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Antibacterial Effect of Silver Nanoparticles *Versus* Chlorhexidine Against *Streptococcus mutans* and *Lactobacillus casei*

Raul Alberto Morales Luckie,
Rafael Lopez Casatañares, Rogelio Schougall,
Saraí Carmina Guadarrama Reyes and
Víctor Sanchez Mendieta

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Abstract

The purpose of the study was to evaluate the antibacterial effect of silver nanoparticles (Ag-NPs) *versus* chlorhexidine (CHX) against *Streptococcus mutans* and *Lactobacillus casei*. Three different reducing agents were used for the synthesis and characterization of Ag-NPs: sodium borohydride (NaBH₄), a chemical method, and *Heterotheca inuloides* (Hi) and *Camellia sinensis* (Cs), two eco-friendly methods. The synthesized substance was deposited on deciduous teeth. Its behavior in dental tissues was evaluated through an energy dispersive X-ray spectroscopy (EDS) analysis, using a scanning electron microscope (SEM). The characterization of Ag-NPs in terms of shape, size, and polydispersity was performed through spectrophotometry of ultraviolet-visible light analysis (UV-vis), as well as by transmission electron microscopy. Isolation and culture of strains *S. mutans* and *L. casei* were done to perform the microbiological analysis.

In Petri dishes, paper discs containing different concentrations of Ag-NPs (synthesized by Hi, and by Cs) were deposited and tested along with paper discs containing CHX. Their antibacterial effect against both bacteria was evaluated by the inhibition zones test. By means of UV-Vis and TEM analysis, it was possible to observe that *Heterotheca inuloides* produced smaller and more stable nanoparticles, also in greater quantities (17.5 nm), when compared to *Camellia sinensis*. EDS analysis through SEM showed a 6.25 average absorption of silver in dental tissues. The microbiological analysis revealed a greater zone of inhibition when the test bacteria were in contact with 20 µl of Ag-NPs, synthesized by Hi, being statistically significant ($p < 0.05$), compared to the growth inhibition zones produced by Cs, and CHX against both strains. We can conclude that eco-friendly methods produced Ag-NPs with an important antibacterial effect in both strains.

Keywords: silver nanoparticles, chlorhexidine, *Streptococcus mutans*, *Lactobacillus casei*, eco-friendly chemistry

1. Introduction

While *Streptococcus mutans* plays an important role in the initiation of dental caries, *Lactobacillus casei*, on the other hand, is considered an important secondary invader; both microorganisms are actively involved in the process of tooth decay [1–5]. Dental practitioners recognize that chlorhexidine has been the gold standard for more than 3 decades [6–8]; it is considered a potent agent against *S. mutans*. Nevertheless, *L. casei*, which dominates among oral Lactobacilli, is relatively resistant [9]. The main concern about its use is its limited period of substantivity (which is the ability of chlorhexidine to bind to tissues and release slowly for a longer time), and some reported cytotoxicity cases [10, 11].

Therefore, we consider it important to find substances with a potent antibacterial effect but with a minor impact on human health and the environment [12].

In recent years, nanotechnology has become an important discipline in the field of biology [13]. A considerable achievement is the ability to form atoms and molecules to further form new structures (*id est.*, one-billion times smaller than what can be observed with the naked eye). Therefore, the new materials and devices can be developed with high atomic precision. Nanoscience involves the use of nanoparticles in a range between 1 and 100 nm to obtain unique and improved properties [14–20]. Nanomedicine is an extremely useful tool because most of the body's natural processes occur at an almost imperceptible level [21, 22].

Due to the aforementioned reasons, the interest in the study and synthesis of nanoparticles has grown recently. Silver nanoparticles (Ag-NP) are broad spectrum microbicide agents widely used in health sciences [23]; they are nanostructured materials based on silver salts. Silver is currently being used to inhibit bacterial growth in a variety of applications, including dentistry [17, 24]. Efforts have been made to explore the properties of Ag-NPs, as an efficient way to provide stability and improve antibacterial effect is the reduction in size [25, 26]. It is known that Ag-NPs exert an antimicrobial effect on Gram+ and Gram– producing lysis in peptides of the membrane of microorganisms [27].

