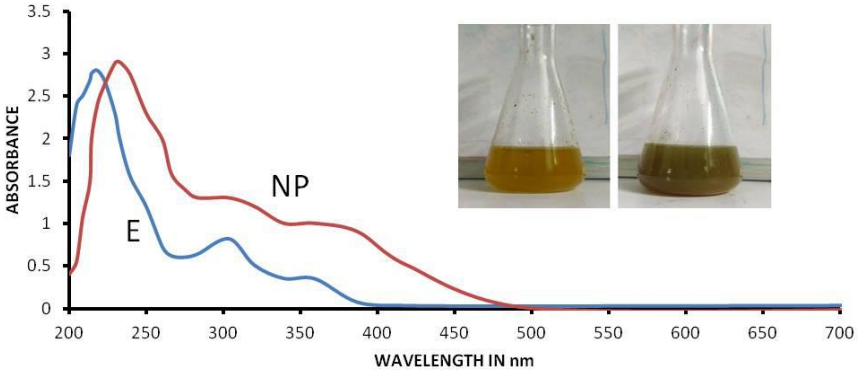


Figure 2. A: Nano particle tracking analysis (NTA) analysis graphs the distribution of FeNPs formed at varying sizes from *Stevia* leaf extract. **B:** SEM image showing the nanoparticle size distribution.

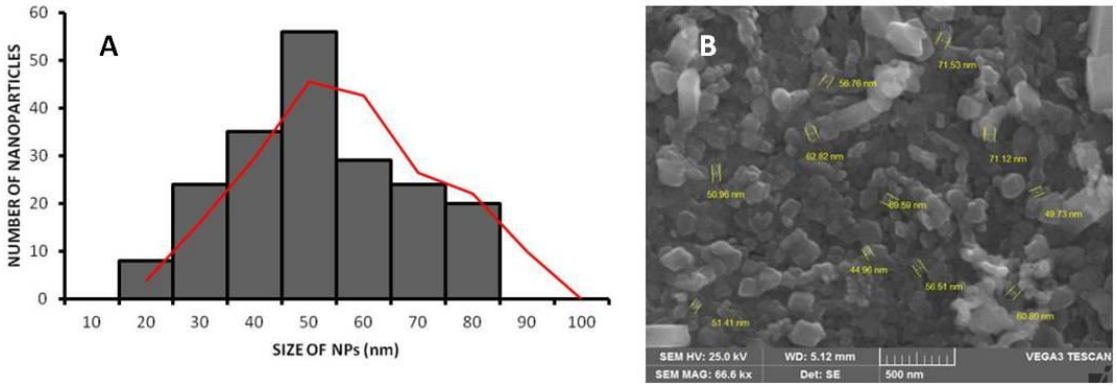


Figure 3. FTIR spectra obtained with Stevia leaf extract and with iron NPs. A: *Stevia* leaf extract; B: Green synthesized FeNPs.

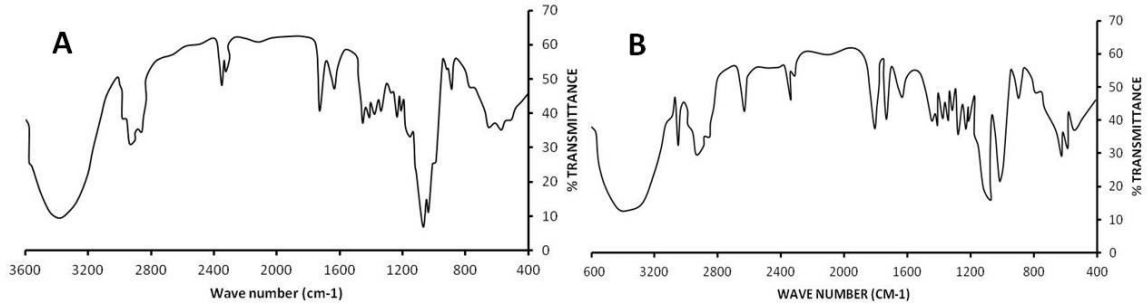


Figure 4. Graph showing the MDR distribution of the isolates.

