Towards a novel toothpaste:
the effect of gum Arabic-silver nanoparticles on the oral biofilm

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Thesis for a Ph.D. Degree in the Department of Restorative Dentistry

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"Success is not an accident. It is hard work, perseverance, learning, studying, sacrifice and most of all, love of what you are doing or learning to do"

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Declaration

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Summary

Background: Oral health is an integral part of overall health and wellbeing of a human being. Over 3 billion people worldwide suffer from poor oral health or dental disease. Oral disease may cause discomfort, pain, disfigurement, tooth loss and loss of function. Untreated oral disease may lead to decreased work productivity in adults, loss of school days for children, economic burden to families, and reduced quality of life. Oral disease includes a range of chronic clinical conditions such as dental caries, periodontal disease and oral cancer. The most prevalent disease in the world is dental caries, which is becoming more prevalent, especially in countries with low and moderate incomes.

Caries has a strong microbial association. Hence, measures of microbial control of microorganisms need to be included in continued research on caries prevention. Multidrug resistant microbes against existing antibiotic drugs is a challenge in health care, including dentistry. The development and evaluation of new technologies is essential to overcome these problems. Nanotechnology has emerged as an important innovation assisting in the provision of health care. When compared to bulk molecules, nanoparticles (NPs) have powerful physical properties. They are solid nanometer-sized particles (100 molecules in size). Recently, interest in the use of nanotechnology has increased in dentistry as cutting-edge techniques for preventing and treating dental caries as well as regulating oral biofilms. It has been established that silver nanoparticles (AgNPs) have the potential to be used to create new antibacterial agents, drug delivery systems, biomaterials, and regeneration materials, in addition to their wide scope of medical applications. The synthesis of AgNPs can either be from inorganic or organic sources. The biosynthesis of AgNPs from organic sources such as plant extract is preferable due to their accessibility, renewability, biocompatibility, environment-benign nature, and cost effectiveness. Consequently, the author investigated the use of gum Arabic (GA) plant extract to synthesize AgNPs because of its ubiquity on the African continent, its sustainability and its known medicinal value.

Aims and objectives: The aim of the study was to evaluate the possibility of developing a novel toothpaste incorporating gum Arabic-silver nanoparticles (GA-AgNPs). The objectives were: to review the literature to establish existing knowledge on antimicrobial activity of topical applications of AgNPs compared to standard antimicrobial agents and particularly to review the efficacy of green synthesized AgNPs in dental therapy; to synthetize GA-AgNPs;

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to evaluate the *in vitro* antimicrobial activity of the GA-AgNPs; to assess their attachment to the tooth enamel *in vitro*; to establish their biocompatibility to oral tissues *in vitro*; and lastly, to incorporate the GA-AgNPs in a toothpaste and test the effect of the combination for biocompatibility and antimicrobial effect on selected micro-organisms *in vitro.*

Methods: Two focused literature reviews were carried out: The first review established the evidence of the antimicrobial effect of topical applications of AgNPs to standard antimicrobial agents using the PRISMA guidelines. Electronic databases: Pubmed, Science Direct, and EBSCOhost were searched for *in vitro* studies in English language published between 2000 and 2020. The second review compared the chemical and green synthesis route of AgNPs in treating dental infections and informed the efficacy of green synthesized AgNPs in dental therapy. Subsequently, a number of laboratory tests were carried out: different fractions of AgNPs (0.1-0.5g) were synthesized using plant extract (GA), sodium borohydride ($NaBH_4$), and their combination as reducing agents. Transmission electron microscopy (TEM), dynamic light scattering (DLS), ultraviolet-visible spectrophotometry (UV-Vis), and Fourier-transform infrared spectroscopy (FT-IR) were used to analyze the AgNPs. Gram positive and Gram-negative bacteria were tested for antibacterial activity using agar well diffusion and microdilution assays, while Caco-2, HT-29, and KMST-6 cells were tested for cytotoxicity using the MTT assay. Furthermore, two fractions of AgNPs (0.1g/0.4g) using plant extract GA were synthesized and characterized as previously mentioned. The GA-AgNPs were assessed for antimicrobial activity on selected oral microbes using the above mentioned methods, and their attachment potential to tooth enamel was evaluated by exposing the enamel blocks to *S. mutans* with and without the GA-AgNPs using scanning electron microscopy (SEM). The cytotoxicity of the two GA-AgNPs fractions was compared on buccal mucosa fibroblast cells and KMST-6 normal skin cell line using MTT assay. Finally, the more potent fraction of GA-AgNPs was incorporated in a commercial toothpaste at a non-active concentration to evaluate its cytotoxicity and antimicrobial potential *in vitro* using the same methods.

Results: The literature revealed that AgNPs may have equivalent antimicrobial activity to conventional antimicrobial agents. However, comparisons are challenging due to heterogeneity in the treatments, microbes and methods used among published studies. Literature also confirmed that green synthesis of AgNPs is an ecological, renewable, biocompatible and cheaper process, which can be included into dental products and employed as antibacterial agents in a variety of dental settings. The synthetized GA-AgNPs were spherical with peaks at

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the expected range. The *in-vitro* experiments revealed that GA-AgNPs had broad-spectrum antibacterial effects on both microorganisms that are Gram-positive and Gram-negative, as well as non-selective cytotoxicity on both healthy and colon cancer cells in the same dosage range. However, these effects were decreased in the combination of GA and $NaBH_4$. The GA-AgNPs_0.4g had more microcidal activity than the GA-AgNPs_0.1g, albeit with higher cytotoxicity and can attach to the enamel. In addition, GA-AgNPs_0.4g maintained its antimicrobial activity when incorporated into an inactive toothpaste (GA-AgNPs_TP) with minimal cytotoxicity in comparison to GA-AgNPs_0.4g alone.

Conclusion: The results suggest that GA-AgNPs have potential to be added to a toothpaste with improved action against oral biofilm. However, reducing cytotoxicity needs further investigation as it is still below the threshold of 80% *in vitro* cell survival.

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Dedication

To my beloved parents whose love sustain my PhD journey. I am a fortunate product of their dream.

To my husband, Hussam (my one and only), for his endless love, patience, and support.

To my child, Ahmed, my comfort and source of motivation.

To my brothers, (my rock) who supported me in this journey, encouraged me all the way and made this endeavor possible.

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May Allah bless them all

The Prophet Muhammad (peace be upon him) said:
"One who treads a path in search of knowledge has his path to Paradise made easy by God"

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Keywords

- Antimicrobial
- Cytotoxicity
- Gum Arabic
- Silver nanoparticles
- Toothpaste

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Acronyms

Acronym	Nomenclature
A. indica	*Azadirachta indica*
ACP	Amorphous Calcium Phosphate
Ag^+	Silver ions
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AgNPs-LA	AgNPs with lipoic acid
AgNPs-PEG	AgNPs with polyethylene glycol
AgNPs-TA	AgNPs with tannic acid
AgNPs-UC	AgNPs without additional capping agents
AgTive®	Central venous catheter impregnated with silver nanoparticles
AMPs	Antimicrobial peptides
AuNPs	Gold nanoparticles
B. subtilis	*Bacillus subtilis*
BHA	Brain heart agar
BHI	Brain heart infusion
BMF	Buccal mucosa fibroblast cell line
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
C. albicans	*Candida albicans*
$Ca(OH)_2$	Calcium hydroxide
Caco-2	Colon carcinoma cells
C-AgNPs	Chemically synthesized AgNPs
CFU	Colony forming units
CH_2	Methylene group
CHX	Chlorohexidine
CLSI	Clinical Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CPP	Casein phosphopeptide
C. sinensis	*Camellia sinensis*

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CTGA-NPs	Chitosan-gum Arabic nanoparticles
CT-NPs	Chitosan nanoparticles
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecoo's phosphate-buffered saline
DPPH	2,2-diphenyl-1-picrylhydrazyl
E . coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EML	Essential medicines list
EPS	Extra-polysaccharide matrix
F. bengalensis	*Ficus bengalensis*
F. nucleaatum	*Fusobacterium nucleatum*
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
FT-IR	Fourier-transform infrared spectroscopy
G. glabra	*Glycyrrhiza glabra*
GA	Gum Arabic
GA-AgNPs	Gum Arabic-silver nanoparticles
GA-AgNPs_TP	Gum arabic-silver nanoparticles toothpaste
GAC-AgNPs	Combination of gum Arabic and chemically synthesized AgNPs
GAE	Gum Arabic extract
GDP	Gross Domestic Product
GTR	Guided tissue regeneration
H. inuloides	*Heterotheca inuloides*
HR-TEM	High-resolution transmission electron microscopy
HT-29	Colon carcinoma cells
IC50	Half-maximal inhibitory concentration
K. pneumoniae	*Klebsiella pneumoniae*
KMST-6	Normal skin fibroblasts
L. acidophilus	*Lactobacillus acidophilus*
L. casei	*Lactobacillus casei*

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L. fermentum	*Lactobacillus fermentum*
L. lactis	*Lactobacillus lactis*
L. rhamousus	*Lactobacillus rhamousus*
LOAEL	Lowest observed adverse effect level
M. luteus	*Micrococcus luteus*
MBCs	Minimum bactericidal concentrations
McF	McFarland standards
MHA	Müeller-Hinton agar
MHB	Müeller-Hinton broth
MIC_{50}	Minimum inhibitory effect that inhibits 50% of the tested bacterial isolates
MIC_{90}	Minimum inhibitory effect that inhibits 90% of the tested bacterial isolates
MICs	Minimum inhibitory concentrations
MNPs	Metallic nanoparticles
MRSA	Methicillin-resistant Staphylococcus aureus
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
$NaBH_4$	Sodium borohydride
NaCL	Sodium chloride
NaF	Sodium fluoride
NaOCL	Sodium hypochlorite
NH	Nitrogen hydride
NOAEL	No observed adverse effect level
NPs	Nanoparticles
NSF	Nanosilver fluoride
NSSF	Nano-silver incorporated sodium fluoride
OH	Hydroxyl group
ORAC	Oxygen radical absorbance capacity
P. aeruginosa	Pseudomonas aeruginosa
P. mirabilis	*Proteus mirabilis*
PBS	Phosphate buffer saline
PDI	Polydispersity index
PICO	Population, intervention, comparison, outcome
PRISMA	Preferred reporting items for systematic reviews and meta-analysis

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PMMA	Polymethyl-methacrylate
RB	Rice bran
RH	Rice husk
RG	Rice germ
PVP	Polyvinyl pyridoline
S. salivarius	*Streptococcus salivarius*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. mutans	*Streptococcus mutans*
S. persica	*Salvadora persica*
S. pyogenes	*Streptococcus pyogenes*
S. sanguinis	*Streptococcus sanguinis*
S = O	Sulfur monoxide
SD	Standard deviation
SDF	Silver diamine fluoride
SEM	Scanning electron microscopy
SilvaSorb®	Nano-silver
SPR	Surface plasmin resonance
TCM	Toothpaste conditioned medium
TEM	Transmission electron microscopy
TPC	Total polyphenolic content
TPs	Toothpastes
TSA	Tryptic soy agar
UV-Vis	Ultraviolet-visible spectrophotometry
WHO	World Health Organization
WSL	White spot lesion
ZnO	Zinc oxide
ZnS	Zinc sulfide
ZnONPs	Zinc oxide Nanoparticles
ZOI	Zones of inhibition
λmax	Maximum absorbance

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Chapter 1: INTRODUCTION

1.1 Executive summary

The introduction chapter gives a general overview regarding oral diseases and preventive strategies. It also reviews the use of nanotechnology for dental treatments and provides a rationale for using GA based on its geographical distribution, chemical composition, and medical application. In addition, the aims, objectives, and hypotheses of the study are provided.

1.2 Overview

1.2.1 Oral diseases

Oral diseases (dental caries and periodontitis) are a major public health problem worldwide (Peres *et al.*, 2019). The worldwide prevalence of oral diseases exceeds 70% for both adults and children. The burden of oral diseases shows unequal distribution, affecting populations with lower to middle economic status more, due to lack of functional facilities within the primary health care system (WHO, 2020). This necessitates disease prevention and oral health promotion as better recourse (WHO, 2017). This was reinforced by the WHO programme (2017-2021) recommending the development of oral hygiene products that are affordable, safe and ecofriendly.

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1.2.2 Prevention

Intensive research and improved preventive strategies to maintain oral health have improved tooth resistance to caries (Janus *et al.*, 2016). The main reason of carious lesions is poor oral hygiene practices which leads to deposition of dental plaque on tooth surfaces (Guven *et al.*, 2019). The efficiency of plaque removal by mechanical means such as tooth brushing and flossing depends on using the correct technique, skill and compliance (Jafer *et al.*, 2016; Valkenburg *et al.*, 2016; Figuero *et al.*, 2017). Toothpastes and mouthrinses are added to the oral hygiene routine, based on their potential role as antimicrobials (Jafer *et al.*, 2016; Figuero *et al.*, 2017; Doméjean, Muller-Bolla and Featherstone, 2018). Various chemical agents have been added to toothpastes such as triclosan and fluoride (Ullah and Zafar, 2015; Spolarich and

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Panagakos, 2017; Doméjean, Muller-Bolla and Featherstone, 2018). Even though these chemical agents have shown antibacterial efficiency, the complexity of toothpastes with different constituents (abrasive, detergents, flavouring, preservatives, humectants) may decrease or increase the antibacterial activity of the chemical agents due to interactions among the different ingredients (Auschill, Hellwig and Arweiler, 2007; Guven *et al.*, 2019). Therefore, if antimicrobial agents are incorporated in toothpastes, they should act synergistically with the other components (Prasanth, 2011; Ahmed *et al.*, 2019).

1.2.3 Efficacy of existing toothpastes

Goldman *et al.(2008)* reported the relationship among diet, globalization, tooth decay and significant inequalities in the affordability of essential preventive health care products. They found that the poorer countries spend a significant portion of household income on "low-cost toothpaste" that may not be as efficacious as more expensive ones (Goldman *et al.*, 2008). This was confirmed by Vorster's work (Vorster, 2019) in South Africa, Tanzania (Kikwilu, Frencken and Mulder, 2009) , and in other countries including Cambodia, Japan, Hong Kong (China), Central African Republic, Trinidad & Tobago, Saint Kitts and Nevis, Cambodia, and New Zealand (Gkekas *et al.*, 2022). Variation in dentifrice products was found on the market: quality is associated with price and could result in low-income population using cheaper and less effective toothpaste (Gupta *et al.*, 2021). This is an important motivation to develop a toothpaste that is affordable and yet effective. Moreover, it is of concern that oral microbes are developing resistance to existing antimicrobial agents (Sousa *et al.*, 2020).