“Eco-friendly” chemistry seeks to reduce waste, and eliminate pollution and environmental damage; it promotes the creation of products that are environmentally and economically sustainable [12]. Different forms of syntheses have been sought, in which natural and renewable reducing agents are used during the chemical processes. The principles of green chemistry are oriented to the search for new ways of synthesizing substances, not only minimizing the costs but also the damage to human health and reducing environmental pollution, while taking advantage of the benefits and properties of plants such as *Heterotheca inuloides* (Hi) and *Camellia sinensis* (Cs), which have shown antimicrobial and inhibitory activity, as well as anti-oxidant and cytotoxic properties against oral bacteria [28, 29].

Mexican medicinal plants have enormous potential [30]. *Heterotheca inuloides* (Arnica) is a Mexican plant widely used due to its medicinal properties; it has shown anti-inflammatory and analgesic effect [31]. The plant grows abundantly in the Mexican region and has been used as part of folk medicine for the topical treatment of contusions, bruises as well as for the treatment of skin wounds and injuries [28, 32].

This medicinal herb has been reported to exhibit antimicrobial activity, cytotoxic, and anti-oxidative properties [33], which has led the World Health Organization (WHO) to recognize its use in medicine [34].

Several constituents of *H. inuloides* have been identified, mainly, flavonoids, sesquiterpenoids, triterpenoids, and sterols. The composition of the essential oil has also been described. Recently, four sesquiterpenoids of *H. inuloides* were identified as antimicrobial agents [35].

The dried flowers of *H. inuloides* have been used for the treatment of postoperative thrombophlebitis, and externally for acne, bruises, and muscle aches in Mexico. Previously, sesquiterpenoids, 7-hydroxy-3,4-dihydrocadalin and 7-hydroxycadalin, were characterized as antibacterial agents from the dried flower of *H. inuloides*. The flavonoids, quercetin, kaempferol, and their glycosides were also isolated from the same source and showed tyrosinase inhibitory activity [36].

2. Experimental details

2.1. Synthesis of silver nanoparticles

Ag-NPs were synthesized from silver nitrate salts with the use of sodium borohydride (NaBH_4), a conventional chemical reducing agent. Pursuing the same purpose, two eco-friendly agents, were separately used as green reducers: *Heterotheca inuloides* and *Camellia sinensis*.

For chemical synthesis, NaBH_4 (in a concentration of 1×10^{-2}) was weighed on an analytical balance (Explorer Pro, model EP213C, OHAUS, USA), and then dissolved in a flask with distilled water.

A 10 mM silver nitrate solution (AgNO_3 , Sigma-Aldrich) was prepared and mixed along with the NaBH_4 solution, in a 1:2.5 ratio to generate Ag-NPs.

The resulting solution was placed in a beaker on a heating grate (Thermo Scientific Cimatec). Using a magnetic stirrer, the incorporation of powder was achieved. The mixture was centrifuged with filter paper, allowing obtaining the smallest particles (i.e., nanoscale).

Water (H_2O) and alcohol (ETOH) were separately used as diluents.

The ecofriendly synthesis was performed by collecting dried flowers of Hi and Cs.

The leaves were mashed to a powder and mixed to obtain a homogeneous sample (both powders were used separately for the synthesis). One gram of each powder was immersed in

100 mL of distilled water; it then underwent a boiling process. Afterward, the solution was filtered through a filter paper.

2.2. Characterization of Ag-NPs

Following Ag-NPs formation, UV-vis analysis was carried out every hour, for the next 6 hours. UV-vis spectra measurements were recorded on a Cary 5000 UV-vis scanning spectrophotometer using quartz cells. The wavelength ranges from 300 to 600 nm.