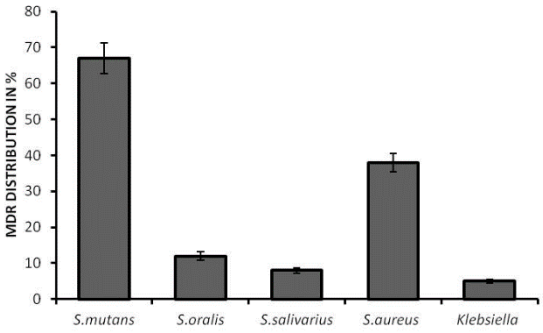


Figure 5. Broth dilution. Graph showing the %inhibition in the growth of the respective cultures under the treatment with their respective susceptible antibiotics. **A:** *S. mutans*; **B:** *S.oralis*; **C:** *S. aureus,* and **D:** *Klebsiella.*

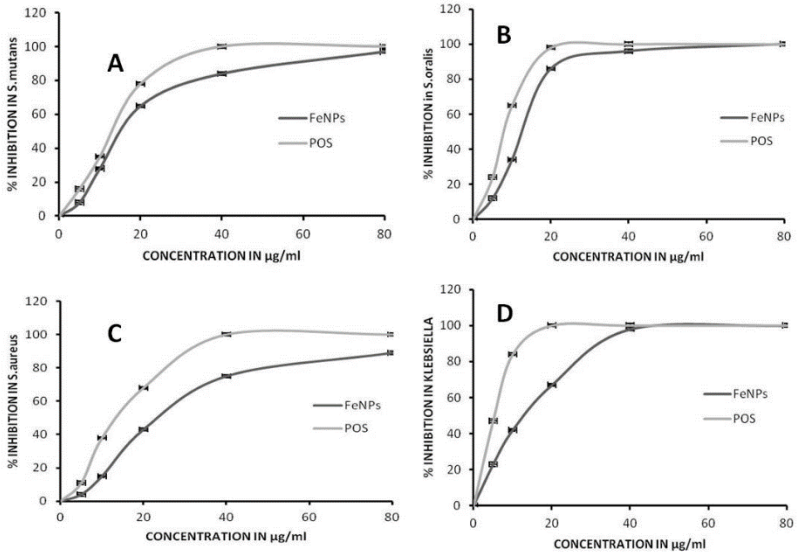


Figure 6. Bacterial drug resistance assay. MIC was measured from passages 0 to 10. Erythromycin was used as a positive control for *S. mutans* and *S. oralis*. Ampicillin was used as a positive control for *S. aureus* and *Klebsiella*. **A:** *S. mutans;* **B:** *S. oralis,* **C:** *S. aureus, and* **D:** *Klebsiella.*

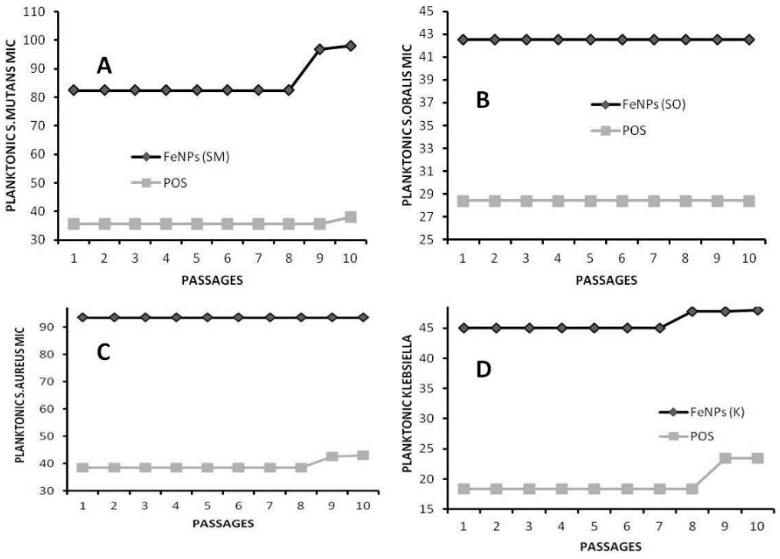


Figure 6. Plates showing the Planktonic drug resistance assay. SM: *S.mutans*; SA: *S.aureus*; K: *Klebsiella*; P: Positive control; C: Control; N: Nanoparticles.