In their systematic review of randomized clinical trials, Nachu, Ravoori and Pachava (2022) reported lack of strong evidence of anti-plaque efficacy of toothpastes, with poor evidence in relation to herbal dentifrices and moderate evidence associated with toothpastes containing triclosan/copolymer with fluoride (Nachu, Ravoori and Pachava, 2022). Previously, in a comprehensive review by Divya *et al*. (2021), various herbs included in dentifrices were evaluated, and the authors concluded that toothpaste comprising different herbs are considered to be relatively safe, readily accessible, with good antimicrobial potential similar to that of conventional toothpaste (Divya, Suresh and Meenakshi, 2021). Furthermore, Ahmed *et al*. (2019) reported some evidence of superior efficacy of nanoparticle based toothpaste on oral microbes over a fluoride containing toothpaste (Ahmed *et al.*, 2019). However, there is still paucity in literature linking the efficacy of nanoparticles-based toothpaste on oral microbiota

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to their cytotoxic potential. Most of these initiatives were unsuccessful because antibacterial chemicals rapidly degrade and released quickly (particularly when carried by nanoparticles), which results in low effectiveness and safety concerns (Saafan *et al.*, 2018). Hence, there is a need to discover new and more effective treatment methods for this condition that may be used extensively, safely, and affordably (Nascimento, 2017). Therefore, adopting cutting-edge technology like nanoparticles as capping agents instead of delivery systems may expand the possibility of managing the microflora in the mouth, especially when using inexpensive and biodegradable materials, making preventive dental treatment accessible to all populations.

1.2.4 Use of nanotechnology in dentistry

Nanotechnology is the synthesis and application of nanosized structures through a bottom-up or top-down design at a nanometer scale (1 nm to 100 nm) (Schwass *et al.*, 2018; Song and Ge, 2019; Yin *et al.*, 2020). Metallic nanoparticles having distinctive physico-chemical characteristics, such silver nanoparticles (AgNPs), have been created and successfully applied in several medicinal applications (Noronha *et al.*, 2017; Bapat *et al.*, 2018; Burdușel *et al.*, 2018; Fernandez *et al.*, 2021). Also, for applications in the field of dentistry, novel nanotechnology is being investigated extensively. In dentistry, silver nanoparticles have been used for their antimicrobial activity at a low concentration. They have shown low cytotoxicity and immunological properties making them an ideal option for biomedical applications, including the inhibition of biofilm formation (Adapa, 2020). Silver nanoparticles possess important biological qualities including their effectiveness as anti-bacterial agents against a wide range of bacteria, including types resistant to antibiotics. Consequently, AgNPs application in dentistry have been used in restorative, prosthetic, endodontic, orthodontic as well as periodontal treatments, and dental implant therapy (Bapat *et al.*, 2018; Saravanan *et al.*, 2018; Yin *et al.*, 2020).

AgNPs can be made using a variety of physical and chemical processes (Klaus-Joerger *et al.*, 2001; Chugh, Viswamalya and Das, 2021). However, these methods have shown drawbacks such as the creation of poisonous chemicals that are bad for the environment and cells. Green synthesis methods were created to lessen these hazardous effects by using natural materials as reducing and stabilizing substances, such as plant extracts and microorganisms (such as bacteria, yeasts, and algae) (Iravani *et al.*, 2014; Aboyewa *et al.*, 2021; Nqakala *et al.*, 2021). The use of plant material in green synthesis is often favored over the microbial synthesis

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method as it is simple, cheaper, environmentally friendly, and more effective (Langmuir *et al.*, 2011; Zargar *et al.*, 2011). Plant extracts are easily obtained, and they generate phytochemicals that reduce and stabilize Ag+ into bioactive AgNPs (Chugh, Viswamalya and Das, 2021). Recently, plant extract-mediated AgNPs have been applied in dentistry as therapeutic agents against drug-resistant microbes due to their outstanding antimicrobial agents (Arokiyaraj *et al.*, 2014; Murugan *et al.*, 2014; Rodríguez-luis *et al.*, 2016; Srikar *et al.*, 2016).

In the context of this study, the synthesis of AgNPs using naturally occurring gum Arabic is extensively explored and reported.

1.2.5 Gum Arabic

1.2.5.a General introduction

Gum Arabic (acacia gum) is one of the oldest most well-known natural gums for more the 5000 years (Barak, Mudgil and Taneja, 2020; Habibu *et al.*, 2021; Prasad *et al.*, 2022). It was utilized by the ancient Egyptians to create hieroglyphs and the ancient inscriptions known as kami as a mineral pigment binder and glue in paints. It was also used as a binder in cosmetics and inks and as an adhering agent to make flaxen wrapping for embalming mummies (Habibu *et al.*, 2021). In addition, Ebers Papyrus, which is an Egyptian medical manual, was compiled with some work containing recipes of soot and gum Arabic mixture as tooth cleaning preparations (Kirtley and Kirtley, 2022). Gum Arabic, named after its country of origin, was transported into Europe through several Arabian ports (Habibu *et al.*, 2021).

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1.2.5.b Geographical distribution of gum Arabic

The Acacia gum is one of the natural agricultural resources that is predominantly located in the gum belt region of African countries namely in the westward are Mauritania, Mali, and Senegal; northward and centrally are Burkina Faso, Niger, Nigeria, Benin, Nigeria, Chad, and Cameroon; eastward are Sudan, Somalia, and Eritrea; and southward are Mozambique, Zimbabwe, Botswana, Namibia, and South Africa. It can also be found outside of Africa in Pakistan (Sindh and Baluchistan), Oman and India (Rajasthan, Haryana, Gujrat, MP, UP and Punjab) (Patel and Goyal, 2015; Barak, Mudgil and Taneja, 2020; Prasad *et al.*, 2022). The global production output and main source of gum Arabic is sourced from Sudan. For more than 15 years, Sudan has supplied 80% to 90% of the world's supply of gum Arabic, making it the

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top producer and exporter in the world (Patel and Goyal, 2015; Musa *et al.*, 2018; Barak, Mudgil and Taneja, 2020; Eisa and Elhadi, 2022). In addition, Sudan gum Arabic contributes approximately 15% to the national gross domestic product (GDP) including 15.3 % and 10% economic contribution to households specifically rural inhabitants for survival and during famers off season period (Prasad *et al.*, 2022). In Sudan, the gum Arabic varieties that are cultivated includes the species named *Acacia senegal* and *Acacia seyal* which are both locally referred to as hashab (i.e., hard gum) and Talh (i.e. friable gum) respectively, with *Acacia senegal* being reported to enjoy high commercial importance (Eisa and Elhadi, 2022). According to the Sudanese National Forestry Corporation, it was estimated that the gross area with gum Arabic plantation accounts for 1/5th of Sudan (520,000 km^2), directly providing employment opportunity to more than 5 million people. Hence, the economic impact of gum Arabic trade is significant (Hamad *et al.*, 2021). The Gum Arabic Belt spans 11 states in Sudan, including the Nile regions (White Nile, Sennar, and Blue Nile), the Kordofan regions (North Kordofan, Western Kordofan, and South Kordofan), the Darfur regions (Western Darfur, North Darfur, South Darfur, and East Darfur), and lastly in Gadarif, which produces 5% of Sudan's gum Arabic (Daoub *et al.*, 2018). North Kordofan is the main state which accommodate a huge gum Arabic market in the world (Yasseen, Salih and Ahmed, 2014). It is therefore imperative that beneficiation of gum Arabic requires additional research as in the case of this study.

1.2.5.c Chemical composition of gum Arabic

Gum Arabic is a plant product that is acquired from a variety of Acacia trees with more than 500 hundred species (Patel and Goyal, 2015; Musa *et al.*, 2018; Verma and Quraishi, 2021; Eisa and Elhadi, 2022). Gum Arabic is a spread-chain polysaccharide and often exists as neutral or acidic and it is a blend of salts cations with calcium, magnesium, and potassium constituent (Musa *et al.*, 2018). According to Musa *et al*. (2018), arabinogalactan, glycoprotein, and the arabinogalactan protein complex make up the structure of GA (Musa *et al.*, 2018). Five monomer components galactopyranose, rhamnopyranose, arabinofuranose, arabinopyranose, and uronic acid combine to form the polysaccharide structure. There are also minor amounts of proteins, primarily hydroxyproline, proline, and serine (Patel and Goyal, 2015; Musa *et al.*, 2018; Habibu *et al.*, 2021). The source, or the botanical origin, age of the trees from which it was harvested, climatic and soil conditions, all affect the chemical makeup of gum Arabic (Khalil Azzaoui *et al.*, 2014; Prasad *et al.*, 2022).

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1.2.5.d Physico-chemical properties of gum Arabic

Studies on the properties of gum Arabic have shown its versatility especially in processing and modification for specific applications, regardless of the species (Daoub *et al.*, 2018; Yebeyen and Haile, 2018; Barak, Mudgil and Taneja, 2020). Gum Arabic has no taste, smell, or transparency, and it doesn't interact with any chemicals (Musa *et al.*, 2018). It also has additional characteristics that affect how it is used, including as moisture content, specific optical rotation, tannin presence, ash concentration, and content in proteins (Daoub *et al.*, 2018). These properties may vary significantly with its source (Prasad *et al.*, 2022). For instance, gum Arabic has greater solubility in water (cold and hot) up to 50% w/w, and low viscosity in comparison with other polysaccharides . Hence, significant variation was observed with moisture content and viscosity depending on the climate conditions at the time of storage and the different preparation procedures (temperature, laboratory conditions) at the time of processing (Prasad *et al.*, 2022). On the other hand, ash, protein and nitrogen content of gum were the same irrespective of their origin and climatic condition (Barak, Mudgil and Taneja, 2020).

1.2.5.e Gum Arabic belt in Sudan and its economic and medical application

Sudan's gum Arabic production results in several benefits including: revenues from foreign exchange, the development of sustainable agroforestry, preventing desertification and income for rural communities (Tutu *et al.*, 2019; Habibu *et al.*, 2021; Hamad *et al.*, 2021). The economic importance of gum Arabic production in Sudan enjoys institutional and government recognition, hence there is concerted efforts to develop the gum Arabic sector through research development and government funding (Hamad *et al.*, 2021).

It has been widely reported that gum arabic has therapeutic uses in the food and pharmaceutical sectors as stabilizers, emulsifiers, thickeners, and film-forming agents (Musa *et al.*, 2018; Habibu *et al.*, 2021). It is important to note that the choice of gum Arabic species is determined by the type of application. For example, *Acacia senegal* enjoys preferential use because it is known to form better emulsions and suspensions (Daoub *et al.*, 2018).

Gum Arabic is used in drug delivery by either oral or transdermal process (Kipping and Rein, 2015). It has been used to reduce cholesterol levels, triglyceride and LDL in patients with hyperlipidemia (Mohamed, Gadour and Adam, 2015), in treatment of chronic renal failure (Ali *et al.*, 2008), and in diabetic patients were it lowers the blood glucose levels (Omaima Nasir,

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2016). Furthermore, GA has antimicrobial (used as natural antibiotics for the treatment of various infectious disease) (Bnuyan *et al.*, 2015; Alawi, Hossain and Abusham, 2018), anti-inflammatory (Kamal *et al.*, 2018) and antioxidant (Abd-Allah *et al.*, 2002; Ahmed *et al.*, 2016; Abd-El-Hafez *et al.*, 2017) properties. Because it contains cyanogenic glycosides and numerous enzymes like oxidases and peroxidases as well as preventing the early formation of plaque and inhibiting the growth of periodontal bacteria, GA is regarded as a caries preventative agent in the oral cavity (Jaafar, 2019). In addition, GA acts as a remineralization agent even in fluoride-free conditions (Onishi *et al.*, 2008; Philip, 2019).

Why develop another toothpaste for an already inundated dentifrice market? It is important to continuously consider novel materials and formulations, and assess their antimicrobial efficacy and cytotoxicity potential, as well as their affordability. In the case of the current study, the reducing agent (gum Arabic) is ubiquitous, biodegradable, and interacts with metallic surface to form highly stable chelating agents, hence is environmental-friendly (Hindi *et al.*, 2017; Alwaan, Jafar and Allebban, 2019; Verma and Quraishi, 2021).

1.3 Aim, objectives, and research questions

1.3.1 Aim

The aim of this study was working towards a toothpaste containing GA-AgNPs and assess its antimicrobial activity and cytotoxicity.

1.3.2 Objectives

The objectives were:

a. To conduct focused literature reviews. The first literature review compares the effect of topical application of AgNPs on oral microorganisms to standard antimicrobial agents; the second literature review describes the efficacy of green synthesized AgNPs in dental therapy.

b. To synthesize and characterize GA-AgNPs agent.

c. Assessing the impact of the GA-AgNPs agent on the *in vitro* development of specific oral bacteria.

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d. To determine whether GA-AgNPs agent inhibits Streptococcus mutans growth and adhesion to the tooth surface *in vitro*.

e. To estimate the *in vitro* cytotoxicity of GA AgNPs on the buccal mucosa fibroblast cells and the KMST-6 cells.

f. To incorporate the synthesized GA AgNPs into an inert commercial toothpaste and to determine its antimicrobial and cytotoxicity activities *in vitro*.

1.3.3 Research questions

a. Part one: What is the effect of topical application of AgNPs on dental plaque or oral biofilm as compared to standard antimicrobial agents? Part two: Is chemical or green synthesized AgNPs are more effective in treating dental infections?

b. Can GA be used to synthesize silver nanoparticles?

c. Do GA-AgNPs have any effect on the *in vitro* growth of selected oral microbes?

d. Do GA-AgNPs adhere to the tooth surface *in vitro*?

e. Is there any in *vitro* cytotoxic effect on immortalized cell line due to GA AgNPs?

f. Does the addition of GA AgNPs to an inert toothpaste affect its antimicrobial and cytotoxicity properties *in vitro*?

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Chapter 2: RESEARCH FRAMEWORK AND METHODOLOGY FOR THE OVERALL STUDY

2.1 Executive summary

This chapter presents the body of the dissertation. It is divided into independent sections and follows a logic sequence of the tasks at hand. Each section addresses some of the objectives and research questions as listed in chapter 1. Every section contains an independent set of tasks and experiments and were dependent on the information gained from the preceding studies. Due to material and premise development, they have their own set of propositions and self-determining conclusions.

