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Eco-Friendly Production of Silver Nanoparticles from *Vernonia amygdalina* and *Citropsis articulata*: An Assessment of Antibacterial Properties against Oral Bacteria

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ABSTRACT

Introduction: Traditional chewing sticks from *Vernonia amygdalina* and *Citropsis articulata* have been used for oral hygiene in African rural communities. This study pioneers an eco-friendly approach to silver nanoparticle (AgNP) synthesis using stem extracts from these medicinal plants, addressing environmental concerns associated with conventional methods. The antibacterial properties of the AgNPs against oral bacterial strains are assessed, offering a sustainable solution for oral health care. **Methods**: AgNPs were synthesized using aqueous and ethanolic stem extracts of *V. amygdalina* and *C. articulata*. Characterization was performed using UV-visible and FTIR spectroscopy. Phytochemical analysis revealed a diverse profile of bioactive compounds, with ethanolic extracts showing greater diversity. The AgNPs were tested against 100 bacterial isolates from dental caries patients at the Federal Medical Center, Abeokuta. **Results:** Molecular identification revealed three prevalent bacterial isolates: *Bacillus fungorum* (strain CUAB-AKINTOLA01), *Klebsiella pneumonia* (strain CUAB-AKINTOLA02), and *K. pneumonia* (strain CUAB-AKINTOLA03). The extracts from *V. amygdalina* and *C. articulata*, as well as the biofabricated AgNPs, showed significant antibacterial activity against these oral pathogens. Notably, AgNPs from V. *amygdalina* exhibited higher zones of inhibition, with *B. fungorum* being the most susceptible. These findings suggest the potential of these eco-friendly AgNPs as an effective antibacterial agent against oral bacterial infections. **Conclusion:** This study highlights the potent antibacterial efficacy of *V. amygdalina* and *C. articulata* stem extracts, as well as the silver nanoparticles biosynthesized from these extracts, against oral bacterial pathogens. While these findings are promising, further investigations are necessary to fully elucidate the therapeutic potential of these eco-friendly agents in the prevention and treatment of dental plaque-associated diseases.

INTRODUCTION

Despite the growing global awareness of oral and dental hygiene, the prevalence of dental caries and other oral disorders continues to rise, which can be attributed to changes in dietary preferences and increased sugar consumption across various age groups. Interestingly, natural products have been used for oral hygiene since ancient times, with records indicating their frequent utilization by various cultures, including the Israelites, Ethiopians, and Egyptians [1].

Previous studies have shown that extracts from *V. amygdalina* and *C. articulata*, commonly used as chewing sticks, exhibit antibacterial activity against oral microbiota [2]. This suggests that these plants may offer additional benefits beyond their traditional use for oral hygiene, such as reducing microbial load, which is a key goal of modern oral care practices. This property makes them an interesting area of study for the development of eco-friendly antibacterial agents. Ndukwe *et al.* [3] repoted

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the inhibitory effects of extracts from different chewing sticks on the growth of selected bacterial species, highlighting the potential of these plant-based agents in oral health care. Although previous studies have shown that more than half of the plant extracts exhibited antibacterial activity against oral bacteria, there is a need to investigate the traditional claim that chewing sticks made from these plants can strengthen teeth roots [2].

While certain bacteria are part of the normal oral microbiota, their overgrowth in the oral cavity can lead to plaque formation. Several oral bacterial pathogens, including *Streptococcus mutans* and *Bacteroides gingivalis*, are associated with dental caries and oral infections. Additionally, specific anaerobic bacteria like *Fusobacterium* and *Eubacterium*, as well as other species like Lactobacillus, have been shown to be susceptible to extracts from chewing sticks, such as *V. amygdalina* and *C. articulata* [3].

The use of chewing sticks has been linked to a lower prevalence of dental caries and tooth decay in certain regions of Nigeria, where they are widely used. In 2000, the World Health Organization (WHO) recognized chewing sticks as a beneficial oral hygiene practice in their Consensus Report on Oral Hygiene [2-4]. Chewing sticks are widely used by up to 90% of the rural population in Nigeria, due to their easy accessibility, affordability, and efficacy. Their beneficial effects can be attributed to two main mechanisms: the mechanical cleaning action facilitated by their fibrous structure, and the release of phytochemicals such as alkaloids, flavonoids, and phenolic acids [3-5]. These compounds may act synergistically to produce the observed benefits.

According to the literature, the antibacterial efficacy of chewing stick extracts against pathogens can be enhanced by combining them with metal nanoparticles, such as silver, selenium, and gold. This approach has shown promise in increasing the extracts' antibacterial properties. Silver nanoparticles, in particular, have been widely explored for their antibacterial potential, and their use can be traced back to ancient times [6]. Silver exhibits potent antibacterial properties, effectively inhibiting a wide range of microorganisms implicated in oral infections and other diseases. AgNPs can be synthesized through biological methods, such as plant extracts, or biomimetic approaches, which reduce silver ions to form nanoparticles. This results in decreased toxicity and significantly enhanced antimicrobial activity compared to their ionic counterparts [7]. Liao *et al*. [7] highlighted the unique properties of AgNPs, which have made them a valuable resource for treating microbial diseases. AgNPs exhibit a unique mechanism of action, disrupting bacterial cell membranes and inhibiting cellular processes, which reduces the likelihood of bacterial resistance through mutation [8].

The emergence of resistant microbial strains has led to a decline in antibiotic efficacy, prompting a search for alternative antimicrobial agents. Metallic nanoparticles, particularly AgNPs, have garnered significant attention due to their effective antimicrobial properties, which are attributed to the release of silver ions [9]. The alarming rise of antibiotic resistance in bacterial infections, particularly in oral health, has been exacerbated by the emergence of multidrug-resistant strains that are resistant to conventional antibiotics. A recent study by Stracy *et al.* [10] highlighted the re-emergence of this issue, underscoring the need for alternative strategies to combat oral bacterial infections.

Conventional chemical and physical methods for silver nanoparticle synthesis have significant drawbacks, including high costs, environmental toxicity, and potential biological risks [11]. Consequently, the use of harmful organic solvents and strong reducing agents in traditional silver nanoparticle synthesis methods results in the generation of toxic byproducts, including heavy metals and organic pollutants, which pose significant environmental risks. Furthermore, these methods are time-consuming and unsustainable. An eco-friendly alternative is the biosynthesis of silver nanoparticles using plant extracts, which eliminates the release of toxic substances into the environment and provides a sustainable solution [6]. The plant-mediated synthesis of AgNPs offers several advantages, including reduced biohazard risk and simplified protocols, compared to microorganism-based approaches. Moreover, using natural products like crude extracts of chewing sticks and their nano-synthesized counterparts may help combat antibiotic resistance in bacteria, particularly oral bacteria, which is a growing concern in dental health [7, 9].

The widespread use of *V. amygdalina* and *C. articulata* stem extracts as chewing sticks in Africa makes them a promising starting point for eco-friendly synthesis of silver nanoparticles. Investigating these nanoparticles' antibacterial properties could lead to innovative applications in dental care, such as preventing oral bacterial infections and promoting oral health [2, 4].