To observe the size and shape of solutions, transmission electron microscopy (TEM) analysis was also carried out with a JEOL JEM-2100-Tokyo, Japan Microscope.

Scanning electron microscope (SEM) analysis was performed in a JSM-6510-LV microscope (JEOL) at 20 kV of acceleration, using secondary electrons.

The two eco-friendly substances were deposited in 20 deciduous teeth to analyze, through EDS (energy dispersive spectroscopy), the behavior of silver in dental tissues.

2.3. Microbiological analysis

Strains of *S. mutans* and *L. Casei* were isolated and cultivated in specific mediums for their growth (Gold *mitis salivarius* and Rogosa). Discs embedded with Ag-NPs synthesized by the two eco-friendly reducing agents, at different concentrations (10, 20, and 30 μ l), were placed in Petri dishes. Some discs were used as blank control, and others were embedded with 2% CHX (Consepsis, Ultradent products Inc). Petri dishes were incubated at 37°C for 48 hours. The inhibitory halos of each substance were measured in millimeters to compare the antibacterial effect at different doses.

3. Results

The synthesis with H₂O provided more stable Ag-NPs because the chemical polarity of a molecule of water is greater than that of ETOH, and through the boiling process (applying heat to the mixture), the active ingredients are extracted from the infusions. UV-vis analysis determined, through the formation of the plasmon (maximum peak where light is absorbed), that Ag-NPs synthesized by Hi showed more stable and smaller nanoparticles in greater quantities compared to Cs but bigger than NaBH₄, because this is a more drastic reducing agent (**Figure 1**).

UV-vis shows that the plasmon wavelength lies between 440 and 456 nm in Ag-NPs synthesized by *Heterotheca inuloides* (**Figure 1A**). While the plasmon wavelength lies between 355 and 448 nm in Ag-NPs synthesized by *Camellia sinensis* (**Figure 1B**); which, unambiguously, indicates the presence of silver nanoparticles under the specific conditions of each synthesis.