This chapter has the following sections:

- Two focused literature reviews address objective a) and research question a) and are provided in **Section 2.2**. The literature reviews address the effect of topical application of AgNPs on oral microorganisms compared to standard antimicrobial agents and to review the efficacy of green synthesized AgNPs in dental therapy.
- A series of *in vitro* tests address objectives b), c), d) and e) and research questions b), c), d) and e) and are given in **Sections 2.3**. These tests describe the synthesis and characterization of the GA-AgNPs, including antibacterial and cytotoxicity testing.
- A final section, **Section 2.4**, that addresses objective f) and research question f) and describes the incorporation of GA AgNPs in a commercial toothpaste including antibacterial and cytotoxicity testing of the modified toothpaste.

2.2. Literature reviews

2.2.1. Executive summary

In this section, two literature reviews are presented, each with a different focus.

Review (A): This review addresses part one of objective (a) and answered part one of the research question (a). The purpose of this review was to carry out a thorough and methodical analysis of the literature to gather data, evaluate the available evidence, and compare topical AgNP application to conventional drugs in terms of antibacterial activity. Using a defined

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search strategy, 208 articles were retrieved after scrutinizing titles for suitability, out of which 26 studies were included for analysis. The analysis revealed significant diversity and heterogeneity among studies including outcomes which are related to the concentration and composition of the interventions, species, and genus variation, as well as comparators. This made conclusive comparison difficult.

Review (B): Following review A, the literature search was narrowed towards green synthesis of AgNPs in comparison to chemical synthesis of AgNPs to evaluate their antimicrobial and cytotoxicity actions (review B). This review reported on part two of objective (a) and retorted part two of the research question (a). The focus of this paper was to provide a literature review of plant-mediated synthesis of AgNPs for therapeutic use in oral disease, since it is practical, biocompatible, and readily available everywhere. The literature describes various limitations connected to chemical synthesis. Dental products may contain this plant based AgNPs, but caution must be exercised due to their potential cytotoxicity and antibacterial effects. **STATUS: This review is published in the Journal of Pharmaceutics. Manuscript ID: 14020380**

2.2.2. Review (A): Antimicrobial Effect of Silver Nanoparticles on Oral Microorganisms compared to Standard Topical Antimicrobial Agents: A Review of *In Vitro* Studies

Summary

The control of dental plaque is essential for prevention of oral diseases. Antimicrobial agents may be used to supplement or replace mechanical removal of plaque. The objective of the study was to discover data on the antibacterial properties of chemically and environmentally friendly produced silver nanoparticles (AgNPs) in comparison to traditional antimicrobial treatments on dental plaque by analyzing published literature. Electronic databases: Pubmed, Science Direct, and EBSCOhost were searched for literature published between 2000-2020. The inclusion criteria were in-vitro studies published in English language. Data extraction was done, and bias assessment was done. 208 records were retrieved from the "MeSH terms". 26 studies were selected after removing duplications, and screening of abstracts and full texts using inclusion and exclusion criteria. The results demonstrated antimicrobial activity of AgNPs. However, comparison of antimicrobial effects on dental plaque or oral biofilm of AgNPs against standard antimicrobial agents were not possible due to heterogeneity in

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reducing agents, particle size, and concentrations among the included studies. Therefore, for future comparisons, it is recommended that there be a standardization of key parameters.

Keywords: silver nanoparticles, dental plaque, oral biofilm, antimicrobials, standard antimicrobial agents, oral application.

Introduction

The human oral cavity is colonized by different types of microbial species [1]. These microbes aggregate in a fixed self-generated matrix of extracellular polymeric substance to form dental plaque (biofilm) [2]. Dental plaque is polymicrobial in nature, dominated by bacteria, but it may contain yeasts, protozoa, *Archaea* and viruses [1]. In a healthy individual, dental biofilm maintains a balanced composition of microbial species. Commensal's microbes prevent the development of pathogenic bacteria by occupying the space and absorbing all the available nutrients [3]. When the ecology of the oral flora is disturbed, the microbes that create the biofilm community during states of health change, results in an increase of pathogenic flora in dental plaque [e.g., increase of *Streptococcus mutan*s (*S. mutans*) compared to other commensals Streptococci]. This promotes the growth of oral infections such dental caries, periodontitis and can further develop into systemic infections if left untreated [4].

Due to the advent of bacteria that are resistant to multiple drugs, pathogenic oral biofilm prevention and treatment are challenging. Many oral biofilm structures have become resistant to antibiotics [5]. To date, agents for restricting oral biofilms are limited to broad-spectrum antimicrobial drugs such as chlorhexidine, which has some side effects including calculus development and tooth discoloration and cannot be used on a long-term daily basis. Alternative antibiofilm agents include naturally occurring substances including terpenoids, essential oils, and flavonoids. These substances prevent the formation of cariogenic biofilms, although their antibiofilm efficiency is limited by deficient drug solubility, extra-polysaccharide matrix (EPS) diffusion, and substantivity [6]. Therefore, there is a need to find additional and more effective methods of treating chronic diseases that can be used broadly, safely, and affordably [7].

Nanotechnology has found application in the biomedical sciences to the advancement of antimicrobial resistance to standard antibiotics [8]. Nanotechnology is the process of creating various materials on an atomic and molecular scale [9]. These nanomaterials have high surface area to volume ratio which allow them to exert action even at low concentrations [10]. Advances in nanotechnology and its diverse range of applications is changing the face of dentistry. Silver nanoparticles (AgNPs) are a group of silver atoms at a nanoscale from 1 to

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100 nm. They can exist in different shapes such as nanospheres, nanopores, nanocapsules, nanotubes, and nanoshells [11]. AgNPs, are antimicrobial agents that have been incorporated into various biomaterials to reduce biofilm formation and thus giving a biotic state [12]. AgNPs have been used in restorative dentistry, prosthetic dentistry, endodontics, implantology, oral malignancies, and periodontology with the goal of inhibiting microbial development and so improving oral health [11]. The mechanism of action of AgNPs is related to the amount and discharge rate of Ag^{+} ions. The metallic silver is inert, however when it comes into contact with fluids or moisture it becomes ionized. The ionized silver perforates the bacterial cell membrane, interacts with different structures in the bacterial cell including tissue proteins, plasma membrane, bacterial DNA, and respiratory processes leading to modifications to the bacterial cell wall and nuclear membrane's structure, and subsequently cell necrosis and death [11,12].

Due to their high antimicrobial efficacy, AgNPs have been combined into dentifrices, mouthwashes and even on bristles of toothbrushes. Ahmed *et al*. (2019) indicated that commercially synthesized AgNPs have shown higher antibacterial activity than fluoride and chitosan toothpaste when tested against *S. mutans* [13]. In addition, nanosilver fluoride (NSF) toothpaste demonstrated lower minimum inhibitory concentration (MICs) compared to sodium fluoride (NaF) in preventing bacterial adhesion and caries development. A randomized clinical trial by Al-Sharani *et al*. (2018) examined the activity of AgNPs and chlorhexidine mouthwashes in reducing plaque, gingival, and papilla bleeding scores. They concluded that CHX was better in reducing plaque scores than AgNPs [14]. Furthermore, AgNPs have been synthesized from Miswak and added to xylitol for the formulation of a mouthwash. These AgNPs' antimicrobial activity was tested against *Candida albicans*, *Lactobacillus sp., Staphylococcus aureus*, and *S. mutans* and the results showed efficient zone of inhibition against *S. aureus* and *S. mutans* and a very strong antifungal activity. Therefore, miswak-mediated silver nanoparticles mouthwash along with xylitol maybe an effective substitute for commercial mouthwashes [15]. Thus, AgNPs may be an applicable treatment to conventional mouthwash particularly in those with impaired immunity as it is alcohol free, and non-irritating to patients with mucosal sensitivity and inflammation [12]. Although a lot of work has been done on oral applications of silver nanoparticles, there seems to be disparities in the use of the test organisms, interventions, concentrations, measure of the effect and controls. Hence, the aim of this review is to contrast the antimicrobial effect of AgNPs with standard antimicrobial agents on dental plaque or oral biofilm. The objectives were to (1) To determine the antimicrobial effect of AgNPs (2) To compare the effect of topical application of silver nanoparticles (AgNPs) on dental plaque or oral biofilm to standard antimicrobial agents.

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Methods

The review was performed approached systematically as per the guidelines for PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analysis) [16]. Studies were included in the PICO format for review: Population (P) were the microorganisms associated with dental plaque or biofilm; Intervention (I) were all studies that described a topical application of AgNPs; Comparator (C) were standard antimicrobial agents such as Amoxicillin or Chlorohexidine. Outcome (O) is eradication or reduction of oral biofilm/ dental plaque/ implicated microorganisms. Only full articles published in scientific journals in English between 2000-2020 were included.

Exclusion criteria included case/reports, letters to the editor, experimental/exploratory research, opinion pieces, conference abstracts, and papers written in languages other than English. Narrative and systematic reviews were also excluded.

The focus question was: "What is the effect of topical application of AgNPs on dental plaque or oral biofilm as compared to standard antimicrobial agents?"

Search strategy: EBSCO Host, Science Direct, and Pubmed databases were all searched, and additional records from other sources as well as reference mining were used. Free terms and medical subject headings (MeSH terms) were used in the following search strategy: (("silver"[MeSH Terms] OR "silver"[All Fields]) AND ("nanoparticles"[MeSH Terms] OR "nanoparticles"[All Fields])) AND (("dental plaque"[MeSH Terms] OR ("dental"[All Fields] AND "plaque"[All Fields]) OR "dental plaque"[All Fields]) OR (("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields]) AND ("biofilms"[MeSH Terms] OR "biofilms"[All Fields] OR "biofilm"[All Fields])))) AND ((("mouth"[MeSH Terms] OR "mouth"[All Fields] OR "oral"[All Fields]) AND application[All Fields]) OR mouthrinses [All Fields] OR ("mouthwashes"[Pharmacological Action] OR "mouthwashes"[MeSH Terms] OR "mouthwashes"[All Fields] OR "mouthwash"[All Fields]) OR ("toothpastes"[MeSH Terms] OR "toothpastes"[All Fields] OR "toothpaste"[All Fields]) OR gel[All Fields] OR ("chewing gum"[MeSH Terms] OR ("chewing"[All Fields] AND "gum"[All Fields]) OR "chewing gum"[All Fields])) in Pubmed (Medline).

Studies were selected according to the criteria outlined below:

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- **Study designs**

All included studies were *in vitro* studies.

- **Participants**

All studies that report on the usage of oral microorganisms ('participants') that control dental plaque biofilm formation *in vitro* were included. All studies were from peer reviewed scientific journals.

- **Intervention(s)/ exposure(s)**

All studies that describe a topical application of silver nanoparticles which were developed for preventing or controlling dental plaque or oral microorganisms implicated in dental plaque formation were included.

- **Comparator(s)/ control**

Studies on interventions which made any of the following comparisons were added:

  - One intervention versus the other intervention (e.g., silver nanoparticles versus standard antimicrobial agents).
  - One combination of interventions versus another combination of interventions (e.g., combination of different types of synthesized silver nanoparticles and combination of synthesized silver nanoparticles with standard antimicrobial agents).
  - One intervention versus placebo.

- **Outcome (s)**

Complete eradication or reduction of oral biofilm/ dental plaque/ implicated microorganisms/ change in the gingival index or plaque index.

- **Timing**

All the articles included were for the period from 2000 to 2020.

- **Language**

All included articles reported in English languages.

Screening of titles for eligibility was done by 3 collaborators independently. An inclusive approach was followed: when in doubt, the publication was included but may be excluded at the next stage. A three-stage screening process was adopted: Initial screening of titles was performed, followed by the abstract. Duplicate articles were removed manually.

Two researchers separately extracted the data using a predesigned data extraction method. The data extraction tool included author information, publication location and date, pertinent data

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pertaining to the research question, and PICO (AgNPs synthesis, size, shape, and concentrations used, type of controls and comparisons utilized, microbes involved, and the measures used to determine the effect), statistical analysis performed, and the results of the study. Finally, an appropriate critical appraisal tool was applied to the full texts to determine eligibility and final inclusion and exclusion. The review only includes papers that were deemed eligible by all reviewers. A PRISMA diagram was prepared to keep track of the search and screening process. Disagreements were resolved by discussion between all investigators.

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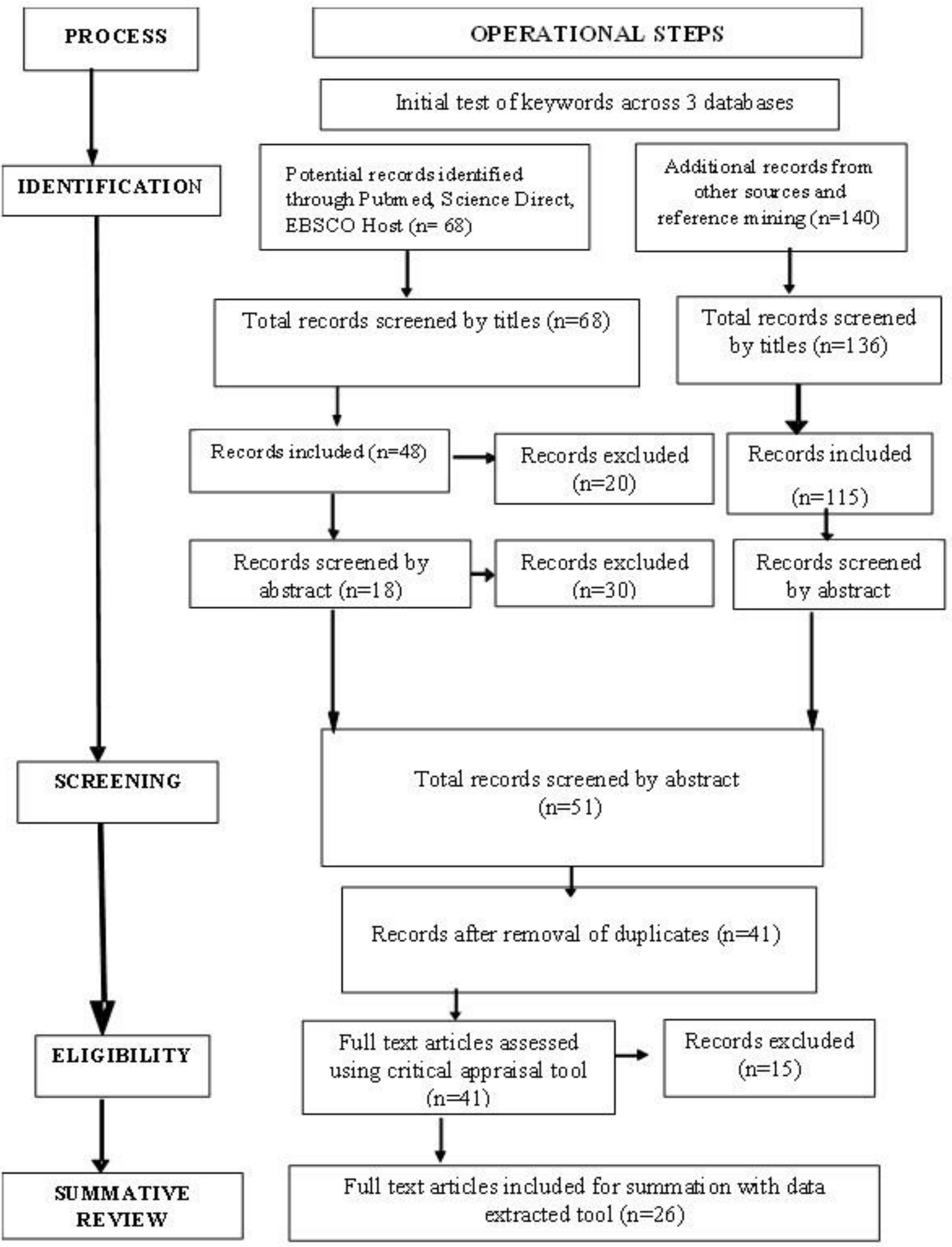

Fig. 1 PRISMA flowchart depicting the workflow of the review

A tailored risk of bias tool was created based on prior evaluations for the Risk of Bias (ROB) assessment [17]. Studies with a low ROB were those that did not record missing items 1-3, those with a moderate ROB were those that reported missing items 4-6, and those with a high ROB were those that reported missing items greater than 6.