In this study, we investigate the antibacterial properties of crude extracts from *V. amygdalina* and *C. articulata*, as well as their AgNPs synthesized using an eco-friendly approach, against selected oral bacterial isolates. We aim to compare the antibacterial efficacy of both the crude extracts and their nano-synthesized counterparts.

MATERIAL AND METHODS

Preparation of plant extracts and materials. Fifteen stem varieties of chewing sticks commonly used by rural communities in Nigeria were purchased from the Lafenwa Market in Abeokuta, Ogun State, Nigeria. These stem varieties were subsequently authenticated at the Forest and Weeds Laboratory of the Federal University of Agriculture in Abeokuta (FUNAAB), and their local names and authenticated identities are listed in Table 1.

Preparation of ethanol extracts. The chewing sticks were washed with distilled water to remove any

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impurities, then cut into small pieces and air-dried at room temperature (30-50% relative humidity) for three days. After drying, they were soaked in 50% ethanol for a specified period, following a modified method described by Ahmad and Sharma [12]. The solution was soaked for 7 days, then filtered using Whatman No. 1 filter paper (11 µm pore size) to remove any impurities. The resulting filtrate was concentrated on a rotary evaporator to remove excess solvent. Finally, the concentrated filtrate was collected and stored at 4°C for use in the synthesis of silver nanoparticles and hybrids.

Preparation of aqueous plant extracts. To prepare the chewing stick extracts, 10 g of ground dried stem material was mixed with 100 mL of distilled water in a 500 mL beaker. The mixture was stirred at 200 rpm for 15 min at 25 °C to facilitate extraction. Then, it was incubated at 25 °C for 30 min, followed by a 30-min settling period to allow particles to settle. The supernatant was then filtered through Whatman filter paper (11 µm pore size), and the resulting filtrate was stored at 4 °C for further use.

Table 1. Authentication of chewing sticks at the Herbarium Laboratory, FUNAAB

S/N	Local Name	Botanical Name	Family Name	UAHA* No/ ID No
1	Pako Meyinro	*Serindeia warnekei*	*Asteraceae*	UAHA/ID No; 0021/6/001
2	Aayan	*Distemonanthus benthamianus*	*Leguminosae*	UAHA/ID No; 0021/6/002
3	Pako Ewuro	*V. amygdalina*	*Asteraceae*	UAHA/ID No; 0021/6/003
4	Orin dudu	*Anogeissus leiocarpus*	*Combretaceae*	UAHA/ID No; 0021/6/004
5	Eme/ Emi	*Butyrospermum paradoxum*	*Sapotaceae*	UAHA/ID No; 0021/6/005
6	Orin ata	*Zanthoxylum zanthoxyloides*	*Rutaceae*	UAHA/ID No; 0021/6/006
7	Orin pupa	*Monodora tenuifolia*	*Annonaceae*	UAHA/ID No; 0021/6/007
8	Pako Orogbo	*Garcinia kola*	*Clusiaceae*	UAHA/ID No; 0021/6/008
9	Botunje	*Jatropha curcas*	*Euphorbiaceae*	UAHA/ID No; 0021/6/009
10	Pako Dongoyaro	*Azadirachta indica*	*Maliaceae*	UAHA/ID No; 0021/6/0010
11	Madunmaro	*Gongronema latifolium*	*Asclepiadaceae*	UAHA/ID No; 0021/6/0011
12	Ifon/Ironyi	*Olax subscorpioidea*	*Olacaceae*	UAHA/ID No; 0021/6/0012
13	Atapari obuko	*C. articulata*	*Rutaceae*	UAHA/ID No; 0021/6/0013
15	Efirin	*Ocimum basilicum*	*Lamiacea*	UAHA/ID No; 0021/6/0014
15	Igi ira	*Bridelia feruginea*	*Phyllanthaceae*	UAHA/ID No; 0021/6/0015

*UAHA: University of Agriculture Herbarium Abeokuta

Phytochemical screening of plant extract. The presence of bioactive phytochemicals, including alkaloids, carbohydrates, proteins, tannins, phenols, saponins, flavonoids, terpenoids, steroids, and glycosides, in the extracts of each plant was determined using the method described by Yasmin *et al*. [13]. The phytochemical composition of the extracts was then compared using one-way analysis of variance (ANOVA) to determine any significant differences among the extracts.

Synthesis of silver nanoparticles (Ag-NPs) using plant extracts. For the synthesis of silver nanoparticles, 10 mL of each chewing stick extract was added to 100 mL of aqueous silver nitrate solutions at concentrations of 0.5, 1.0, and 2.0 mM. The resulting mixture was transferred to a 250 mL conical flask and stirred continuously on a shaker incubator at 25°C for 72 h, until the solution exhibited a brownish coloration, indicating the formation of silver nanoparticles. To monitor the bioreduction of Ag+ to Ag0 ions, the UV-Vis absorption spectra of the reaction mixture were measured at regular intervals (5, 10, 15, 20, 30, 45, and 60 minutes) using a UV-Vis spectrophotometer, within the wavelength range of 350-700 nm.

The AgNPs synthesized using the plant extracts were characterized using UV-visible spectroscopy. The AgNP solution was transferred into a quartz cuvette, and the absorption spectrum was recorded in the wavelength range of 350-700 nm with a resolution of 1 nm. This characterization step confirmed the presence of AgNPs, which were reduced and stabilized by the plant extracts, serving as both reducing and capping agents, as reported by Ahmad and Sharma [12].

Characterization of silver nanoparticle. The AgNPs synthesized using the plant extracts were characterized using UV-visible spectroscopy (UV-Vis) in the wavelength range of 350-700 nm [14]. Additionally, Fourier-transform infrared (FTIR) spectroscopy was employed to analyze the surface chemistry of the AgNPs in the wavenumber range of 200-4500 cm^{-1} [15].

Collection of oral samples. Dental samples were collected from participants using sterile oral swabs, which were gently rubbed over the inner cheeks, teeth, tongue, gums, and oral cavity for approximately one min. The swabs were then aseptically removed, sealed in sterile containers to prevent contamination, and labeled for identification. The collected oral samples were immediately transported to the Microbiology Laboratory at the Department of Biological Sciences, Crescent University, and stored at 4°C until processing. The oral swab samples were inoculated onto freshly prepared nutrient agar, blood agar, chocolate agar, and MacConkey agar plates, followed by incubation at 37°C for 24 h under aerobic conditions, using aseptic techniques to prevent contamination.

Isolation of pure cultures. Pure bacterial cultures were obtained from the mixed populations on the agar plates using a modified streak plate technique [16]. The resulting

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pure bacterial cultures were maintained at 4°C for further analysis. The streak plate technique was employed due to its effectiveness in isolating individual bacterial species from mixed populations, allowing for further characterization and study.