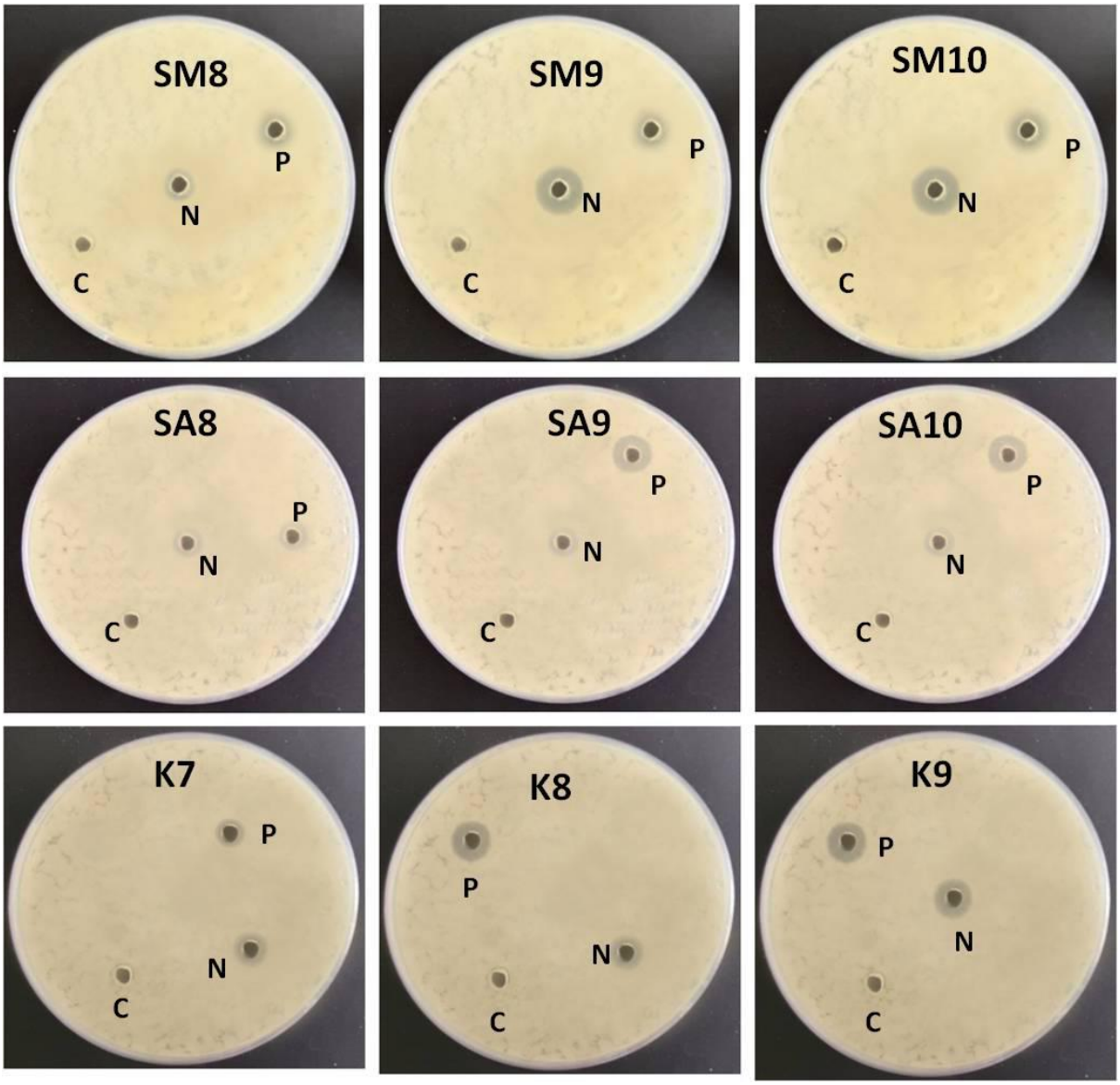
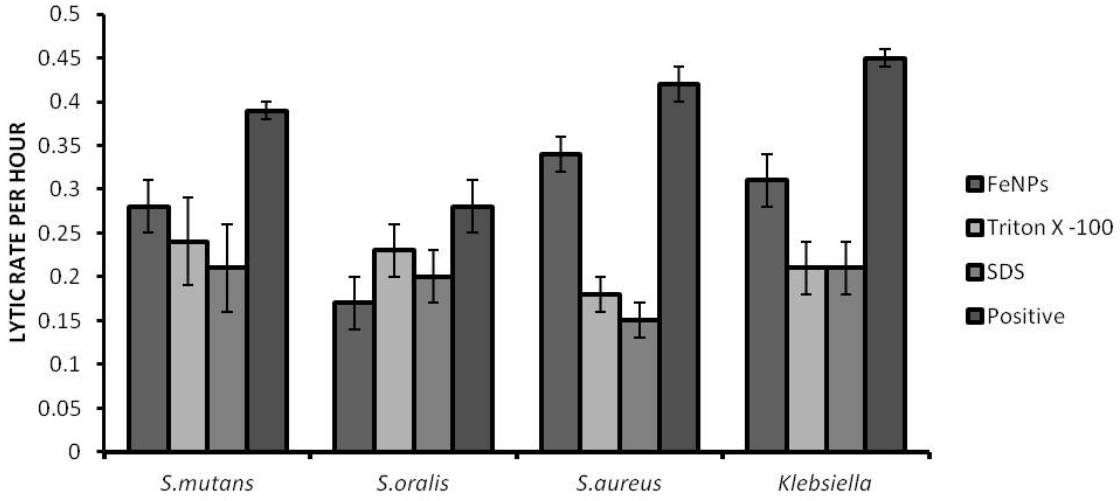