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Results

Study selection: There were 208 studies in total found after the initial database search [68 studies from Pubmed, Science Direct, EBSCO Host and 140 studies from other sources and reference mining in line with step one in Fig. (1), PRISMA P]. On screening the titles, 20 studies were removed from UWC databases, and 21 studies were removed from the additional records. The total records screened by abstract were 51 (Fig. 1). After the removal of duplicates, the remaining were 41 studies. Full text articles were examined in detail using a critical appraisal tool (Fig. 1, eligibility). All investigators agreed to exclude studies that did not meet the inclusion criteria [18–32] (Table 1). Twenty-six full text articles were included for summation with data extracted tool and are shown in Table 2.

Study characteristics: After the screened papers were examined, 26 articles that met the inclusion requirements were added to the review. 26 studies total were analyze; two were from China [33,34], three from India [13,35,36], three from Brazil [37–39] and ten from Iran [40–48]. The remaining eight articles were from New Zealand [49], United Kingdom [50], South Korea [51], Czech Republic [52], Egypt [53], United States of America [54], Saudi Arabia [55] and Mexico [56]. All studies used bacterial cultures for detection. Most of the studies included *Streptococcus mutans* (16 articles), some included *Enterococcus faecalis* (18 articles), 25 included *Candida albicans*, 6 included *Lactobacillus acidophilus* and *Lactobacillus fermentum*. The studies employed different AgNP concentrations and different particle sizes.

Risk of bias assessment: The findings of the ROB evaluation are shown in Fig. 2 and 3 adapted from the comprehensive ROB table available on request (Addendum). ROB estimation was based on the reporting of random sequence generation (preference bias, incubation period, incubation conditions, and adjustment to Mcf); blinding (blinding of participants, blinding of outcomes, and tools for measurement); attrition bias (loss of participants); selective reporting, which can affect AgNPs' antibacterial activity, as well as the study's methodology. Of the 26 included studies 3 which were judged as having a high ROB, 7 having an intermediate ROB, while 16 studies had a low ROB.

Outcomes: All research demonstrated a decrease in the number of bacteria using either zones of inhibition (ZOI) or minimum inhibitory and bactericidal concentrations (MICs, MBCs), with only a few studies using live/dead labeling with confocal laser scanning microscopy (CLSM), and colony forming units (CFU). However, no investigation demonstrated a total eradication

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of the microorganisms when the AgNPs were employed in isolation. Table (1) summarizes the information gained from the studies.

Fig. 2 and 3 provide information on the ROB estimation of the 26 included publications.

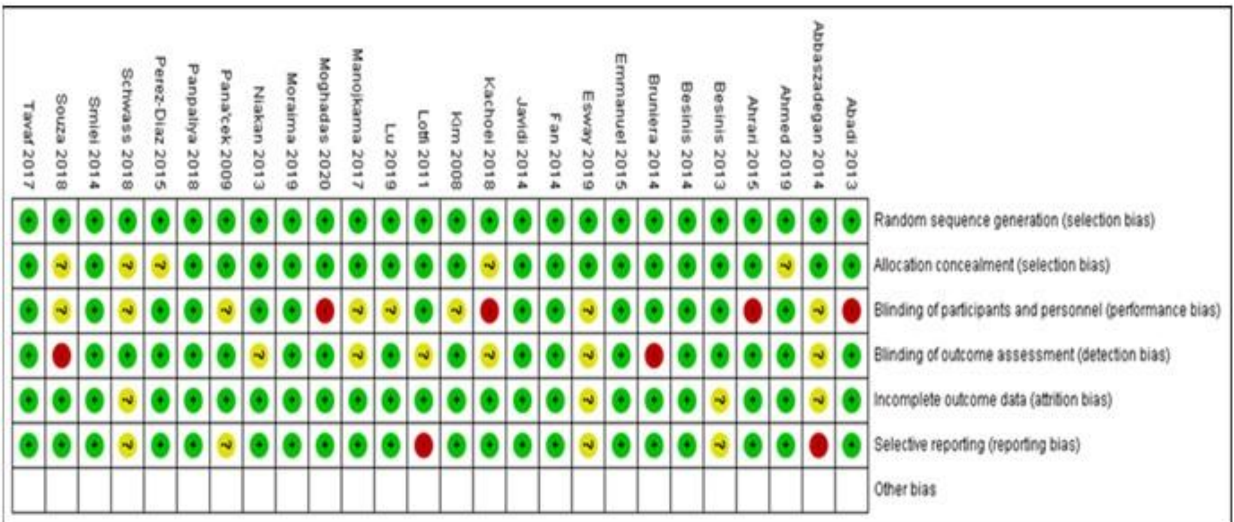

Fig. 2 Risk of bias summary: review author's judgments about each risk of bias item for each included study

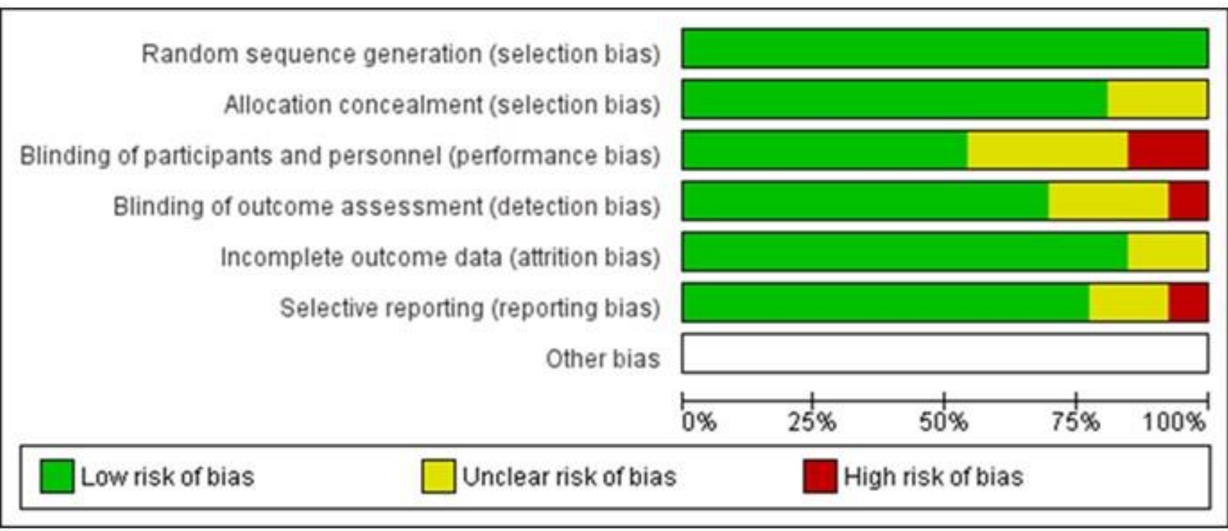

Fig. 3 Review author's judgments about each risk of bias item presented as percentages across all included studies

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Table (1): Excluded articles

Excluded articles	Reason for exclusion	Citation
Evaluation of the efficacy of three antimicrobial agents used for regenerative endodontics: An in vitro study	Synthesis was not mentioned and there was no positive control used	Sadek et al, 2019 [18]
Capping agent-dependent toxicity and antimicrobial activity of silver nanoparticles: An in vitro study	Synthesis was not mentioned	Niska et al, 2016 [19]
Antibacterial effect of silver nanoparticles against Enterococcus faecalis	Synthesis was not mentioned	Alabdulmohsen & Saad, 2017 [28]
The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold	No positive control was used	Hernández-Sierra et al, 2008 [29]
Antimicrobial and cytotoxicity evaluation of colloidal chitosan – silver nanoparticles – fluoride nanocomposites	No positive control was used	Freire et al, 2016 [30]
Silver Colloid Nanoparticles: Synthesis, Characterization, and Their Antibacterial Activity	Not appropriate positive control was used	Panáček et al, 2006 [31]
A new "silver-Bullet" to treat caries in children - Nano Silver Fluoride: A randomised clinical trial	The only randomized control trial study	Dos Santos et al, 2014 [32]
Ultrasonic-assisted green synthesis of flower like silver nanocolloids using marine sponge extract and its effect on oral biofilm bacteria and oral cancer cell lines	Not appropriate positive control was used	Inbakandan et al, 2016 [20]
Antibiofilm efficacy of silver nanoparticles as a vehicle for calcium hydroxide medicament against Enterococcus faecalis	Synthesis was not mentioned	Afkhami et al, 2015 [21]
Study on how nanosilver-based inorganic antibacterial agent functions on biofilm formation of Candida albicans, inside the oral cavity	Synthesis was not clear and no appropriate positive control was used	Wang & Xie, 2016 [22]
Microbial diversity of the supra- and subgingival biofilm of healthy individuals after brushing with chlorhexidine- or silver-coated toothbrush bristles	The only study that used healthy individuals	Do Nascimento et al, 2015 [23]
Evaluation of the antibacterial efficacy of silver nanoparticles against Enterococcus faecalis biofilm	Synthesis was not mentioned	Wu et al, 2014 [24]
Antimicrobial Efficacy of Mineral Trioxide Aggregate with and without Silver Nanoparticles	Synthesis was not mentioned, and the source of strains was not mentioned	Samiei et al, 2013 [25}
Size-dependent antibacterial activities of silver nanoparticles against oral anaerobic pathogenic bacteria	Not appropriate positive control was used	Lu et al, 2013 [26]
Green synthesis and characterization of silver nanoparticles using banana peel extract and their antimicrobial activity against representative microorganisms	Some of the instruments for measuring the outcome was not mentioned, and statistical analysis was unclear	Ibrahim, H.M., 2015 [27]

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Table (2): Characteristics of included studies

Author's Name	Participant's/ Population	Intervention	Controls	Measurments
Chávez-Andrade et al, 2019 [37]	*E. faecalis* (ATCC 29212) *C. albicans* (ATCC 10231)	Chemical	NaOCl 1% Farnesol 4%	MICs MBCs Crystal violet assay SEM CFU
Ahmed et al, 2019 [13]	*S. mutans* (MTCC 890)	Commercial	Fluoride toothpaste (Oral B Pro Health)	ZOI
Lu et al, 2018 [33]	*S. mutans* (ATCC 25175)	Chemical	CHX 10 µg/mL	MICs MBCs Live Dead Kit Growth Kinetics
Souza et al, 2018 [38]	*S. mutans* (ATCC 25175) C. albicans (ATCC 10231)	Green	CHX 180 mg/mL	MBCs CFU
Schwass et al, 2018 [49]	*S. mutans* (UA 159) *E. faecalis* (JH22)	Chemical	CHX Silver diamonde fluoride Isopropanol (70%)	MICs MBCs ZOI
Besinis et al, 2014 [50]	*S. mutans* (NCIMB 702062)	Commercial	CHX 1% (v/v)	Live Dead Kit SEM
Lotfi et al, 2011 [40]	*E. faecalis* (ATCC 2367)	Chemical	MICs: CHX 2% NaOCl 5.25% NaOCl 0.33% ZOI: Vancomycin papers	MICs ZOI
Kim et al, 2008 [51]	*C. albicans* (ATCC 90028)	Chemical	Amphotericin B	MICs
Panáček et al, 2009 [52]	*C. albicans* (I and II) *C. tropicalis* *C. parapsilosis*	Chemical	Itraconazole Fluconazole Amphotericin B Voriconazole Caspofungin	MICs Growth kinetics
Esawy et al, 2019 [53]	*S. mutans*	Green	Forcetex (Cefepime)	MICs ZOI
Panpaliya et al, 2019 [35]	*S. mutans* (MTCC 497) *L. acidophilus* (MTCC 10307) *L. fermentum* (MTCC 903) *C. albicans* (MTCC 183)	Commercial	CHX 2% Perioshield mouth wash	MICs MBCs
Tavaf et al, 2017 [41]	*S. mutans* (PTCC 1683)	Green	Ampicillin 0.03-2µg	MICs MBCs Crystal violet assay Growth kinetics
Javidi et al, 2014 [42]	*E. faecalis* (ATCC 29212)	Not mentioned	$Ca(OH)_2$	CFU
Abbaszadegan et al, 2014 [43]	*E. faecalis* (AGH 011)	Chemical & Green	CHX 0.2% NaOCl 2.5%	MICs
Fan et al, 2014 [34]	*E. faecalis* (ATCC 292120	Chemical	$Ca(OH)_2$	Live Dead Kit CFU SEM

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Samiei et al, 2014 [44]	*E. faecalis* (PTCC 1237)	Chemical	CHX 2% NaOCl 2.5%	MICs Efficacy of SCHNC on disinfecting root dentine
Bruniera et al, 2014 [39]	*S. mutans* (ATCC 25175) E. faecalis (NCTC 775)	Chemical	CHX 0.12%	MICs MBCs
Moghadas et al, 2020 [45]	*E. faecalis* (ATCC 29212)	Chemical	NaOCl 5.25%	CFU
Besinis et al, 2014 [54]	*S. mutans* (NCIMB 702062)	Commercial	CHX 0.5%	MICs
Abadi et al, 2013 [46]	*S. mutans* (ATCC 25175) *C. albicans* (ATCC 10231)	Commercial	Ethanol 30,000 µg/mL	MICs MBCs CFU
Kachoei et al, 2018 [47]	*S. mutans*	Chemical	CHX 0.2% Sodium fluoride 0.05% Mixture of sodium fluoride 0.05% plus CHX 0.2%	ZOI
Niakan et al, 2013 [57]	*S. mutans* (ATCC 35668)	Chemical	Amoxicillin 25 µg/disc	ZOI
Ahrari et al, 2015 [48]	*S. mutans* (PTCC 1683)	Commercial	CHX 0.2% Sodium fluoride mouth washes 2.0%	MICs MBCs
Emmanuel et al, 2015 [55]	*S. mutans* *L. acidophilus* *C. albicans*	Green	Azithromycin/ Clarithromycin	MICs ZOI
Pérez-Díaz et al, 2015 [56]	*S. mutans* (Clinical isolate)	Chemical	Oxacillin	MICs
Manojkama et al, 2017 [36]	*E. faecalis* *C. albicans*	Green	$Ca(OH)_2$	ZOI

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Tables 3 to 5 provide characteristics and the effect measures for reducing agents used in the chemically synthetized, green synthesized and commercially available AgNPs.