Characterization and identification of bacterial isolates. The presumptive identification of the bacterial isolates was performed using a combination of physical, morphological, and biochemical tests [17]. These tests included motility, oxidase, citrate utilization, urease production, catalase activity, coagulase production, indole production, and Gram staining, which provided a comprehensive characterization of the isolates. The motility test determined whether the bacterial isolate was motile or non-motile, while the oxidase, citrate utilization, urease production, catalase activity, and coagulase production tests detected the presence of specific enzymes. The indole production test differentiated members of the *Enterobacteriaceae* family, and Gram staining classified bacteria as Gram-positive or Gram-negative based on their cell wall composition.

Molecular identification of bacterial isolates. The three most prevalent bacterial isolates were identified using DNA sequencing techniques to confirm their identities. Specifically, DNA extraction and polymerase chain reaction (PCR) amplification of the *16S rRNA* gene were performed at Inqaba Biotec West Africa, a subsidiary located in Moniya, Ibadan, Oyo State, Nigeria, following established protocols [18, 19]. This approach enabled the precise identification of the bacterial isolates.

The purified PCR amplicons were subjected to Sanger sequencing, followed by quality control measures to ensure accuracy. These measures included evaluating the quality of base calls and verifying sequence accuracy. The obtained sequences were then aligned, and a phylogenetic tree was constructed using MEGA11 software. This tree revealed the evolutionary relationships among the bacterial isolates, which were inferred using the neighbor-joining method. Genetic distances were calculated based on the p-distance model, providing a clear understanding of the relationships among the isolates [20].

Plasmid mapping of the three bacterial isolates was performed using the MB DNA Analysis software, a specialized tool for DNA sequence analysis and plasmid mapping. The restriction map positions were determined by analyzing the nucleotide sequences of each isolate, and the resulting maps were generated using the software's plasmid mapping module [21]. The plasmid maps clearly displayed the restriction sites and their positions, providing a visual representation of the genetic organization of each isolate.

Antibacterial susceptibility. The antibacterial activity of the crude extracts from *C. articulata* and *V. amygdalina* (100 µL each) and the AgNPs synthesized using these extracts (100 µL each) was evaluated against selected bacterial strains using the agar well diffusion method. The controls used were AgNO3, ethanol, and distilled water [22]. The interpretation of the antibacterial activity was based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for antimicrobial susceptibility testing [23]. The antibacterial activity was evaluated based on the diameter of the inhibition zone, with values of ≥18 mm indicating susceptibility, 14-17 mm indicating intermediate susceptibility, and ≤13 mm indicating resistance. The experiment was performed in triplicate, and the inhibition zone diameters were measured at four different points and expressed in millimeters. The reported values represent the mean of the triplicate measurements, providing a clear and accurate assessment of the antibacterial activity.

Statistical Analysis. Duncan's multiple range test was used to determine the statistical significance ($P \leq 0.05$) of differences among the means of various treatment groups. The results were expressed as the mean ± standard deviation of three independent replicates, providing a clear and accurate representation of the data.

Ethical considerations. The research project was approved by the Health Research Ethics Committee of the Federal Medical Centre Abeokuta (NHREC/08/10/2015). Before collecting dental samples from participants, written informed consent was obtained from each individual, as outlined in the approved research protocol (see Supplementary Materials).

RESULTS

Phytochemical composition in chewing sticks

Ethanol extract of chewing sticks. The ethanolic extracts of the plant samples were analyzed for their phytochemical content, with the results shown in Fig. 1 (a-e). The extracts contained varying levels of tannins, saponins, flavonoids, phenols, and alkaloids. Notably, the extract of *V. amygdalina* had the highest content of tannins (3.76%), saponins (2.65%), flavonoids (3.09%), and phenols (3.12%), followed by *C. articulata* and *B. ferruginea*.

Table 2 presents the levels of glycosides, steroids, and terpenoids in the ethanolic extracts of the plant samples. The ethanolic extract of *V. amygdalina* contained the highest levels of glycosides (3.78%) and terpenoids (3.93%). The extract of *C. articulata* contained 2.51% glycosides and 2.79% terpenoids, while the extract of *S. warneckei* contained 0.88% glycosides and *O. subscorpioidea* contained 0.16% terpenoids.

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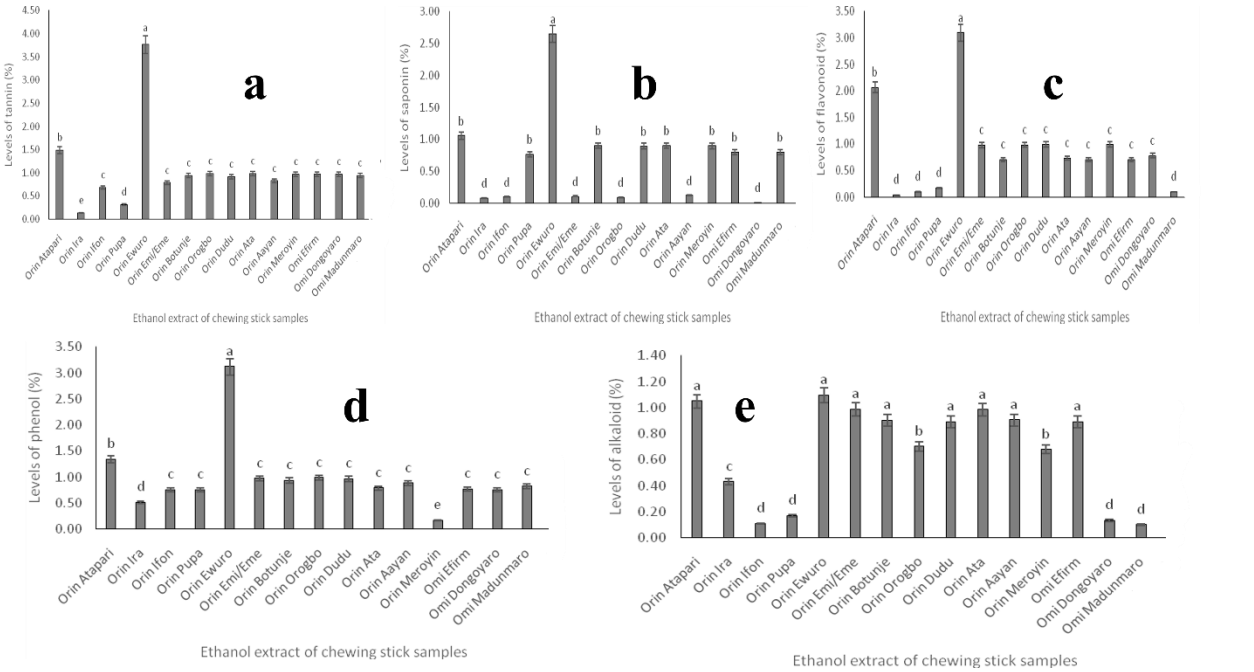

Fig. 1. Level of phytochemicals in the ethanol extracts of chewing sticks (a-e)

Table 2. Levels of glycoside, steroid and terpenoid in ethanol extract of chewing stick samples