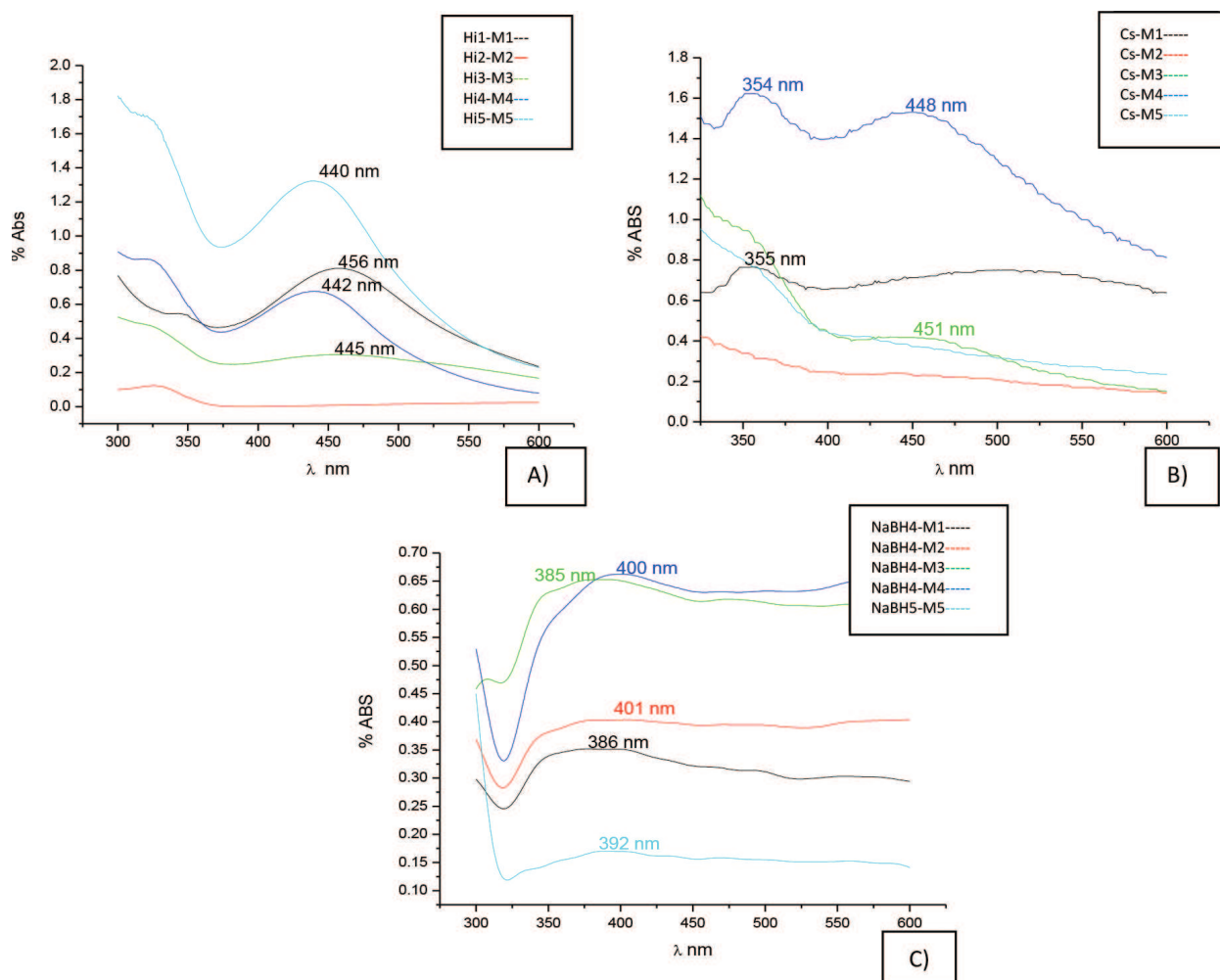


Figure 1. UV-visible spectroscopy analysis of Ag-Np synthesized by (A) *Heterotheca inuloides*, (B) *Camellia sinensis*, and (C) sodium borohydride.

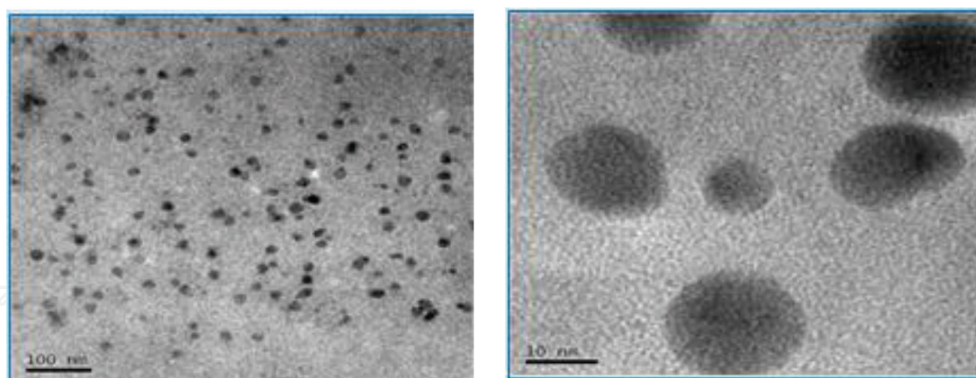
The plasmon wavelength lies between 385 and 401 nm in Ag-Np synthesized by sodium borohydride because this is a more drastic reducing agent (**Figure 1C**).

TEM allowed us to see that Hi showed smaller nanoparticles than *Camellia sinensis* while sodium borohydride were smaller (**Figure 2**).