Table (3): Reducing agents/ Capping agents used in chemical synthesis of AgNPs and their effect measure on microbial growth

Author's Name	Microorganisms	Reducing Agents (RA)/ Capping Agents	Particle size	Shape	Measure of the Effect
Chávez-Andrade et al, 2019 [37]	*C. albicans* (ATCC 10231)	**Poly-vinyl alcohol**	4-11 nm	Not mentioned	**MICs**= 27.5 µM **MBCs**=37.5µM
Kim et al, 2008 [51]	*C. albicans* (ATCC 90028)	**Nitric acid with sodium chloride**	3 nm	Spherical	**MICs**=2µg/ml
Panáček et al, 2009 [52]	*C. albicans* (I)	**D-maltose**	25 nm	Not mentioned	**MICs**=0.42mg/L
Panáček et al, 2009 [52]	*C. albicans* (II)	**D-maltose**	25 nm	Not mentioned	**MICs**=0.21mg/L
Panáček et al, 2009 [52]	*C. parapsilosis*	**D-maltose**	25 nm	Not mentioned	**MICs**=1.69mg/L
Panáček et al, 2009 [52]	*C. tropicalis*	**D-maltose**	25 nm	Not mentioned	**MICs**=0.84mg/L
Abbaszadegan et al, 2014 [43]	*E. faecalis* (AGH 011)	**Sodium borohydride**	7.5-10.1 nm	Not mentioned	**MICs50**: PC AgNPs=5.7×10-15 mol/L **MICs90:** PC AgNPs=5.7×10-10 mol/L **MICs50:**NC AgNPs=9.7×10-8 mol/L **MICs90**:NC AgNPs= -
Chávez-Andrade et al, 2019 [37]	*E. faecalis* (ATCC 29212)	**Poly-vinyl alcohol**	4-11 nm	Not mentioned	**MICs**=42.5 µM **MMCs**=0.85%
Moghadas et al, 2020 [45]	*E. faecalis* (ATCC 29212)	**Sodium thioglycolate + Sodium thiosulfate**	1-100 nm	Not mentioned	**CFU**=0 CFU/mL
Fan et al, 2014 [34]	*E. faecalis* (ATCC 29212)	**Tetraethyl orthosilicate**	2-4 nm	Spherical	**Live Dead Kit** **CFU** **SEM**
Schwass et al, 2018 [49]	*E. faecalis* (JH22)	**Sodium borohydride**	6.7-9.2 nm	Not mentioned	**MICs**=19.2µg/mL **MBCs**=38.4µg/mL **ZOI**=3mm
Bruniera et al, 2014 [39]	*E. faecalis* (NCTC 775)	**Sodium borohydride**	1-100 nm	Spherical	**MICs**=7100ng/mL **MBCs**=7100ng/mL
Samiei et al, 2014 [44]	*E. faecalis* (PTCC 1237)	**Sodium borohydride**	20-30 nm	Spherical	**MICs**=15ppm

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Kachoei et al, 2018 [47]	*S. mutans*	**Photo reduction**	20-50 nm	Hexagonal	**ZOI:** (Ag/ZnO10)(mg/100mL)]= 16.60±0.49mm (Ag/ZnO15)(mg/100mL)]= 14.50±0.50mm (Ag/ZnO20)(mg/100mL)]= 13.80±0.40mm (Ag/ZnO10+NaF0(mg/100mL)] = 15.7±0.46mm (Ag/ZnO10+CHX)(mg/100mL)] = 15.30±0.46mm
Bruniera et al, 2014 [39]	*S. mutans* (ATCC 25175)	**Sodium borohydride**	1-100 nm	Spherical	MICs=7100-3600ng/mL MBCs=7100-3600ng/mL
Lu et al, 2019 [33]	*S. mutans* (ATCC 25175)	**Tetraethyl orthosilicate**	2-5 nm	Spherical	**MICs**: Ag-MSNs at CHX=12.5µg/ml **MBCs**: Ag-MSNs at CHX=25 µg/mL **MBIC**=50µg/mL **Live Dead Kit** **Growth Kinetics**
Niakan et al, 2013 [57]	*S. mutans* (ATCC 35668)	**Polyaldehyde**	2-6 nm	Not Mentioned	**ZOI**=8.5 ± 0.5/7± 0mm
Pérez-Díaz et al, 2015 [56]	*S. mutans* (Clinical isolate)	**Gallic acid**	9-99 nm	Spherical	**MICs:** AgNPs size: 9.5±1.1= 4.0±00ppm 25.9±2.6= 8.0±00ppm 78.7±19.2= 16.0±0ppm
Schwass et al, 2018 [49]	*S. mutans* (UA 159)	**Sodium borohydride**	6.7 -9.2 nm	Spherical	**MICs**=12.8µg/mL **MBCs**=38.4µg/mL **ZOI**=6mm

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Table (4): Reducing agents used in green synthesis of AgNPs

Author's Name	Microorganisms	Reducing Agents	Particle size	Shape	Measure of the Effect
Souza et al, 2018 [38]	*C. albicans* (ATCC 10231)	***Punica granatum*** **peel**	50 nm	Spherical	**MBCs:** Peel mg/L; AgNPs+CaGP=156.2 AgNPs=312.5 Seeds mg/L ; AgNPs+CaGP=312.5 AgNPs=>1250.0 Leaves mg/L; AgNPs+CaGP=9.8 AgNPs=4.9
Emmanuel et al, 2015 [55]	*C. albicans*	***Justicia glauca*** **leaf**	10-20 nm	Spherical	**MICs:** AgNPs=25 ± 0.45µg/mL **ZOI:** AgNPs=26mm AZM (50 µg) + AgNPs (50 µg)=28mm CLR (50 µg) + AgNPs (50 µg)=27mm
Manojkama et al, 2017 [36]	*C. albicans*	***Plectranthus ambionicus***	Not mentioned	Not mentioned	**ZOI:** 1000µg=11±0.72mm 2000µg=16.35±0.46mm 3000µg=22.15±0.48mm
Abbaszadegan et al, 2014 [43]	*E. faecalis* (AGH 011)	**Rice**	7.5-10.1 nm	Not mentioned	**MICs50:** Neut AgNPs =4.0×10-9 mol/L **MICs90:** Neut AgNPs= _____
Manojkama et al, 2017 [36]	*E. faecalis*	***Plectranthus ambionicus***	Not mentioned	Not mentioned	**ZOI:** 1000µg=10.15±0.38mm 2000µg=14.5±0.75mm 3000µg=19.35±0.95mm
Souza et al, 2018 [38]	*S. mutans* (ATCC 25175	**Pomegranate**	50 nm	Spherical	**MBCs:** Peel mg/L; AgNPs+CaGP=156.2 AgNPs=78.1 Seeds mg/L; AgNPs+CaGP=156.2 AgNPs=625.0 Leaves mg/L; AgNPs+CaGP=625.0 AgNPs=125.0

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Tavaf et al, 2017 [41]	*S. mutans* (PTCC 1683)	**Bacterial (*E.coli*)**	50-100 nm	Regular	**MICs:** 10±0.2µg **MBCs:** 14±1µg **Crystal violet assay** **Growth kinetics**
Emmanuel et al, 2015 [55]	*S. mutans*	***Justicia. glauca* leaf**	10-20 nm	Spherical	**MICs:** 75 ± 0.25µg/mL **ZOI:** AgNPs=25mm AZM (50 µg) + AgNPs (50 µg)=28mm CLR (50 µg) + AgNPs (50 µg)=27mm
Esawy et al, 2019 [53]	*S. mutans*	***Punica granatum* peel**	4-27 nm	Spherical	**MICs:** AgNPs PGPC, AgNPs PGPM=100µg/mL **ZOI:** AgNPs PGPC=16.20±0.20mm AgNPs PGPM=18.03±0.15mm **Mouthwash:** AgNPs PGPC=20.1 ± 0.36mm AgNPs PGPM=18.03 ± 0.25mm
Emmanuel et al, 2015 [55]	*L. acidophilus*	***Justicia glauca* leaf**	10-20 nm	Spherical	**MICs:** 50 ± 0.24µg/mL **ZOI:** AgNPs=25mm) AZM (50 µg) + AgNPs (50 µg)=28mm CLR (50 µg) + AgNPs (50 µg)=27mm

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Table (5): Origin of the reducing agents used in commercial synthesis of AgNPs

Author's Name	Microorganisms	Origin of the Reducing Agents	Particle size	Shape	Measure of the Effect
Abadi et al, 2013 [46]	*C. albicans* (ATCC 10231)	**Nano Nasb ParsCo. (Tehran, Iran)**	10-40 nm	Spherical	**MICs:** 0.78µg/ml **MBCs:** 1.56µg/mL
Panpaliya et al, 2018 [35]	*C. albicans* (MTCC 183)	**Nano-world company (Calcutta, India)**	3-50 nm	Not mentioned	**MICs:** 2.82µg/mL **MBCs:** 48µg/mL
Lotfi et al, 2011 [40]	*E. faecalis* (ATCC 2367)	**Nano-world company (Calcutta, India)**	35 nm	Not mentioned	**MICs:** **AgNPs;** 6H=12.5µg/ml 18H/24H/28H=50µg/ml **ZOI:** 4000µg/ml=14mm 50µg/ml=13mm 25µg/ml=10mm
Panpaliya et al, 2018 [35]	*L. acidophilus* (MTCC 10307)	**Nano-world company (Calcutta, India)**	3-50 nm	Not mentioned	**MICs:** 15µg/mL **MBCs:** 9µg/mL
Panpaliya et al, 2018 [35]	*L. fermentum* (MTCC 903)	**Nano-world company (Calcutta, India)**	3-50 nm	Not mentioned	**MICs:** 90µg/mL **MBCs:** 119.6µg/mL
Abadi et al, 2013 [46]	*S. mutans* (ATCC 25175)	**Nano Nasb ParsCo. (Tehran, Iran)**	10-40 nm	Spherical	**MICs:** 3.12 µg/mL **MBCs:** 6.25µg/mL
Panpaliya et al, 2018 [35]	*S. mutans* (MTCC 497)	**Nano-world company (Calcutta, India)**	3-50 nm	Not mentioned	**MICs:** 60µg/mL **MBCs:** 18.5µg/mL
Ahmed et al, 2019 [13]	*S. mutans* (MTCC 890)	**TruCare-TruCare Nanosilver Toothpaste**	Not mentioned	Not mentioned	**ZOI:** AgNPs (Mean ± SD) mm =20.14±0.96 SEM= 0.21.
Besinis et al, 2013 [50]	*S. mutans* (NCIMB 702062)	**Sigma-Aldrich**	Not mentioned	Not mentioned	**Live Dead Kit SEM**
Besinis et al, 2014 [54]	*S. mutans* (NCIMB 702062)	**Sigma-Aldrich**	56.8 nm	Not mentioned	**MICsL:** 50mg/L
Ahrari et al, 2015 [48]	*S. mutans* (PTC 1683)	**Fanavaran Araz Tajhiz Co., Iran**	5-50 nm	Not mentioned	**MICs/ MBCs:** 25µg/mL

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Discussion

As per the present review, PRISMA guidelines (Fig. 1) were followed. All the studies included presented in table (2) having excluded studies in table (1) demonstrated antimicrobial activity of AgNPs against the microbes tested. However, comparison of antimicrobial effect on dental plaque or oral biofilm of AgNPs against standard antimicrobial agents was difficult to be done due to the diversity in reducing agents, particle size, and concentrations of AgNPs as well as diversity within the conventional antimicrobial among the included studies.

The analysis in this review was stratified according to population (micro-organisms associated with dental plaque biofilm). From the 26 included studies, there were 7 population groups made up of four genera namely *Candida, Enterococcus, Lactobacillus and Streptococcus*. Twenty-five studies use *Candida* species, 18 studies use *Enterococcus*, six studies used *Lactobacillus*, and 16 studies used *Streptococcus* (Table 2). The genus *Candida* was the most heterogenous genus group, with three species (*C. albicans*/ *C. parapsilosis*/ *C. tropicalis*), followed by the *lactobacillus* genus with two species (*L. acidophilus* and *L. fermentus*), while *Enterococcus* and *Streptococcus* were homogeneous with one species in each genus. There was also diversity within each species used according to the different type strains as well as whether the strains used were clinical isolates.

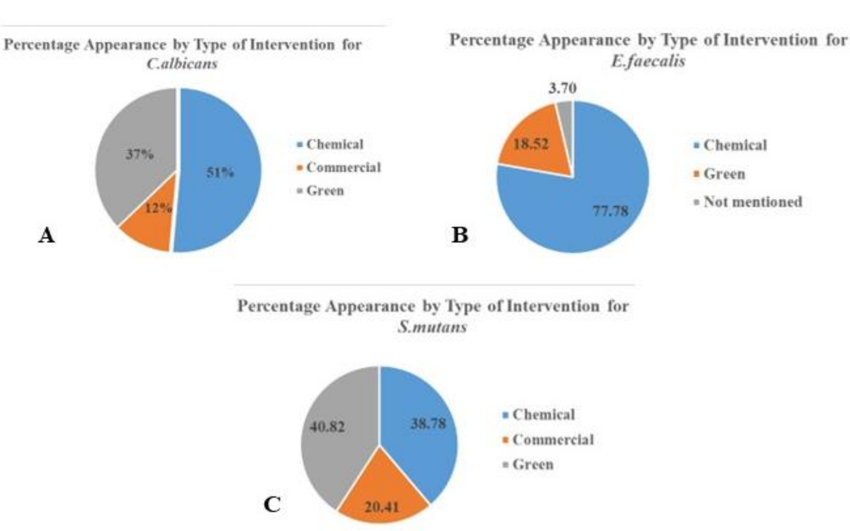

Fig. 4 Comparison between chemical, green, and commercial AgNPs of all microbes

Within the *Candida* genus, there were three types of interventions namely by chemical, green, and commercial AgNPs (Fig. 4A). The studies used different AgNP synthesis methods: chemical (n=18. 51%), followed by green (n=4. 37%), and commercial (n=3. 12%) (Fig. 4A).