Plant species	Glycoside	Steroid	Terpenoid
C. articulata	2.71±0.01[b]	1.84±0.01[a]	2.72±0.03[b]
B. feruginea	0.65±0.04[c]	0.62±0.01[b]	0.94±0.01[c]
O. subscorpioidea	0.77±0.00[c]	0.36±0.01[c]	0.16±0.00[d]
M. tenuifolia	0.13±0.00[d]	0.98±0.01[b]	0.51±0.00[c]
V. amygdalina	3.78±0.11[a]	1.82±0.03[a]	3.93±0.01[a]
B. paradoxum	0.92±0.01[c]	0.78±0.00[b]	0.92±0.01[c]
J. curcas	0.85±0.00[c]	0.95±0.03[b]	0.96±0.01[c]
G. kola	0.81±0.02[c]	0.98±0.01[b]	0.91±0.02[c]
A. leiocarpus	0.92±0.01[c]	0.90±0.01[b]	0.72±0.01[c]
Z. zanthoxyloides	0.99±0.00[c]	0.99±0.01[b]	0.99±0.01[c]
D. benthamianus	0.72±0.00[c]	0.53±0.01[b]	0.92±0.01[c]
S. warnekei	0.88±0.01[c]	0.89±0.01[b]	0.66±0.01[c]
O. basilicum	0.69±0.00[c]	0.89±0.01[b]	0.95±0.01[c]
A. indica	0.96±0.01[c]	0.86±0.01[b]	0.94±0.00[c]
G. latifolium	0.85±0.00[c]	0.93±0.01[b]	0.83±0.01[c]

[abcd]Mean values (±Standard deviation) in the same column having similar superscript are not significantly different ($P > 0.05$)

Aqueous extract of chewing sticks. Figure 2 (a-e) illustrates the quantitative analysis of tannins, saponins, flavonoids, phenols, and alkaloids in the aqueous extracts of the plant samples. The aqueous extract of *C. articulata* exhibited the highest tannin content (0.27 ± 0.01%), followed by *G. kola* (0.18 ± 0.01%) and *V. amygdalina* (0.17 ± 0.01%), respectively.

The saponin levels in the aqueous extracts of *C. articulata* (0.01 ± 0.001%), *B. ferruginea* (0.01 ± 0.001%), *O. subscorpioidea* (0.01 ± 0.001%), *M. tenuifolia* (0.01 ± 0.001%), and *B. paradoxum* (0.01 ± 0.001%) were not significantly different (Fig. 2b). In comparison to the other plant extracts, these samples had relatively higher saponin levels. The saponin content in the remaining plant extracts was significantly lower, with *A. indica* showing no detectable saponins.

The aqueous extract of *D. benthamianus* exhibited significantly higher phenolic content (0.50 ± 0.04%) compared to the other chewing stick aqueous extracts (Fig. 2d). In contrast, the aqueous extract of *S. warneckei* contained the lowest phenolic level (0.002 ± 0.0001%).

Table 3 presents the levels of glycosides, steroids, and terpenoids in the aqueous extracts of the plant samples. The aqueous extract of *C. articulata* exhibited significantly higher glycoside content (0.311 ± 0.001%), followed by *G. kola* (0.200 ± 0.028%) and *V. amygdalina* (0.18 ± 0.003%), respectively. The glycoside levels in the aqueous extracts of the remaining twelve plant samples were lower and did not differ significantly (Table 3).

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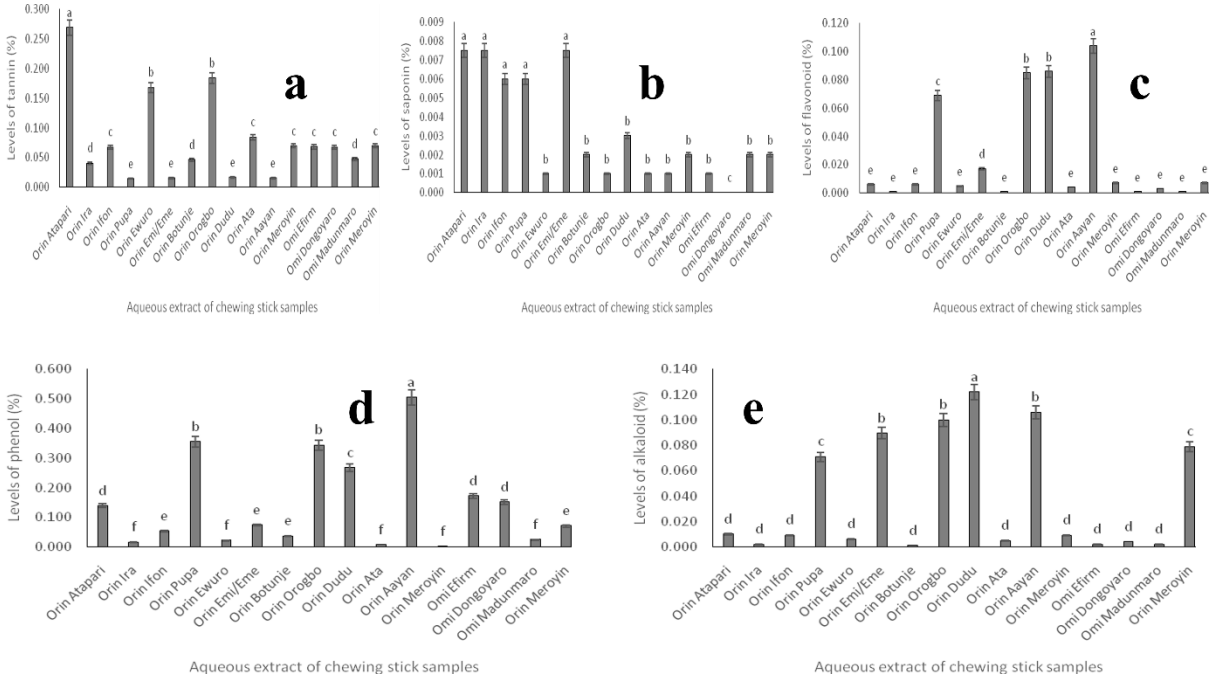

Fig. 2. (a-e) Level of phytochemicals in the aqueous extracts of chewing sticks

The plant extract of *M. tenuifolia* exhibited the highest steroid content (0.38 ± 0.013%), followed by *A. leiocarpus* (0.31 ± 0.014%). In contrast, the plant extract of *Z. zanthoxyloides* contained the lowest steroid level (0.008 ± 0.001%) among the fourteen plant extracts analyzed.