Figure 7. Autolytic rates: Graph showing the autolytic rates of the bacterial strains along with autolytic detergents and respective positive controls.



Discussion

Because dental caries is an ongoing illness that progresses slowly in most people and affects everyone in the world at some point in their lives, the difference may be the result of rising patterns of caries patients. This study, however, was not as high as research done [28], which reveals a prevalence of almost 62.5%. Various factors, including the study setting, sample size, sociocultural disparities, and attitude, could be the cause of these variances. Gram-positive bacteria, such as *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus mitis*, and *Streptococcus salivarius*, are the predominant microorganisms in supragingival plaque [29].

In line with this study, several investigations [30] found that *S. aureus* and *S. mutans* were the main bacterial agents causing dental caries and various associated infections of the cavity. Nevertheless, some surveys emphasized the contribution of *E. coli*, *E. aerogenes*, and other streptococcal species to the development of dental caries, dental plaques in the oral cavity [12]. According to [31], there were 53.40% and 39.70% of *S. aureus* and *S. mutans*, respectively, in the dental caries samples. *S. aureus*, *S. mutans*, and *E. coli* were distributed throughout dental plaques at total percentages of 15%, 19%, and 10%, respectively [32].

In our findings, *S. mutans* bacteria exhibited a high resistance rate to tetracycline (90%), ampicillin (78%), penicillin (70%), amoxicillin (70%), and erythromycin (60%). The *S. oralis* strains that showed more resistance to ampicillin (70%), tetracycline (95%), penicillin (62%), and amoxicillin (50%) were found. High resistance to tetracycline, ampicillin, penicillin, amoxicillin, and erythromycin was present in the isolates. Additionally, a substantial percentage of gentamicin resistance was reported in Gram-negative isolates [33]. Reports [34] also found that both Gram-positive and Gram-negative bacteria isolated from dental caries had high resistance to tetracycline, penicillin, ampicillin, amoxicillin, gentamicin, and erythromycin. According to [35], isolates of *S. mutans* showed excellent sensitivity to ciprofloxacin (71.1%) and amoxicillin (86.6%) but resistance to penicillin (82.2%). The results demonstrated that the *S. oralis* strains exhibited 100% resistance to imperium and 100% sensitivity to gentamycin, ciprofloxacin, cefotaxime, and amoxicillin.

About 20% of the more than 700 distinct bacterial species that make up the complex microbial community that constitutes the human mouth biofilm are oral *Streptococci*. Newborns' oral cavities are colonized by *Streptococcus mutans*, which is also present in the adult oral early microbial population. This bacterium becomes resistant to drugs like erythromycin and tetracycline [36,37,38]. The current study examined three oral species associated with caries: *S. mutans*, *S. oralis*, *S. aureus*, and *Klebsiella*. These bacteria were exposed to FeNPs for 10 passages, and the results showed that different FeNP minimum inhibitory concentrations could successfully suppress bacterial growth without causing the build-up of resistant bacteria.