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There were many different controls across the 25 studies on *Candida*. Thirteen types of controls were used namely Amphotericin B (n=4), Azithromycin (n=1), $Ca(OH)_2$ (n=1), Caspofungin (n=3), CHX (n=2), Clarithromycin (n=1), Ethanol (n=1), Farnesol (n=1), Fluconazole (n=3), Itraconazole (n=3), NaOCl (n=1), Periosheild mouthwash (n=1), Voriconazole (n=3). The apparent variation in the controls could be due to the different species used e.g., Amphotericin B against *C. albicans*, Caspofungin used against *C. parapsilosis*, and Itraconazole against *C. tropicalis*. Although there was a variation in types of controls used, there was more than one control used across all the species. Among the controls used, the conventional antimicrobials were Amphotericin B, Azithromycin, Caspofungin, Clarithromycin, Fluconazole, Itraconazole, and Voriconazole, where Amphotericin B was predominant (n=4).

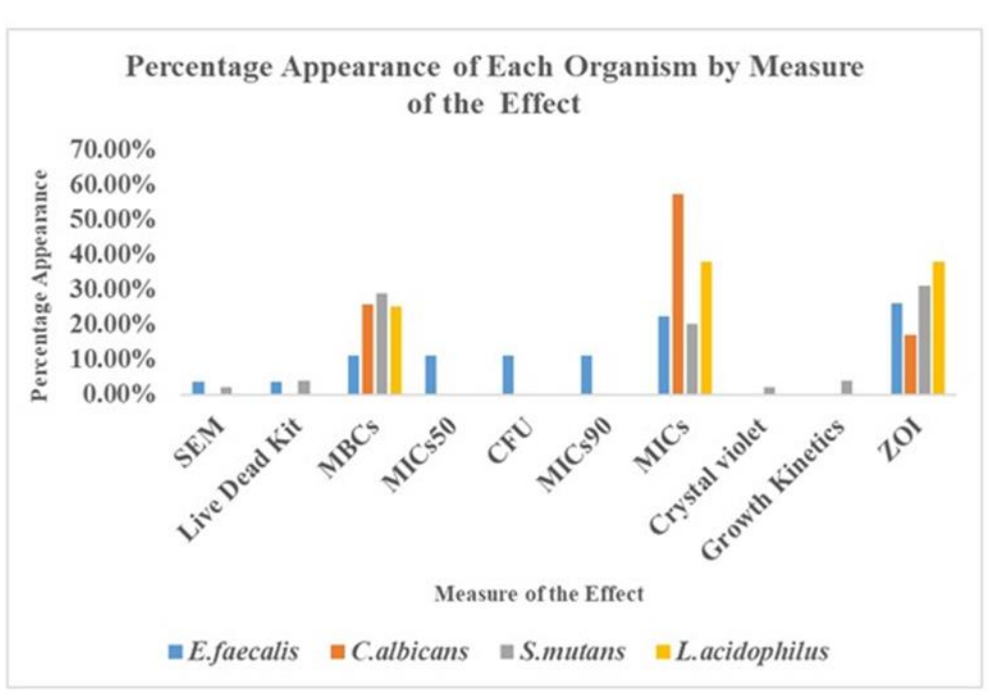

Fig. 5 Percentage appearance for each organism by measure of the effect

There were more similarities in the methods of measuring antimicrobial activity across the 25 studies in the *Candida* species, where MICs predominated (n=19), followed by MBCs and ZOI (n=4 and 2 respectively) (Fig. 5 in % contribution). There was consistency in the units used to present MICs with mg/L (n=16), µg/ml (n=4) and µM (n=2) (Table 3). The µM unit was only used in studies using chemical synthesis (Table 3). The measure of MICs ranges from 0.21-1.69 mg/L, which could be attributed to differences in concentrations of the NPs, differences in species, and their innate resistance profile. The MBCs reported ranged from 4.9-1250 mg/L. This variation comes mainly from one study where different parts of the plant were used [38].

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The ZOI appeared similar across the studies, ranging from 11-26 mm from 4 experimental settings in 2 studies, whilst also differing in concentration [36,55].

With regards to the 18 studies which evaluated *E. faecalis,* there was similarity within the genus but with six different type strains. The studies used different synthesis methods including chemical and green, with the chemical synthesis (n=15, 77.78%), and the green (n=3, 18.52%) (Fig. 4B). In one study the synthesis of the intervention was not mentioned [42]. The comparators within the included studies varied. There were seven types of controls used: $Ca(OH)_2$ (n=3), CHX (n=6), Isopropanol (n=1), Farnesol (n=1), NaOCl (n=6), Silver diamond fluoride (n=1), and Vancomycin (n=1). Out of those controls, the conventional antimicrobials were CHX and Vancomycin. There were contrasts with respect to the concentrations used for CHX and NaOCl. There was uniformity within the outcomes reported, with MICs (n=12), MBCs (n=2), ZOI (n=3), CFU (n=3), and only one study reported in live dead and SEM (Fig. 5 in % contribution) [34]. The units for the measure of the effect in the MICs were dissimilar, with µg/ml, ng/ml, µM, mol/L, ppm. Among the 3 studies that reported ZOI as the measure of the effect, the concentrations ranged between 25-4000 µg/ml resulting in 3-19 mm diameter zones. However, one study reported 19.35mm zones from 3000 µg/ml [36], while another study reported 14 mm resulting from 4000 µg/ml [40]. This discrepancy could be due to different types of strains used, concentrations used, and conditions surrounding the experiment e.g., green vs chemical synthesis.

All studies reported similarities with Streptococcal genus, even though seven different types of strains were used across the 16 studies [13,33,35,41,49,50,57]. Different interventions were used, with green synthesis (n=40.82%), followed by chemical synthesis (n=38.78%), and commercial synthesis (n=20.41%) (Fig. 4C). In addition, different controls were used with 15 types of controls namely Amoxicillin (n=1), Ampicillin (n=1), Azithromycin (n=1), CHX (n=9), Clarithromycin (n=1), Ethanol (n=1), Fluoride toothpaste (n=1), Forcetex (cefepime) (n=2), Isopropanol (1), mixture of sodium fluoride plus CHX (n=1), Oxacillin (n=1), Perioshield mouth wash (n=1), Silver diamine fluoride (n=1), Sodium fluoride (n=2). There were 7 conventional antimicrobials used amongst the controls, that is Amoxicillin, Ampicillin, Azithromycin, CHX, Clarithromycin, Cefepime, and Oxacillin. The results of these studies demonstrated MICs (n=11), MBCs (n=7), ZOI (n=8), Live dead (n=2), Growth kinetics (n=2), SEM (n=1), and Crystal violet (n=1). There was uniformity in the units used in the measure of the effect for MICs and MBCs (µg/ml), with one study reporting ppm. The MICs ranges between 3.12-75 µg/ml, and MBCs ranging from 3.6-125 µg/ml, depending on the conditions

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surrounding the experiments. The ZOI ranges from 6-25 mm in diameter influenced by the synthesis and concentrations of the interventions, plus the strains used.

There were two genera of Lactobacilli among six studies used, with 3 different type strains. Furthermore, there were similarities in the interventions used with 4 studies [35] using commercial synthesis, and 4 studies [55] using green synthesis. The controls used were diverse with 4 types including Azithromycin (n=1), CHX (n=2), Clarithromycin (n=1), and Perioshield mouthwash (n=2). Most of the controls used were conventional antimicrobials except for one study (Perioshield mouthwash). The MICs for these studies (n=3) ranged between 15-50 µg/ml for *L. acidophilus*, and 90 µg/ml for *L. fermentum.* The MBCs (n=2) for *L. acidophilus* was 9 µg/ml, and 119.6 µg/ml for *L. fermentum.* The ZOI was only reported by one study [55], and the diameter of the ZOI for AgNPs was about 25 mm (Fig. 5).

For the interventions, 13 studies [33,37,39,43–45,47,49,51,52,56,57] reported using chemically synthetized AgNPs, where there was more consensus in the use of silver nitrate (n=12) as a precursor (Fig. 5). In 4 studies [39,43,44,49] the reducing agent utilized was sodium borohydride, while D-maltose, poly-vinyl alcohol, and polyaldehyde were used, among other studies [37,52,57](n=1 each) (Table 3). The consistency in the use of silver nitrate was also reflected in the particle size which ranged between 1-30 nm in ten studies, with only two studies that deviated reporting a range of 1-100 nm [39,56]. The shape of the AgNPs was reported as spherical in four out of the 12 studies using silver nitrate as precursor [39,44,49,51]. The shape was not reported in the other studies, with only one study reporting hexagonal shape [47]. Because of the diversity in the population (microorganisms), each form of the intervention was further stratified accordingly within the silver nitrate studies. There were 6 studies [33,39,47,49,56,57] in *S. mutans* in the above-mentioned group, where there was disagreement in MICs, (n=4), and MBCs (n=3), and consistency in the ZOI (n=3 where there was 7 experiments ranging between 6-16mm) (Table 3) [47,49,57]. The disagreement between the studies of MICs and MBCs could emanate from the broad range in particle size used (1-100 nm), while the consistency in ZOI agrees with the particle size ranging from 2-50 nm. With regards to *E. faecalis,* 5 studies reported in MICs [37,39,43,44,49], 2 studies in MBCs [37,49], and only one in ZOI [49], Live dead kit, SEM and CFU [34] (Table 3). Due to inconsistency in reporting comparison could not be done. For an example, MICs were reported at 90% (n=1) and 50% (n=1) [43], while the other studies were non-descriptive. Furthermore, there was inconsistency in the units for the measure of the effect, where one study reported in percentage concentration [37], while other reported in ppm [44]. For *Candida* species, three studies described MICs within the range of 0.21-2 µg/ml, while one study reported MBCs in µM [37].

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This consistency is also reflected in particle size ranging from 3-25 nm (Table 3). There were no reports in chemical synthesis of AgNPs used against *Lactobacillus* species.

In respect to green synthesis of AgNPs, there were 6 studies with disparity in the reducing agents, namely 2 studies used pomegranate [38,53], one used rice [43], one used *Justicia glauca* leaf [55], one used *Plectranthus ambionicus* [36], and one used *E.coli* [41] (Table 4). The particle size ranges from 4-100nm across the seven studies, where 3 studies reported spherical morphology [38,53,55] while the remaining did not mention, with only one study reporting a regular shape of AgNPs [41]. Studies in *S. mutans* demonstrated MICs (n=3) [41,53,55], MBCs (n=2) [38,41], ZOI (n=2) [53,55], and one study in Crystal violet assay and Growth kinetics [41] (Table 4).The MICs ranged between 10-100 µg/ml, this wide range could be due to the diversity in the reducing agents used as well as the particle size. Also, the MBCs had a wide range from 14-625 µg/ml, which can also be attributed to the same circumstances as mentioned above. The ZOI ranges between 16-25mm, this narrow range is consistent with the narrow range of the particle size (4-27nm) in the associated studies (Table 4). For *Candida* species, one study recorded a diversity in MBCs ranging from 312.5-1250 µg/ml, which is due to the use of different parts of the plant [38]. Two studies reported in ZOI ranging from 11-26 mm, with one study reporting the particle size to be between 10-20nm [55] and other one did not report on the particle size [36]. Additionally, only one study demonstrated that the MICs was 25 µg/ml [55] (Table 4). With respect to *E. faecalis*, there were only two studies that used green synthesized AgNPs. The results of these studies were dissimilar, with one study reported on MICs50 and MICs90 with particle size between 7.5-10.1 nm [43], and the other study reported in ZOI (10-19 mm) without mentioning the particle size [36], making it difficult to research a conclusive outcome (Table 4). There was only one study that employed synthetic green AgNPs and tested them against *L. acidophilus,* and the results revealed a spherical AgNPs with particle size in the range between 10-20 nm, and the MICs were 50 µg/ml, and ZOI were 25 mm [55].

Commercially synthesized AgNPs were used in seven studies (Table 5). Among the studies in table (5) these studies, no study indicated the reducing agents used, with four studies reported on the particle size in the range of 3-56 nm [35,46,48,50], while the other two did not mention. There was only one study that reported the shape of AgNPs to be spherical [46]. Investigated studies in *S. mutans* revealed MICs (n=3) between 3-60 µg/ml [46,48,54], while MBCs (n=2) were between 6-25 µg/ml [46,48], and ZOI (n=1) was 20 mm [13]. Only 2 studies examined commercially AgNPs against *C. albicans,* and the findings indicated MICs in the 0.78 to 2 g/ml range, and MBCs between 1.56-48 µg/ml [35,46]. Only one study utilized commercial AgNPs

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against *E. faecalis*, with MICs ranged between 12.5-50 µg/ml, and ZOI that ranged between 10-14mm [40]. In addition, only one study reported on *Lactobacillus* species, and the results indicated MICs of 15 µg/ml, and MBCs of 9 µg/ml for *L. acidophilus*, while for *L. fermentum* the MICs and the MBCs were 90 µg/ml, and119 µg/ml respectively [35].

Despite the diversity in the conventional antimicrobials, there was a consensus in a number of studies where AgNPs had comparable effect to standard antimicrobials [13,39,41,47,50–53,56,57], with a few studies indicating lower activity in the AgNPs group [35,43,44]. Overall, the review data revealed a few studies (n=4) related to topical application [13,47,48,53], with 3 out of the 4 studies that used mouthwash [47,48,53] and only one study used toothpaste [13]. All these studies recommended the use of AgNPs for topical application. However, more studies are needed for comprehensive analysis of beneficial effect with cytotoxicity assays. This may rationally lead to meaningful and safe clinical applications.