Table 3. Levels of glycoside, steroid and terpenoid in aqueous extract of chewing stick samples

Plant species	Glycoside	Steroid	Terpenoid
C. articulata	0.311±0.001^{a}	0.144±0.003^{b}	0.219±0.013^{a}
B. feruginea	0.050±0.006^{c}	0.018±0.001^{d}	0.037±0.003^{b}
O. subscorpioidea	0.072±0.001^{c}	0.056±0.003^{c}	0.059±0.001^{b}
M. tenuifolia	0.032±0.001^{c}	0.379±0.013^{a}	0.012±0.001^{b}
V. amygdalina	0.177±0.003^{b}	0.023±0.003^{d}	0.124±0.006^{a}
B. paradoxum	0.018±0.001^{c}	0.012±0.001^{d}	0.020±0.001^{b}
J. curcas	0.052±0.003^{c}	0.050±0.004^{c}	0.055±0.004^{b}
G. kola	0.200±0.028^{b}	0.177±0.003^{b}	0.202±0.003^{a}
A. leiocarpus	0.018±0.001^{c}	0.310±0.014^{a}	0.025±0.001^{b}
Z. zanthoxyloides	0.091±0.001^{c}	0.008±0.001^{d}	0.093±0.004^{b}
D. benthamianus	0.017±0.003^{c}	0.130±0.004^{b}	0.018±0.001^{b}
S. warnekei	0.079±0.001^{c}	0.085±0.004^{c}	0.056±0.003^{b}
O. basilicum	0.072±0.003^{c}	0.190±0.008^{b}	0.054±0.004^{b}
A. indica	0.070±0.010^{c}	0.158±0.001^{b}	0.043±0.004^{b}
G. latifolium	0.051±0.001^{c}	0.034±0.001^{d}	0.029±0.001^{b}

^{abcd}Mean values (±Standard deviation) in the same column having similar superscript are not significantly different ($P > 0.05$)

Characterization of synthesized plant extracts

Ultraviolet visible spectrophotometer (UV-vis). The UV-visible absorption spectra of AgNPs biosynthesized using *V. amygdalina* and *C. articulata* extracts are presented in Fig. 3 (a and b), respectively. The formation of AgNPs mediated by *V. amygdalina* extract was completed within 72 h, as evidenced by the appearance of a sharp surface plasmon resonance (SPR) band at approximately 455 nm (Fig. 3a). In contrast, the AgNP synthesis using *C. articulata* extract was achieved within 24 h, with a characteristic SPR band observed at around 460 nm (Fig. 3b). The presence of these distinct SPR bands confirms the successful formation of AgNPs, which is a result of the collective oscillation of free electrons in the nanoparticles upon interaction with incident light [24].

Analysis of Fourier Transform Infrared Spectroscopy (FTIR). The FTIR spectra of the raw extracts (aqueous and ethanolic) and the biosynthesized silver nanoparticles are presented in Fig. 4 and Table 4. The broad absorption band above 3447 cm^{-1} is attributed to the O-H stretching vibrations and carbonyl stretching modes. Medium bands observed at approximately 2925 cm^{-1} and 2350 cm^{-1} can be assigned to the C-H stretching vibrations and the stretching mode of CO_2, respectively. The band at 1753 cm^{-1} is characteristic of the stretching vibration of the carbonyl (C=O) functional group,

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commonly found in carboxylic acids and aldehydes. The lower wavenumber bands at 1029 cm^{-1} and 528 cm^{-1} are tentatively attributed to the presence of aliphatic compounds, containing fluorine and iodine, respectively.

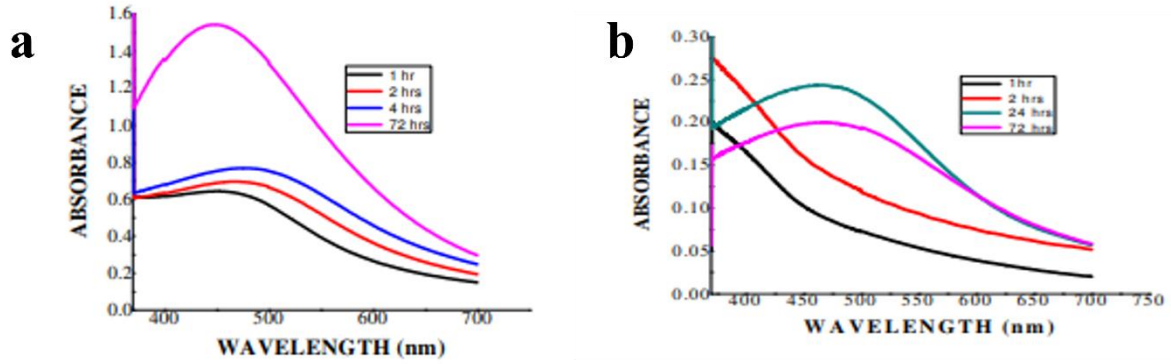

Fig. 3. UV-Visible spectrophotometry analysis of nano-synthesized extracts from *V. amygdalina* and *C. articulate*

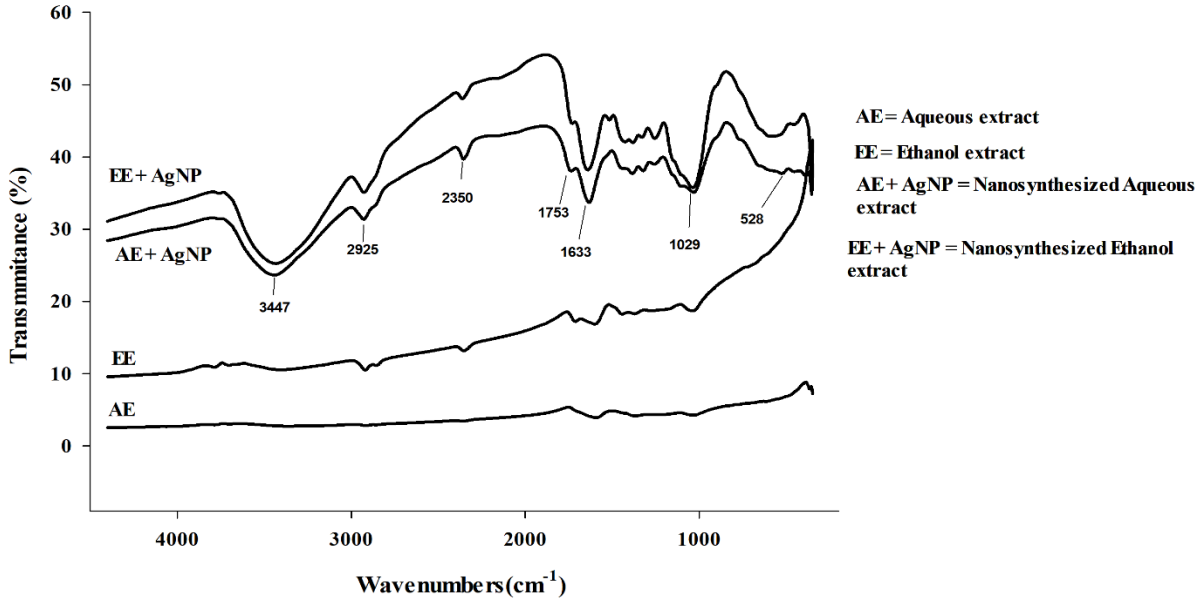

Fig. 4. FTIR spectra of the pristine extracts and biosynthesized extracts

Table 4. Wavenumbers and the detected functional groups

Wavenumber (cm^{-1})	Functional group detected
3447	Normal "polymeric" OH stretch
2925	Methyl C-H asym./sym. Stretch
2350	(Carbon dioxide) O=C=O Stretch
1753	C=O Stretch, which denotes the ester group
1633	Alkenyl C=C stretch
1029	Aliphatic fluoro compounds, C-F Stretch
528	Aliphatic iodo compounds, C-I stretch

Characterization and identification of bacterial isolates. Table 5 presents the biochemical profiles of the bacterial isolates obtained from dental samples collected from patients with dental infections. From the 100 dental samples cultured, eight distinct bacterial isolates were identified: *Bacillus* spp. (30%), *Klebsiella* spp. (22%), *Pseudomonas* spp. (18%), *Streptococcus* spp. (5%), *Staphylococcus* spp. (4%), *Enterobacter* spp. (5%), *Lactobacillus* spp. (10%), and *Escherichia coli* (6%). The percentages indicate the relative abundance of each isolate among the total isolates.