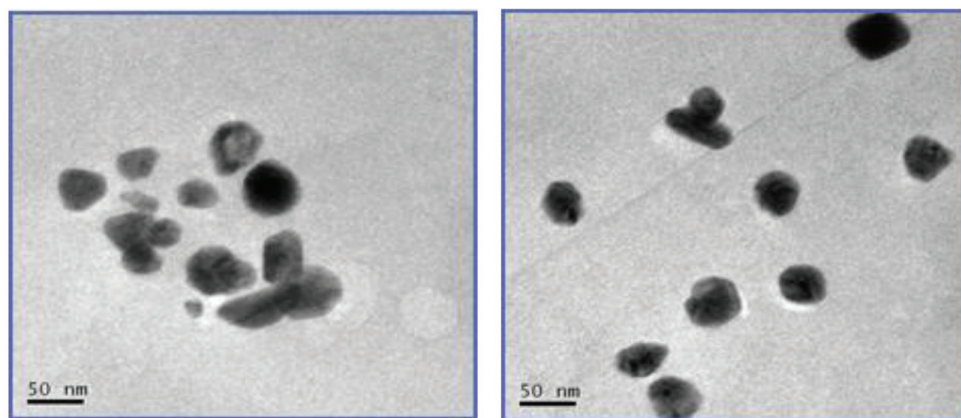
3.1. Characterization of Ag-Np

Assessment of Ag-NPs impregnated teeth was performed through EDS (energy dispersive spectroscopy) analysis. A 6.26 weight percent mean absorption of silver to dental tissues was found among the total percent, meaning that Ag-NPs are compatible with deciduous teeth. A statistical analysis at a confidence level of 95% was set (**Figure 3A and B**).

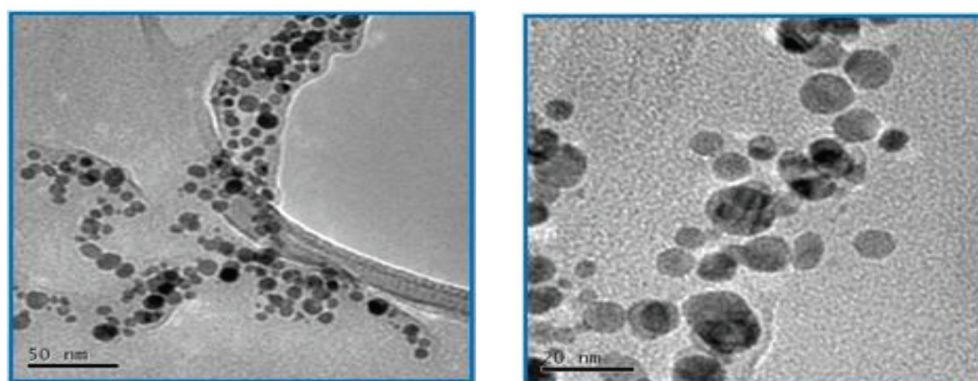
Elemental chemical analysis through EDS by SEM.



A) Ag-NPs synthesized by *Heterotheca inuloides*



B) Ag-NPs synthesized by *Camellia sinensis*



C) Ag-NPs synthesized by Sodium Borohydride

Figure 2. The size of silver nanoparticles synthesized by two eco-friendly reducing agents *Heterotheca inuloides* (17.5 nm) and *Camellia sinensis* (48.2 nm), and sodium borohydride (8 nm).

Student t test for unknown variances was used to establish the average inhibition of bacterial growth. A higher antibacterial effect of Ag-NPs synthesized with Hi, followed by Cs was observed, compared with CHX, particularly at the dose of 20 μ l. The effect on both bacteria was similar. The results were statistically significant to a confidence level of 95% (**Figure 4** and **Table 1**).