On updating the search to date, the system retained 1150 studies with 87 duplicated articles, and 13 relevant articles according to previously set parameters using Covidence. Among these studies, few new articles were considered eligible for consideration in this study. Most of the remaining studies were excluded due to differential comparators and missing information of intervention. The added studies up to 2023 reinforce the previous conviction (Addendum).

Conclusion

In this study, AgNPs demonstrated antimicrobial activity against the microbes reported similar to the standard antimicrobial agents. Within the AgNPs, chemically synthesized AgNPs seemed to have more effect on *Candida* species, while green synthesized AgNPs showed more effect on *S. mutans* and *E. faecalis*. The heterogeneity in the outcomes could have been affected by the concentrations of the intervention and the species variation. Similarly, the heterogeneity in the standard antimicrobials could also have been due to internal factors as well as external factors such as diversity within the controls, concentrations used and species variation respectively.

Recommendations and future line of research

- A need for standardization of protocols with respect to organisms used and the concentration of the nanoparticles along with the comparators.
- The concentration of the nanoparticles may be benchmarked from cytotoxicity studies to avoid aggravation of mammalian tissues for topical application.

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- Furthermore, research could be directed toward green synthesized AgNPs due to their biocompatibility and effect on cariogenic bacteria.

  In future studies a wider search strategy will have to be implemented.

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Critical Appraisal Checklist for *In-Vitro* Studies

Reviewer……………………………………………Date………………………………………..

Author……………………………………………….Year…………….Record number…………

	Yes	No	Unclear	NA
1.Is the study relevant to the research question? *- Is the topic being relevant to one's own field of work*	☐	☐	☐	☐
2.Does the study add anything new? *- A study might increase confidence in the validity of previous research by replicating its findings or might enhance the ability to generalize a study by extending the original research findings to a new population.*	☐	☐	☐	☐
3.Were the study samples taken from the same population? *- The microbes should be isolated from single colony, adjusted to 0.5McF and vortexed before sampling.*	☐	☐	☐	☐
4.Were the study samples described? *- Type of the strains used and source of strains.*	☐	☐	☐	☐
5.Were the settings described? *- Media used, conditions, volumes pipeted.*	☐	☐	☐	☐
6. Enough information on intervention/agent? *-Synthesis of the agent, concentration used.*	☐	☐	☐	☐

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7. Was a control group present? ☐ ☐ ☐ ☐

- *Comparison to gold standard measure.*

8. Was the exposure measured in a valid and reliable ☐ ☐ ☐ ☐

Way?

- *Techniques used.*

9. Were the outcomes measured in a valid and reliable ☐ ☐ ☐ ☐

Way?

- *Instruments for observation.*

10.Was appropriate statistical analysis used? ☐ ☐ ☐ ☐

- *Sample size calculation, quantitative data (mean & standard deviation (SD), CI, P value.*

Overall appraisal: Include Exclude Seek further info

Comments (Including reason for exclusion)

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Addendum

* A comprehensive ROB table is available from the author (3689306@myuwc.ac.za).

	Random sequence generation: selection of species and pre-allocation handling															
Article Name	Random sequence generation															
	Preference bias				Incubation period				Incubation conditions				Adjusment to Mcf			
	Yes	No	Comment	Score	Yes	No	Comment	Score	Yes	No	Comment	Score	Yes	No	Comment	Score
Chávez Andrade et al. 2019		No	(+),(-), fur	Low	Yes		24hr	Low	Yes		37C	Low	Yes		0.5Mcf	Low
Ahmed et al, 2019	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low		No	Not ment	High
Lu et al, 2019	Yes		Title ment	High	Yes		24hr	Low	Yes		37C	Low	Yes		0.5Mcf	Low
Souza et al, 2018	Yes		(+), fungi	Intermedi	Yes		24hr	Low	Yes		35C	Low	Yes		0.5Mcf	Low
Schwass et al, 2018	Yes		(+),(-)	Intermedi	Yes		overnight	Low	Yes		37C	Low		No	Not ment	High
Bedinis et al, 2013	Yes		(+)They u	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		OD-0.1	Low
Lotfi et al, 2011	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		OD-0.11	Low
Kim et al, 2008	Yes		Fungi rele	Intermedi	Yes		48hr	Low	Yes		28C	Low	Yes		2x104	Low
Panáček et al, 2009	Yes		Fungi rele	Intermedi	Yes		36hr	Low	Yes		37C	Low	Yes		10^8	Low
Esawy et al, 2019	Yes		Although I	High	Yes		24hr	Low	Yes		37C	Low	Yes		10^8	Low
Pangaliya et al, 2018		No	(+)(-), yea	Low	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Tavaf et al, 2017	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Javidi et al, 2014	Yes		(+)releva	Intermedi	Yes		1day/7day	Low	Yes		37C	Low	Yes		Mentione	Low
Abbaszadegan et al, 2014	Yes		(+)they c	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Fan et al, 2014	Yes		(+)they u	Intermedi	Yes		7days	Low	Yes		37C	Low	Yes		Mentione	Low
Samiei et al, 2014	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Bruniera et al, 2014	Yes		(+)(-) no f	Low	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Moghadas et al, 2020	Yes		(+)	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Bedinis et al, 2012	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Abadi et al, 2013		No	(+)(-), yea	Low	Yes		24hr/over	Low	Yes		37C	Low	Yes		Mentione	Low
Kachoei et al, 2018	Yes		(+)	Intermedi	Yes		48hr	Low	Yes		37C	Low		No	Amount c	High
Niakan et al, 2013	Yes		(+)(-) no f	Intermedi	Yes		18hr	Low	Yes		35C	Low	Yes		Mentione	Low
Ahrari et al, 2015	Yes		(+)releva	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Emmanuel et al, 2015		No	(+)(-) fung	Low	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low
Pérez Díaz et al, 2015	Yes		(+)clinical	Intermedi	Yes		24hr	Low		No	Not mentd	High		No	Not ment	High
Manojkanna et al, 2017	Yes		(+), yeast,	Intermedi	Yes		24hr	Low	Yes		37C	Low	Yes		Mentione	Low

	Binding									Attritiob Bias: Loss of subjects				Selective Reporting: reporting in al, treat			
Article Name	Binding of Participants: prevention of participants from knowing certain information that may									Attrition Bias				Selective Reporting			
	Binding of Participants				Binding of outcomes: Tool for measurment												
	Yes	No	Comment	Score	Automate	Manual	Not mentioned	Comment	Score	Yes	No	Comment	Score	Yes	No	Comment	Score
Chávez Andrade et al. 2019	Yes		Vortexing	Low	Yes			Antimicrobial: CFU Antibiofilm: Spectrophotometer/SEM	Very low (two methods are mentioned)		No	All stains	Low		No	Reporting	Low
Ahmed et al, 2019	Yes		All the tre	Low	Yes			Vernier C.	Low		No	No loss of	Low		No	Reporting	Low
Lu et al, 2019	Yes		Mixing un	Intermedi	Yes			Live/Dead	Low		No	All concen	Low		No	All treatm	Low
Souza et al, 2018	Yes		Centrifug	Low		Yes		Scraping c	Intermediate		No	No loss of	Low		No	All treatm	Low
Schwass et al, 2018			Unclear. E	High	Yes			4 points m	Low		No	All organi	Low		No	All treatm	Low
Bedinis et al, 2013	Yes		Although	Low	Yes			SEM/Live,	Very low (3 method		No	All strains	Low		No	All treatm	Low
Lotfi et al, 2011	Yes		Mixing wa	Low		Yes		Spectroph	Intermediate		No	No loss of	Low	Yes		CHX was c	Intermedi
Kim et al, 2008		No	Vortexing	High	Yes			ELISA rea	Low			No loss of	Low		No	All treatm	Low
Panáček et al, 2009		No	Vortexing	High	Yes			ELISA rea	Low		No	No loss of	Low	Yes		Not all co	High
Esawy et al, 2019	Yes		Vortexing	Intermediate			Not mentioned	Measuring	High		No	No loss of	Low		No	All treatm	Low
Pangaliya et al, 2018	Yes		Samples v	Intermedi	Yes			Spectroph	Low		No	No loss of	Low		No	All treatm	Low
Tavaf et al, 2017	Yes		Vortexing	Intermedi	Yes			Microplat	Low		No	No loss of	Low		No	All treatm	Low
Javidi et al, 2014	Yes		Vortexing	Intermedi	Yes			CFU	Low		No	No loss of	Low		No	All treatm	Low
Abbaszadegan et al, 2014	Yes		Mixing wa	Intermedi	Yes			CFU	Low		No	No loss of	Low	Yes		Selective	High
Fan et al, 2014	Yes		Vortexing	Intermedi	Yes			Fluoresc	Low		No	No loss of	Low		No	All treatm	Low
Samiei et al, 2014	Yes		Centrifug	Low		Yes		CFU	Intermediate		No	No loss of	Low		No	All treatm	Low
Bruniera et al, 2014	Yes		Vortexing	Intermediate		Yes		Visual	High		No	No loss of	Low		No	All treatm	Low
Moghadas et al, 2020		No	Vortexing	High	Yes			CFU/Steril	Low		No	No loss of	Low		No	All treatm	Low
Bedinis et al, 2012	Yes		Vortexing	Intermedi	Yes			Microtitre	Low		No	No loss of	Low		No	All treatm	Low
Abadi et al, 2013		No	Vortexing	High		Yes		Visual/CF	Intermediate		No	No loss of	Low		No	All treatm	Low
Kachoei et al, 2018		No	Vortexing	High			Not mentioned	Tool used	High		No	No loss of	Low		No	All treatm	Low
Niakan et al, 2013	Yes		Vortexing	Intermediate		Yes		Special u	Intermediate		No	No loss of	Low		No	All treatm	Low
Ahrari et al, 2015	Yes		Vortexing	High	Yes			Spectroph	Low		No	No loss of	Low		No	All treatm	Low
Emmanuel et al, 2015	Yes		Vortexing	Intermedi	Yes			Antibiotic	Intermediate		No	No loss of	Low		No	All treatm	Low
Pérez Díaz et al, 2015	Yes		Vortexing	Intermedi	Yes			Instrumen	Intermediate		No	No loss of	Low		No	All treatm	Low
Manojkanna et al, 2017	Yes		Vortexing	Intermediate-High			Not mentioned	Measuring	High		No	No loss of	Low		No	All treatm	Low

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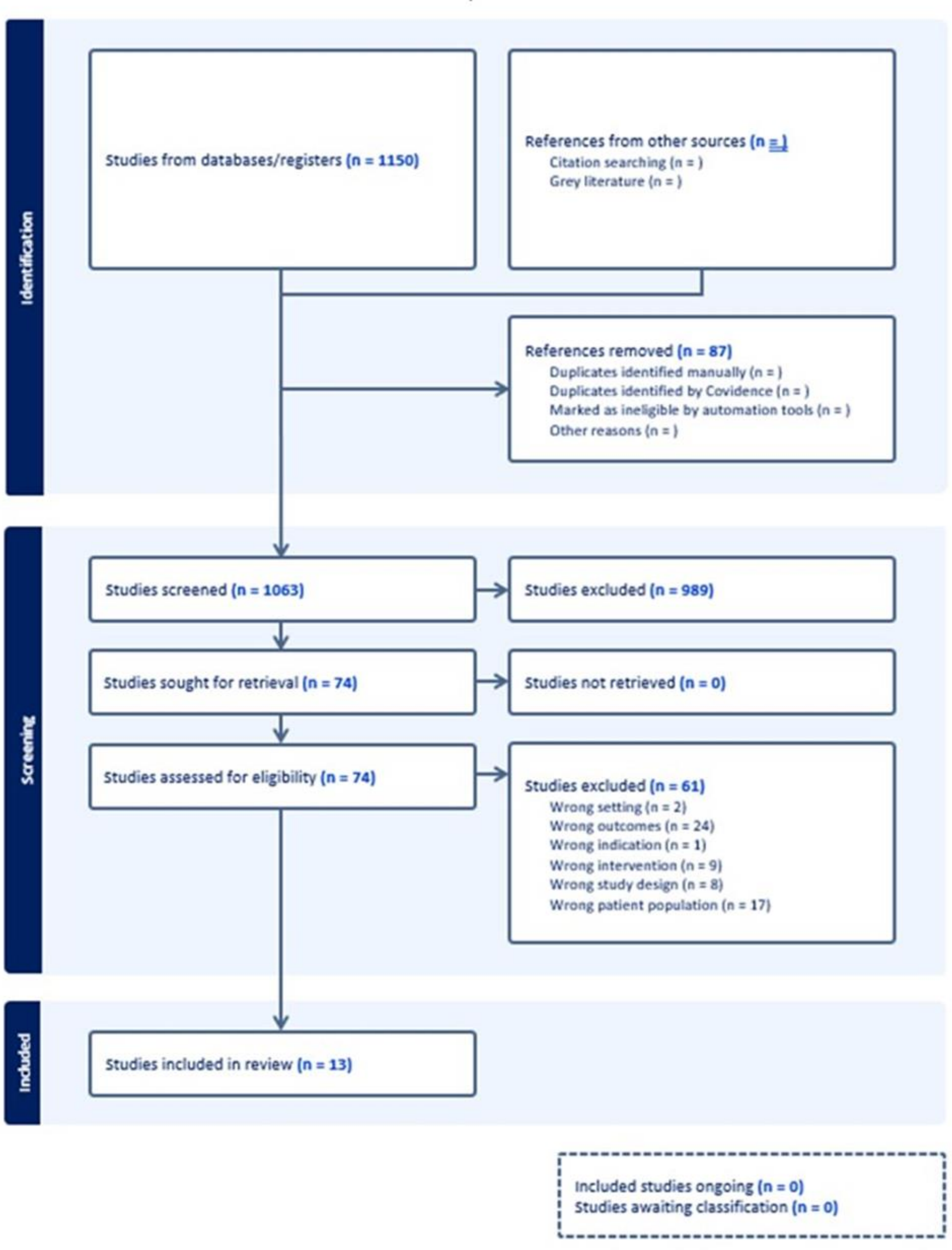

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2.2.3. Review (B): Plant extract-synthesized silver nanoparticles for application in dental therapy.