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Table 5. Biochemical characteristics of bacteria isolated from patients with dental infections

Isolate	Shape	Mot	Ox	Cit	Ur	Cat	Coa	In	H_2S	GR	Probable Isolates	Isolate Frequency (%)
1	R	+	+	+	-	+	-	-	-	+	*Bacillus* spp.	30
2	R	-	-	+	+	+	-	-	-	-	*Klebsiella* spp.	*22*
3	R	+	-	+	-	+	-	-	-	-	*Pseudomonas* spp.	*18*
4	C	-	-	+	+	-	-	-	-	+	*Streptococcus* spp.	*5*
5	C	-	-	+	+	+	-	-	-	+	*Staphylococcus* spp.	*4*
6	R	+	-	-	-	+	-	-	-	-	*Enterobacter* spp.	*5*
7	R	-	-	-	-	-	-	-	-	+	*Lactobacillus* spp.	*10*
8	R	+	-	-	-	+	-	-	-	-	*Escherichia coli*	*6*

Mot: Motility**; Ox:** Oxidase**; Cit:** Citate**; Ur**: Urease**; Cat:** Catalase**; Coa:** Coagulase**; In:** Indole**; GR:** Gram Reaction

Molecular identification of bacterial isolates. The three most prevalent bacterial isolates were selected for molecular characterization through *16S rRNA* gene sequencing analysis. The nucleotide sequences obtained were subjected to a BLAST search against the NCBI (National Center for Biotechnology Information) database. The results revealed that two of the isolates were new strains closely related to *B. fungorum* (a novel species recently isolated in Fangshan District, Beijing, PR China in 2020) [25] and *Klebsiella pneumoniae*, respectively. The *16S rRNA* gene sequences of these isolates were submitted to the NCBI GenBank database, and the accession numbers assigned to the respective strains are presented in Table 6.

Table 6. Molecular identification of bacterial isolates

Isolate ID	Organism Identity	Strain name	Identity (%)	Accession No
TOLA 1	*B. fungorum*	CUAB-AKINTOLA01	94.66	ON881240
TOLA 2	*K. pneumoniae*	CUAB-AKINTOLA02	98.47	ON881241
TOLA 3	*K. pneumoniae*	CUAB-AKINTOLA03	99.26	ON881242

Phylogenetic tree and plasmid mapping. The evolutionary relationships among the bacterial isolates were analyzed using the pairwise distance (P-distance) method, which measures the genetic distance between pairs of sequences. The results are presented in Figure 5, which shows the phylogenetic tree constructed based on the P-distance values. Figure 5 presents the phylogenetic tree constructed using the neighbor-joining method and aligned with the MUSCLE algorithm, which shows the evolutionary relationships among the bacterial isolates. Notably, the two *Klebsiella* strains (*CUAB-AKINTOLA02* and *CUAB-AKINTOLA03*) cluster together, indicate a close phylogenetic relationship. Similarly, the *B. fungorum* strain *CUAB-AKINTOLA01* isolated in this study is closely related to the *B. fungorum* strain *BQ41 (OM534588)* from the NCBI database, supported by a bootstrap value of 100%. This high bootstrap value indicates a robust phylogenetic relationship between the two isolates. Figure 6 depicts the restriction endonucleases present in each isolate, which may contribute to their resistance against specific antibacterial agents.

Antibacterial effects of chewing sticks on oral pathogens. The chewing sticks *C. articulata* and *V. amygdalina* were selected for antibacterial assays due to their rich phytochemical composition. Specifically, they exhibited significantly higher levels of bioactive compounds, including tannins (*e.g.*, gallic acid), saponins (*e.g.*, ursolic acid), flavonoids (*e.g.*, quercetin), phenols (*e.g.*, ferulic acid), alkaloids (*e.g.*, berberine), glycosides (*e.g.*, stevioside), steroids (*e.g.*, β-sitosterol), and terpenoids (*e.g.*, limonene), compared to other chewing sticks analyzed.

Extracts of *V. amygdalina* and their synthesized nanoparticles. The antibacterial activities of the aqueous and ethanolic extracts of *V. amygdalina*, as well as the biosynthesized AgNPs using these extracts, are presented in Table 7. The control tests (silver nitrate, distilled water, and ethanol) showed no antibacterial activity against *K. pneumoniae* (*CUAB-AKINTOLA02* and *CUAB-AKINTOLA03*) and *B. fungorum* (*CUAB-AKINTOLA01*). The aqueous extract of *V. amygdalina* did not exhibit any antibacterial activity against *K. pneumoniae* (*CUAB-AKINTOLA02*). In contrast, the ethanolic extract of biosynthesized AgNPs demonstrated the highest antibacterial activity against all three test organisms, followed by the aqueous extract of biosynthesized AgNPs, the ethanolic extract of *V. amygdalina*, and the aqueous extract of *V. amygdalina*, respectively.

***C. articulata* extracts and their synthesized nanoparticles**. Table 8 presents the antibacterial activities of the aqueous and ethanolic extracts of *C. articulata*, as well as the biosynthesized AgNPs using these extracts, against *K. pneumoniae* (*CUAB-AKINTOLA02* and *CUAB-AKINTOLA03*) and *B. fungorum* (*CUAB-AKINTOLA01*). The control tests (silver nitrate, distilled water, and ethanol) showed no antibacterial activity, as did the aqueous and ethanolic extracts of *C. articulata*. In contrast, the ethanolic extract of biosynthesized AgNPs exhibited significantly higher inhibition zones ($P < 0.05$) against all three test organisms compared to the aqueous extract of biosynthesized AgNPs.