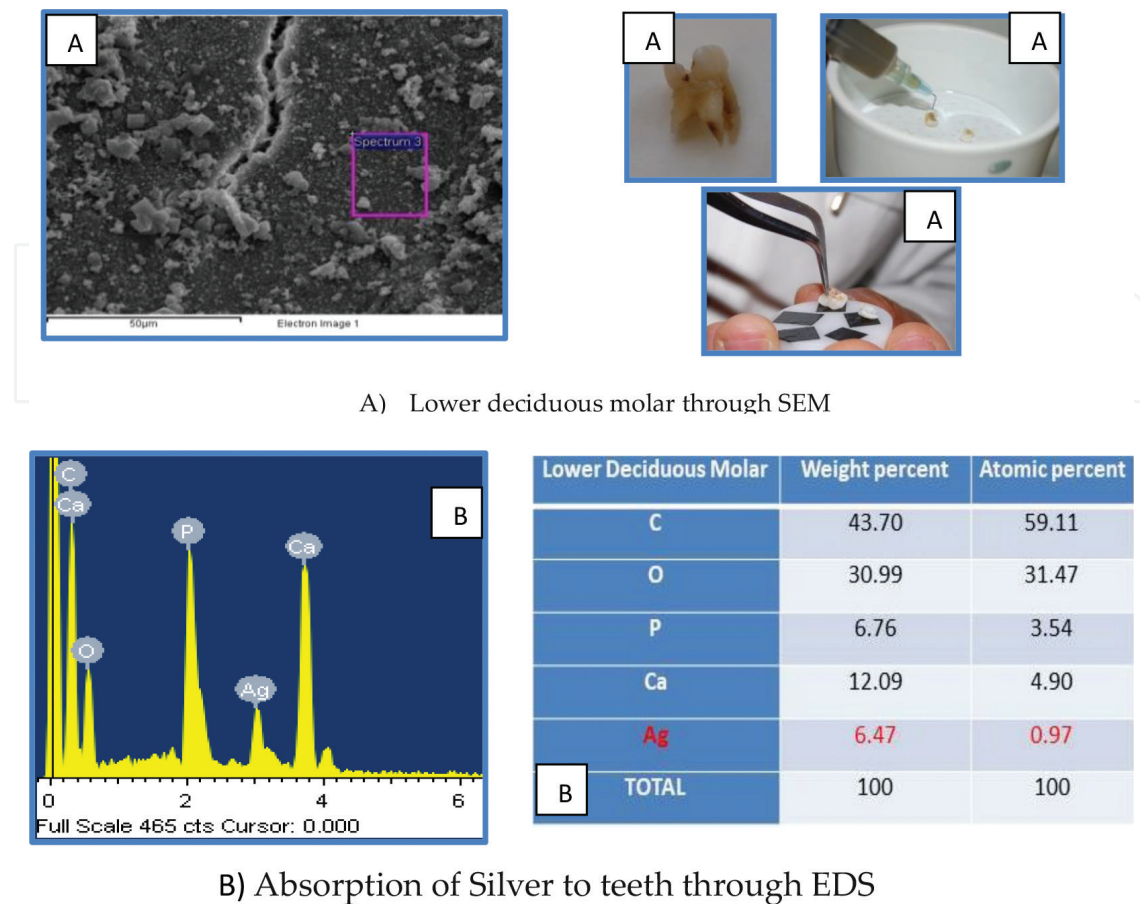


Figure 3. Weight percent of elements on deciduous teeth showing the absorption of silver. (A) Lower deciduous molar through SEM. (B) Absorption of silver to teeth through EDS.