Passage assay was performed on Planktonic forms, under the treatment of FeNPs and corresponding positive controls, and the serial MICs were measured for ten passes. Regarding *S. mutans*, there appears to be a small variation in the resistance patterns between positive and FeNPs. At the ninth and tenth passages, respectively, the MIC value of 82.4µg/ml changed to 96.8 and then 98µg/ml ($P<0.05$). At the tenth passage, positive control also appeared to shift from 35.6 to 38µg/ml. *S. oralis's* MIC values did not change, indicating that the bacteria did not develop resistance to FeNPs or the positive control ($P<0.05$). Regarding *S. aureus*, the resistance patterns appear to remain consistent for FeNPs; nevertheless, a minor variation was seen with positive control during the ninth and tenth passages. At the ninth and tenth passages, respectively, the positive control's MIC value of 38.5µg/ml changed to 42.5 and subsequently 43µg/ml ($P<0.05$). Regarding Klebsiella, there appears to be a small variation in the resistance patterns for both positive and FeNPs. At the eighth, ninth, and tenth passages, the MIC value of 45µg/ml changed to 47.8, 47.8, and 48µg/ml, respectively ($P<0.05$). Additionally, at the ninth passage, positive control appeared to shift from 18.4 to 23.4 ($p<0.05$).

The findings of this investigation, however, were derived from MIC concentrations of FeNPs; additional research is required to verify whether or not the conclusion that FeNPs do not lead to drug resistance in bacteria remains valid when employing varying MIC concentrations of FeNPs.

However, controversial results were seen with a similar type of experiment [1]. Despite the same repeated exposures, different oral bacteria species showed distinct drug resistance qualities. For example, *S. gordonii* developed resistance to antibacterial agents, but *S. mutans* and *S. sanguinis* showed no resistance. But our FeNPs did show potent susceptibility when compared to their respective positive control.

In this case, the makeup of the bacterial wall might not be the sole factor influencing the activity of NPs. The reason for FeNPs' inhibitory effect could possibly be their nanometric size, making them easily stick to the bacterial membrane and penetrate the cellular content. FeNPs can disrupt the bacterial membrane's structure and enhance its permeability as a result. In addition to disrupting cellular functions like respiration and permeability, FeNPs' small size actually makes it feasible for them to adhere to walls and interact with cell membranes, which ultimately leads to cell death. It has been demonstrated that AgNPs bind to DNA and RNA, denaturing the molecules. Thus, the bacterial replication process is halted.

There are further restrictions on the current investigation. Initially, the trials were run simultaneously in triplicate. Additionally, we must schedule the same study for various times. Second, other Gram-negative strains as well as other Gram-positive strains were not evaluated, except for oral *Streptococcal* species. In order to confirm the drug resistance results and explore Gram-negative strains and other Gram-positive strains that are significant in dentistry, more research is required. This research should be repeated on different days. All things considered, this study is among the first to identify the pathogenic bacteria causing tooth cavities as having antibiotic resistance from the Wasit University premises. The few isolated bacteria, the paucity of demographic information about the population under study, and the inability to ascertain the patients' medical histories of gastrointestinal diseases are the only limitations of the findings.

Conclusion:

In this work, the effective synthesis of iron oxide nanoparticles using *Stevia rebaudiana* extract in a single pot using a green technique was confirmed. We studied the percent inhibition via MIC and the antibacterial drug resistance of *S. mutans, S. oralis, S. aureus*, and *Klebsiella.* The MICs varied slightly for *S. mutans, S. oralis, S. aureus*, and *Klebsiella*. *S. mutans* showed slightly different resistance patterns, while *S. oralis* did not acquire resistance. *S. aureus* showed constant resistance patterns, while *Klebsiella* showed slightly different resistance patterns. Finally, the present revealed strong antibacterial activity of FeNPs produced from *Stevia rebaudiana* extract against isolates that are widely prevalent in the study population. The strains exhibited little to no resistance to these FeNPs, which demonstrated their effectiveness as an inducer of autolysis.

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