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Fig. 5. Phylogenetic tree illustrating the evolutionary relationships among the different isolates

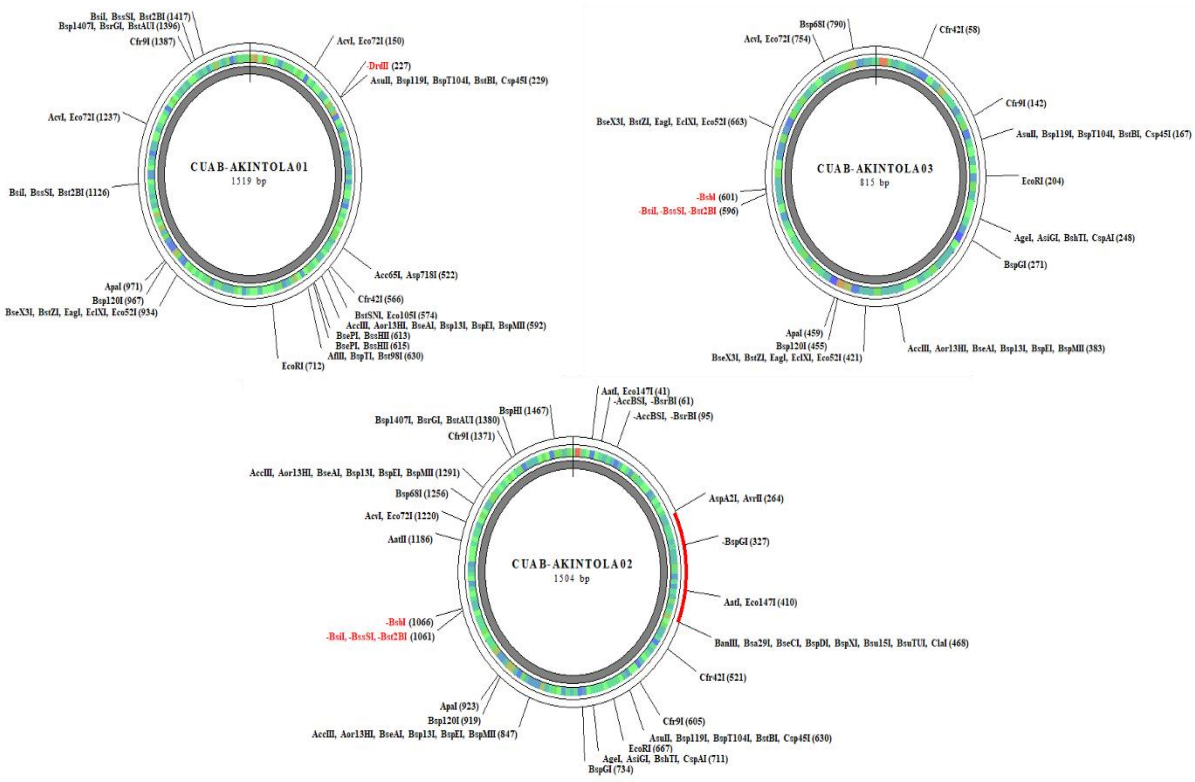

Fig. 6. Plasmid maps of *B. fungorum* and the two strains of *K. pneumonia*

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Table 7. Antibacterial activities (zones of inhibition in mm) of *V. amygdalina* extracts and their synthesized nanoparticles against oral pathogens

Solutions	*K. pneumoniae (CUAB-AKINTOLA02)*	*K. pneumoniae (CUAB-AKINTOLA03)*	*B. fungorum (CUAB-AKINTOLA01)*
Aqueous	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[e]
Ethanol	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[e]
$AGNO_3$	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[e]
Aqueous extract	0.00±0.00[d]	2.33±0.58[d]	4.67±1.15[d]
Ethanol extract	2.67±0.58[c]	5.00±1.00[c]	9.67±1.15[c]
AESNP	7.67±0.58[b]	14.00±1.00[b]	17.33±1.15[b]
EESNP	12.00±1.00[a]	16.33±1.53[a]	20.00±1.00[a]

[abcd]Mean values (±Standard deviation) in the same column having similar superscript are not significantly different ($P > 0.05$); $AGNO_3$: Silver nitrate solution, AESNP: Aqueous extract nanoparticles, EESNP: Ethanol extract nanoparticles

Table 8. Antibacterial activities (zones of inhibition in mm) of *C. articulata* extracts and their synthesized nanoparticles against oral pathogens

Solutions	*K. pneumoniae (CUAB-AKINTOLA02)*	*K. pneumoniae (CUAB-AKINTOLA03)*	*B. fungorum (CUAB-AKINTOLA01)*
Aqueous	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
Ethanol	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
$AGNO_3$	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
Aqueous extract	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
Ethanol extract	0.00±0.00[c]	0.00±0.00[c]	0.00±0.00[c]
AESNP	11.33±1.15[b]	9.00±1.00[b]	11.33±0.58[b]
EESNP	13.00±2.00[a]	14.33±1.53[a]	13.67±1.15[a]

[abcd]Mean values (±Standard deviation) in the same column having similar superscript are not significantly different ($P > 0.05$)); $AGNO_3$: Silver nitrate solution, AESNP: Aqueous extract nanoparticles, EESNP: Ethanol extract nanoparticles.

DISCUSSION

The use of ethanol as an extraction solvent is advantageous due to its ability to extract a significant amount of phytochemicals. However, aqueous extraction offers a contrasting benefit, eliminating the need for organic solvents and requiring less energy consumption during the extraction process. In the present study, the ethanolic extract (EE) of *V. amygdalina* stem contained 3.7% tannins, 2.6% saponins, 3.1% flavonoids, 3.1% phenols, and 1.1% alkaloids. In contrast, the aqueous extract (AE) had lower concentrations of 1.75% tannins, 0.4% phenols, and trace amounts of saponins, flavonoids, and alkaloids. The observed disparity in phytochemical concentrations between the two extracts can be attributed to the limited solubility of bioactive compounds in water, which restricts their extraction into the aqueous extract. This phenomenon has been previously reported by Udochukwu *et al.* [26].

The aqueous extract (AE) of *C. articulata* contained 0.26% tannins, trace amounts of saponins, flavonoids, and alkaloids, and 1.3% phenols, whereas the ethanolic extract (EE) had higher concentrations of 1.5% tannins, 1.1% saponins, 2.0% flavonoids, 1.4% phenols, and 1.1% alkaloids. The presence of these phytochemicals is crucial for treating various chronic diseases, such as oxidative stress, inflammation, and cancer [27-29]. This observation is in accordance with the findings of Acheampong *et al*. [30] and Olushola-Siedoks *et al*. [31] who reported that the use of alkaloid-containing and ethanolic solvents in the extraction of *Z. zanthoxyloides* facilitated better extraction and conversion of insoluble free radicals and compounds, which would not have been possible with aqueous extraction alone. The choice of solvent is crucial, as water may not be suitable for extracting non-polar or moderately polar compounds due to its high polarity.