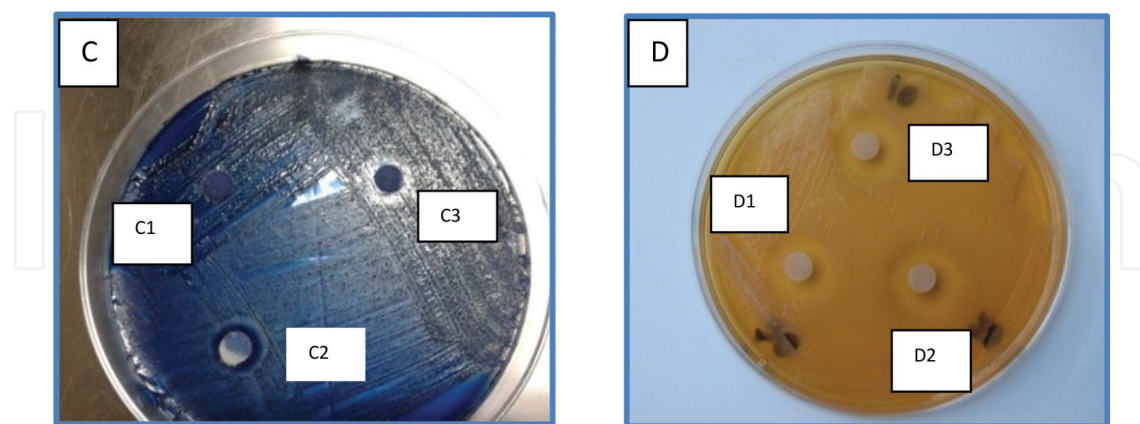


Figure 4. Petri dishes showing inhibitory halos of the discs rinsed with Ag-NPs by two reducing agents (Hi and Cs) *versus* chlorhexidine against *S. mutans* and *L. casei*. C1. Blank disc; C2. Disk containing chlorhexidine; C3. Disk containing Ag-NPs synthesized by *Heterotheca inuloides* *versus* *S. mutans*. D1. Disk containing 30 μ l of Ag-NPs synthesized by *Heterotheca inuloides*; D2. Disk containing 20 μ l; D3. Disk containing 10 μ l *versus* *L. casei*. Values of inhibitory halos of Ag-NPs with two eco-friendly reducing agents and chlorhexidine T.

Doses	Against <i>S. mutans</i>				Against <i>L. casei</i>			
	Mean	S.D	Minimum value	Maximum value	Mean	S.D	Minimum value	Maximum value
10 microliters								
Ag-Np reduced by <i>Camellia sinensis</i>	6.20	0.264	5.9	6.4	6.26	0.404	5.8	6.5
Ag-Np reduced by <i>Heterotheca inuloides</i>	5.43	0.404	5.0	5.8	5.43	0.153	5.3	5.6
Chlorhexidine	5.60	0.264	5.3	5.8	5.56	0.493	5.0	5.9
20 microliters								
Ag-Np reduced by <i>Camellia sinensis</i>	6.38	0.252	6.6	7.1	7.00	0.264	6.8	7.3
Ag-Np reduced by <i>Heterotheca inuloides</i>	6.93	0.416	6.6	7.4	6.93	0.231	6.8	7.2
Ag-Np reduced by Chlorhexidine	6.06	0.305	5.8	6.4	5.83	0.568	5.2	6.3
30 microliters								
Ag-Np reduced by <i>Camellia sinensis</i>	6.03	0.252	5.8	6.3	6.30	0.458	5.8	6.7
Ag-Np reduced by <i>Heterotheca inuloides</i>	6.56	0.737	6.0	7.4	6.50	0.781	6.0	7.4
Chlorhexidine	5.83	0.252	5.6	6.1	5.70	0.700	5.0	6.4

Table 1. As can be seen from the experimental data, with the agents used at different concentrations, the inhibitory halos' size ranged from 5.0 to 7.4 mm. The highest growth inhibition means were observed at doses of 20 μ l. The greatest variability in the size of halos was observed with Ag-NPs synthesized with *Heterotheca inuloides*. T en mm. SD. Standard deviation.

4. Discussion

We consider it important that dental science search for treatment alternatives incorporating products of natural origin, such as green plants. Mexico's varied vegetation allows the implementation of traditional medicine, as an alternative treatment for various diseases, including dental caries. Eco-friendly chemistry may propose a dental practice that is environmentally and economically sustainable. Nanotechnology has moved rapidly toward the improvement of health. The incorporation of silver salts in dental materials allows the dentist to ensure a longer lasting effect against pathogenic microorganisms.

As it is known, the size of bacteria is measured in microns, three orders of magnitude greater than the nanoparticles obtained by any of the three methods used. Therefore, the probability that nanoparticles come into contact with bacteria is higher when the size of Ag-NPs is smaller; hence, Hi turns out to be the best eco-friendly reducing agent.

L. casei has shown some resistance to chlorhexidine [9]. In this study, it is shown that green Ag-NPs inhibited both *L. casei* and *S. mutans*. Ag-NPs at a concentration of 1×10^{-2} showed acceptable antibacterial effect when compared to 2% CHX (containing 20 mg of chlorhexidine gluconate). The concentration used in Ag-NPs is minimal (nm) compared with the various concentrations shown in different presentations of CHX [6, 7, 37–41].

The knowledge of nanoscience and the emergence of new technologies in medical practice can transform the traditional way of attending a patient and promote a new paradigm based not only on clinical experience, but on the use of technological tools [42].

Having not found in our country, a study where Ag-NPs were in contact with deciduous teeth, in order to observe their behavior, the authors would like to propose deeper research in this matter.

The study of tissues from a nanoscale perspective, at molecular and cellular levels, leads to a better understanding of the structure-function-physiological relationship of oral structures, making it possible that diseases can be better understood and thereby prevented.

Ag-NPs have demonstrated efficacy in inhibiting the main microorganisms causing tooth decay. So the authors consider it relevant to continue research in this area. Ag-NPs can be used in various fields of treatment as an effective antibacterial agent; as an example, we can mention, the area of periodontology, where its action as a mouthwash agent can reduce gingival conditions. It is also possible to prepare toothpastes combining the antibacterial effect of silver and the benefits of green plants. In other areas of dental practice, Ag-NPs can be added to traditional dental cements or surgical materials and thereby diminish the risk of a possible postoperative infection.

From the visionary talk "There's Plenty of Room at the Bottom," given by physicist Richard Feynman in the American Physical Society meeting at Caltech in the late 1950s, from the "nanorobots" used in medicine, nanodentistry can really improve dental practice and treat common problems, such as dental hypersensitivity and implant placement; it can provide more durable and biocompatible restorations and more precise orthodontic treatments and reduce the appearance of gum and bone diseases.

5. Conclusions

Interpretation of the UV-vis spectra and images obtained through TEM showed that the formation of silver nanoparticles is more effective when the active ingredients are extracted from the bioreducers in the boiling process. This shows that the polarity of the active ingredients is similar to that of water, and that temperature plays an important role by increasing solubility and antibacterial effect. It is also concluded that nanoparticles obtained by Hi have a very good size (17 nm), in sufficient quantity, and with a narrow particle size distribution.

Ag-NPs can be an alternative for treatment, not only against dental caries, but also to prevent the formation of pathogenic bacteria affecting the balance of the oral cavity. They may be an option to reduce the harmful effect of the major pathogens, to diminish postoperative infectious processes, while also representing an attractive option as part of the eco-friendly substances, which would result not only in less costly drugs, but also in substances with a minor risk to human health and the environment.

Conflict of interest

The authors declare that they have not conflict of interest.

Author details

Raul Alberto Morales Luckie^{1*}, Rafael Lopez Casatañares¹, Rogelio Schougall², Sarai Carmina Guadarrama Reyes² and Víctor Sanchez Mendieta¹

*Address all correspondence to: ramoralesl@uaemex.mx

1 Joint Center for Research in Sustainable Chemistry (CCIQS), Autonomous University of the State of Mexico, Toluca, State of Mexico, Mexico

2 School of Dentistry, Autonomous University of the State of Mexico, Toluca, State of Mexico, Mexico

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