The ethanolic extracts from both *V. amygdalina* and *C. articulata* were utilized for the green synthesis of AgNPs. Silver nanoparticles were chosen for this study due to their well-established antibacterial properties and low toxicity compared to other metal nanoparticles, making them a suitable choice for oral applications. The biosynthesized AgNPs exhibited a brownish color in aqueous medium, a characteristic typical of AgNPs due to the surface plasmon resonance phenomenon [32], which confirms their successful synthesis. Similar color changes have been observed in previous studies, confirming the successful reduction of silver ions and the formation of AgNPs. For example, in our study, the reaction between the *V. amygdalina* and *C. articulata* extracts and the silver nitrate ($AgNO_3$) solution resulted in the formation of AgNPs, which was facilitated by stirring the solution at 500 rpm at room temperature [32]. The UV-Vis spectroscopic analysis confirmed the successful green synthesis of silver nanoparticles from the *V. amygdalina* and *C. articulata* extracts, revealing an increase in nanoparticle formation over time. This increase in nanoparticle formation can enhance the activities of the bioactive compounds present in the extracts, as reported previously. The enhanced activities can be attributed to the increased surface area, improved durability, and enhanced permeability of the nanoparticles, which can improve their antibacterial properties [33].

The FTIR analysis revealed significant differences in the functional groups between the pristine plant extracts and their biosynthesized nanoparticle counterparts. Specifically, the biosynthesized nanoparticle extracts

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exhibited additional functional groups, including O-H stretching, methyl C-H stretching, O=C=O stretching, ester group C=O stretching, alkenyl C=C stretching, aliphatic iodo compound C-I stretching, and aliphatic fluoro compound C-F stretching, which were not detected in the pristine extracts. These additional functional groups could explain the enhanced antibacterial activities observed with the biosynthesized nanoparticle extracts against the tested organisms. The variation in functional group composition may be responsible for the improved antibacterial properties of the biosynthesized nanoparticle extracts compared to the pristine extracts.

Consistent with our findings, Odeniyi *et al*. [34] reported similar results, highlighting the importance of functional group composition in determining the antibacterial properties of biosynthesized nanoparticles. In a related study, Aderibigbe *et al*. [35] demonstrated that the choice of extraction solvent, such as chloroform, can also influence the detection of additional functional groups in *V. amygdalina* extracts. These studies collectively underscore the significance of optimizing extraction conditions and functional group composition to achieve enhanced antibacterial properties in biosynthesized nanoparticles [35].

In the present study, eight different bacterial genera were isolated, with *Bacillus* spp. and *Klebsiella* spp. being the most prevalent. *B. fungorum*, isolated in this study, is a facultatively anaerobic, Gram-positive, spore-forming bacterium. In addition to *Bacillus* spp., other bacterial isolates identified as potential oral pathogens and tested for their susceptibility to the biosynthesized silver nanoparticles included *Klebsiella* spp., *Pseudomonas* spp., *Streptococcus* spp., *Staphylococcus* spp., *Enterobacter* spp., *Lactobacillus* spp., and *E. coli*. Consistent with our findings, previous studies have also isolated oral bacteria from dental biofilms, such as *Lactobacillus sakei* and *Streptococcus salivarius* [36]. These bacteria are commonly found in oral environments and are relevant targets for the antibacterial properties of the biosynthesized silver nanoparticles. Furthermore, the diversity of oral bacteria isolated in our study is consistent with previous findings, which have also reported the presence of *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, and other organisms in the oral cavity of patients with removable dentures [37]. These studies collectively highlight the complexity of the oral microbiome and the need for effective antibacterial agents, such as the biosynthesized silver nanoparticles, to combat oral infections.

To further characterize the bacterial isolates, phylogenetic analysis was performed to elucidate their evolutionary relationships with closely related species in the NCBI GenBank database. This analysis provided valuable insights into the taxonomic classification and diversity of the oral bacteria, which is essential for understanding their susceptibility to the biosynthesized silver nanoparticles and the potential applications of these nanoparticles in oral healthcare. The bootstrap consensus tree constructed for the isolates was supported by bootstrapping analysis with bootstrap values ranging from 66 to 100%. This analysis allowed us to investigate the diversity and relationships among the bacterial isolates, including *B. fungorum*.

Previous studies have employed phylogenetic analysis to explore bacterial diversity and relationships, including investigations of *K. pneumoniae* (Ejaz *et al*., [38]) and *Lentilactobacillus fungorum* (Tohno *et al*., [39]). These efforts provide valuable insights into bacterial diversity, which is crucial for understanding the potential impact of antibacterial agents like silver nanoparticles.

The antibacterial activities of *V. amygdalina* extracts against the isolated bacterial strains were further evaluated by measuring the zones of inhibition to assess their ability to inhibit bacterial growth. The ethanolic extract (EE) showed better efficacy against all the test organisms compared to the aqueous extract (AE). However, the biosynthesized silver nanoparticle extracts (AESNP and EESNP) displayed significantly higher antibacterial activities, with larger zones of inhibition compared to their corresponding crude extracts. The EESNP of *V. amygdalina* demonstrated a significantly higher antibacterial activity against the isolates. This enhanced activity can be attributed to the silver nanoparticles facilitating the delivery of the active compounds present in the *V. amygdalina* extract to the bacterial cells, leading to increased inhibition of bacterial growth. Similar findings have been reported by Desalegn *et al*. [40], Ramanathan *et al*. [41], and Vijayakumari *et al.* [42].

Neither the aqueous extract (AE) nor the ethanolic extract (EE) of *C. articulata* exhibited antibacterial activity against the tested bacterial strains. In contrast, the biosynthesized silver nanoparticle extracts of *C. articulata* demonstrated potent antibacterial activity, inhibiting the growth of all three test organisms. The EESNP exhibited the most potent antibacterial activity, which could be attributed to the higher concentration of silver nanoparticles and bioactive compounds, such as flavonoids and alkaloids, extracted from *V. amygdalina* compared to the AE, as previously demonstrated by the phytochemical analysis results. This enhanced antibacterial activity is consistent with previous literature reports that have shown that silver nanoparticles can enhance the delivery and efficacy of bioactive compounds against oral bacteria [43, 44]. Notably, the antibacterial activity of the biosynthesized silver nanoparticles against oral bacteria can be influenced by various factors, including incubation time, nutrient concentration, growth phase of the bacterial strains, and initial bacterial concentration. These factors can affect the efficacy of the nanoparticles in inhibiting bacterial growth and should be considered in the design of future studies [8, 11].

In conclusion, both the pristine and biosynthesized silver nanoparticle extracts exhibited varying degrees of

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antibacterial activity against the isolated oral bacterial pathogens, with *V. amygdalina* demonstrating superior activity. The novel bacterial species isolated, *Lentilactobacillus fungorum* (formerly known as *Bacillus fungorum*), showed significantly increased sensitivity to *V. amygdalina* extracts, with a higher zone of inhibition compared to the other bacterial strains. This observation could be attributed to the bioactive compounds present in *V. amygdalina*, which may interact with the thick peptidoglycan layer of this Gram-positive bacterium, ultimately leading to cellular lysis and death.

While the pristine extracts of *V. amygdalina* and *C. articulata*, as well as their biosynthesized silver nanoparticle counterparts, exhibit promising antibacterial properties, further research is warranted to comprehensively evaluate their potential applications. In particular, *in vivo* studies are necessary to assess the potential toxicity and liver-related effects of these extracts, including any adverse effects on liver function or damage, before recommending their use in clinical settings.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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