2.2.3.a: Publication

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Review

Plant Extract-Synthesized Silver Nanoparticles for Application in Dental Therapy

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Abstract: Oral diseases are the most common non-communicable diseases in the world, with dental caries and periodontitis causing major health and social problems. These diseases can progress to systematic diseases and cause disfigurement when left untreated. However, treatment of oral diseases is among the most expensive treatments and often focus on restoration of form and function. Caries prevention has traditionally relied on oral hygiene and diet control, among other preventive measures. In this paper, these measures are not disqualified but are brought into a new context through the use of nanotechnology-based materials to improve these conventional therapeutic and preventive measures. Among inorganic nanomaterials, silver nanoparticles (AgNPs) have shown promising outcomes in dental therapy, due to their unique physicochemical properties and enhanced anti-bacterial activities. As such, AgNPs may provide newer strategies for treatment and prevention of dental infections. However, numerous concerns around the chemical synthesis of nanomaterials, which are not limited to cost and use of toxic reducing agents, have been raised. This has inspired the green synthesis route, which uses natural products as reducing agents. The biogenic AgNPs were reported to be biocompatible and environmentally friendly when compared to the chemically-synthesized AgNPs. As such, plant-synthesized AgNPs can be used as antimicrobial, antifouling, and remineralizing agents for management and treatment of dental infections and diseases.

Keywords: dental caries; silver nanoparticles; green synthesis; antimicrobial agents; periodontitis; phytochemicals

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1. Introduction

Oral diseases such as dental caries (tooth decay) and periodontitis (diseases of the dental supporting tissues) are the most common noncommunicable diseases and affect people of all ages, causing pain, discomfort, tooth loss and disfigurement [1,2]. Dental caries is estimated to affect 60–90% of schoolchildren and nearly 100% of adults worldwide [3]. Dental caries is considered to be the principal cause of tooth loss in children and young adults, and also the primary cause of tooth root breakdown in the elderly [4]. Periodontal disease is classified into four stages, of which severe periodontitis was ranked the sixth most common health condition between 1990 and 2010 [5], and estimated to affect 10% of the global population [6]. Dental caries and periodontitis can progress to systemic infections, leading to infective endocarditis, diabetes, and respiratory infections

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such as pneumonia [2] if proper prevention and management is not practiced. Both dental caries and periodontitis are polymicrobial in their aetiology and result when the supra- and subgingival microbial communities experience a homeostasis breakdown and undergo dysbiosis, forming a biofilm (plaque) on the teeth and surrounding tissues [7]. Plaque protects the pathogenic microorganisms from the host defence mechanisms, resulting in dental infections [8]. Oral hygiene through the use of antimicrobial and remineralization agents, in addition to mechanical removal of plaque, maybe used to treat and prevent dental diseases [4]. In severe cases, artificial materials are used in restorative procedures (cavity filling, root canal, dental implants, bridges, crowns, dentures) to restore and ensure full function of the oral cavity [8]. However, these strategies are not always successful and restorative procedures could be costly for individuals in low- and middle-income countries. Oral diseases are the fourth most expensive disease to treat, worldwide [3]. In high income countries, the cost of treatment can be covered by patients, with or without the assistance from medical insurance. In contrast, for patients from low- and middle-income countries, treatment may be unaffordable [3]. This, therefore, leaves oral disease prevention and health promotion as the most viable options [9]. The World Health Organization (WHO) Essential Medicines List (EML) programme (2017–2021) recommended that research should focus on developing affordable, safe and environmentally friendly oral hygiene products [10].

In recent years, there is an increasing interest in the use of green synthesized nanomaterials to be included in dental care products. Among the various existing nanomaterials, silver nanoparticles (AgNPs) have gained attention owing to their distinctive physical and bio-chemical properties, as well as their pronounced antimicrobial effects [11]. Moreover, AgNPs due to their larger surface area can be combined with other therapeutic agents to exhibit superior antimicrobial properties in the oral cavity [12]. This review will be devoted to the use of green synthesized AgNPs for the development of novel and improved dental agents to combat oral pathogens, with the goal to arrest and prevent the development of dental caries.

1.1. Dental Caries

Dental caries is a chronic oral disease caused by accumulation of microbial biofilm (plaque) on the tooth surface; it occurs as a result of acid build-up that was converted from the free sugars consumed from food products. The acids slowly destroy the tooth enamel and dentine over time, leading to demineralization and subsequently the development of caries. Dental caries has a negative impact on oral-health-related quality of life, and in the advanced stage can lead to tooth loss and systemic infections [6,13]. Among oral diseases, dental caries has a high rate of morbidity across all populations. There is an association between socio-economic and demographic conditions and the incidence of dental caries across all populations [14]. Dental caries not only affects oral health, but can also be associated with systemic and inflammation-related diseases, such as diabetes and respiratory diseases [2,4]; thus, indicating that the prevention and treatment of dental caries are of utmost importance to mitigate this global health problem [4].

Pathophysiology of Dental Caries

The oral cavity is a microbiological medium that hosts over 1000 bacterial species [15] that are responsible for metabolic activities in the oral cavity. Primary colonizers are predominantly the anaerobic Gram-positive oral streptococci (e.g., *Streptococcus oralis*, *Streptococcus sanguinis*, and *Streptococcus mitis*), followed by Gram-positive rods, such as *Actinomyces* species [16]. The oral cavity requires a constant state of equilibrium between the commensal microbial communities to maintain a healthy oral environment [7]. Many factors, which include poor oral hygiene, change in the microflora (cariogenic bacteria), low salivary flow, insufficient exposure to fluoride, diet (high sugar consumption), and immunodeficiency, can alter the oral microbiome homeostasis, and consequently lead to the development of infections [13,14]. The shedding of the oral epithelial lining occurs three

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times a day, which is an effective method to reduce bacterial adhesion, while the non-shedding surfaces, such as teeth, dentures, and dental implants, are prone to biofilm formation [17].

Dental caries progress when acidogenic bacteria and fermentable carbohydrates interact with host factors such as teeth and saliva. The bacteria adhere to the dental surfaces via adhesion receptors and charge interactions among other mechanisms [16]. The initial attachment of bacteria to dental surfaces is preceded by the formation of the pellicle which consists of glycoproteins (mucins), phosphoproteins, histidine-rich proteins, proline-rich proteins, α-amylase, bacterial glucosyltransferase, etc. [18]. The formation and composition of the pellicle vary from one surface to another [19]. Imbalance in these factors causes fluctuations in plaque pH as a result of increased bacterial acid production and decreased buffering action from saliva and the surrounding tooth structure. Thus, there is a dynamic balance between the tooth surface and its surrounding environment. If this balance is disturbed, the pH drops below a critical value (< 6.5–5.5) and the demineralization of tooth structures (enamel, dentine, or cementum) occurs [20]. Demineralization results in dissolution of the major component of tooth enamel and dentine (hydroxyapatite) leading to enamel softening. This appears as a chalky white spot lesion (WSL) on the tooth. At this point, the enamel surface is sound, and the lesion is reversible. If the minerals continue to be lost because of increased acid production, the lesion changes into a black discoloration and subsequent cavitation. If the lesion progresses, large areas of the tooth can be lost [20]. Demineralization is preceded by remineralization, a natural repair process in which minerals such as calcium, phosphate and fluoride from saliva are deposited into the damaged area [21]. However, the natural remineralization process is not always as successful, especially for the larger lesions [22]. Moreover, fluoride-mediated salivary remineralization is limited to the outer tooth structure [23], and remineralization is typically up to 10 times slower than the demineralization process. It is estimated that about 5 h of remineralization are needed to repair a 0.5 h demineralization episode [24]. Additional extrinsic sources of calcium and phosphate ions are then required to accelerate the natural remineralization process. Both demineralization and remineralization processes happen concurrently during the day, with the long-term disequilibrium between the two processes resulting in the development of dental caries [25].

1.2. Prevention of Dental Caries

Strategies intended to prevent and arrest the formation and avoid recurrence of carious lesions and cavities include good oral hygiene, diet control, use of saliva substitutes, fluoride, and non-fluoridated and antimicrobial agents [26,27]. These preventive strategies are summarized in Table 1. Proper oral hygiene by itself can neither fully protect against caries or eliminate the biofilm, because biofilm formation starts developing immediately after tooth brushing. It might take time for the microorganisms that are responsible for demineralization to be removed by brushing alone [28]. Regular mechanical plaque control through proper tooth brushing twice daily, can help maintain the composition of the oral microbiome in a healthy state [26], remove plaque in proximal caries [27], and reduce caries. However, mechanical plaque control measures without fluoride are not successful for the prevention of dental caries [29]. Hence, the inclusion of fluoride or agents that delay oral biofilm formation or its symbiosis in the toothpaste may be useful [30]. Fluoride prevents damage to the tooth structure by inhibiting dental caries through remineralization of early lesions [24]. It exerts its antimicrobial effect by forming hydrogen fluoride that diffuse into the bacterium and inhibits its metabolism [28]. When used at the recommended levels—0.7 mg for toddlers, 3 mg for adult women and 4 mg for adult men [31]—fluoride is safe and effective for the prevention of dental caries [27]. Furthermore, the WHO recommended that fluoride toothpaste should be included on the core list of the EML, with fluoride concentration of 1000–1500 ppm, because of its proven activity in this range [32].

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Table 1. Preventive strategies for dental caries.

Caries Prevention Agents	Examples	Formulation Type/Tools	Action	Limitation	Refs
Oral hygiene	Toothbrush Dental floss Mini-brushes Oral irrigators		Delay or prevent oral biofilm formation	Flossing alone may not be effective in reducing caries Personal skills influence the outcome Must be used in combination with toothpaste	[27,29,30]
Diet modification	Sugar reduction	Food	Control biofilm formation by eliminating plaque Lowers the rate of dental caries	Reducing sugar content is less successful Dietary control of sugar is a challenge	[33,34]
Saliva substitutes			Cleans the oral cavity and the pH buffering capacity		[35]
Fluoride		Toothpaste Varnish Gel Mouthwash Tablets Drops Chewing gum Lozenge	Remineralization	Fluoride classified as a neurotoxicant Continuous use can lead to fluoride-resistant strains Excessive use can lead to tooth fluorosis, discoloration, and gastrointestinal problems Skeletal fluorosis can cause disabilities such as chronic joint pain, stiffness of joint, sporadic pain, calcification of ligaments, and osteosclerosis	[24,36,37]
Non-fluoridated (cal-cium-based) agents	Amorphous Cal-cium Phosphate (ACP)	Added into a fluoride toothpaste	Remineralization	Reduce root caries Unstabilized ACP promote dental calculus deposition on the teeth ACP interferes with remineralization by removing free fluoride ions in the oral environment	[21,38]

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	Casein Phosphopeptide- ACP (CPP-ACP)				
Antimicrobial Therapies	Chlorohexidine (CHX)	Mouthwash	Antimicrobial agent	Brownish staining of the teeth, removable by discontinuing the product Altered taste, persist for several hours after use Oral burning Development of lesions and ulcerations of the gingival mucosa Has a strong, unpleasant taste	[39]
	Probiotics Prebiotics	Probiotics: dairy products, fermented vegetables, sourdough bread, drops, tablets, and lozenges containing various strains	Reduce harmful gastrointestinal microorganisms, discomfort, and stimulate the immune system		[26,40]
		Prebiotics: chewing gum, oral rinse	Stimulate growth and/or activity of bacteria already resident in the host colon Inhibit the attachment of pathogenic bacteria Alters pH of the environment Stimulate the immune system		[41,42]

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Sugar substitute (sweeteners), e.g, Stevia, Xylitol	Chewing gum	Stevia: reduces plaque formation Acts as a healing agent at the periodontium level	[26]
		Xylitol: reduces plaque formation, and bacterial adherence Inhibits enamel demineralization (i.e., reduces acid production)	[43]

Fluoride toothpastes that contain lysozyme, lactoferrin and proteins can modulate oral health by generating hydrogen peroxide and hypothiocyanite, which can reduce pathogenic microorganisms and promote the proportion of bacteria associated with good gum health [44]. However, continuous use of fluoride can drive the development of fluoride resistance in bacteria, such as *S. mutans* [45], *S. salivarius,* and *S. sanguinis* [46], and are not adequate in highly cariogenic oral environments [47]. Moreover, fluoride is classified as an "unapproved new drug" by the US Food and Drug Administration, and as of January 2012, a memorandum to end water fluoridation worldwide was signed [31]. Additionally, fluoride toothpaste, rinses and varnish applications are not universally affordable [31].

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Non-fluoridated agents, amorphous calcium phosphate (ACP) and casein phosphopeptide-ACP (CCP-ACP), have been proposed as alternatives to fluoride for the prevention of caries. ACP accumulates on the tooth surface, which then buffers the free calcium and phosphate ion activities, preventing demineralization and enhancing remineralization [48]. CPP have antibacterial and buffering effects on plaque by inhibiting the growth and adherence of *S. mutans* and *S. sorbinus* [27,49]. CPP-ACP products have improved remineralization and anticaries effects when compared to fluoride-containing products [50–54]. However, recent clinical trials revealed that some of these agents should be used in conjunction with fluoride rather than as an alternative [27].

Dietary modification by reducing sugar content to ≤ 10% of total energy intake [9] can dramatically reduce caries incidence [32,55]. Dietary restrictions require a lifelong commitment and are seldom adhered to by patients [24,56]. Insufficient production of saliva as a result of aging, systemic medical conditions, or chemo/radiotherapy lead to delay in the removal of sugary or acidic foods, and consequently biofilm dysbiosis and overgrowth of *Candida* species [57]. In cases of dry mouths, saliva substitutes are used to stimulate saliva secretions to clean the oral cavity and increase the pH buffering capacity [35].

Antimicrobial agents, such as chlorhexidine (CHX), sweeteners, antimicrobial peptides (AMPs), pre- and probiotics, prevent dental caries by altering the dental plaque to be less cariogenic. CHX prevents dental caries by suppression of salivary *S. mutans* [26]. A major reduction in caries in high-caries-risk adults was observed by using 0.12% CHX mouth rinse in conjunction with daily fluoride toothpaste [27]. Sweeteners such as Stevia and Xylitol have anticariogenic properties. Stevia is active against *S. mutans*, *S. sorbinus*, *L. acidophilus*, and *C. albicans*. The indications for using Stevia in the prevention of caries is still under consideration [49]. Xylitol is a natural anti-cariogenic derived from plants and agricultural materials. It acts by disrupting the energy production processes of *S. mutans*, leading to cell death [43]. AMPs became popular due to their selective activity against *S. mutans* and maintaining a balanced oral microbiota for long-term caries protection. Their exact mechanism is not well understood, but membrane disruption and subsequent interference with intracellular targets are thought to be the main processes [58]. AMPs can reduce *S. mutans* count in plaque and saliva without affecting the non-cariogenic oral streptococci [59]. The clinical effect of AMPs is currently limited due to toxicity to normal cells [26].

Pre- and probiotics are used to support microbial diversity in order to obtain a sustained caries-preventive effect. Food products supplemented with live microorganisms, such as *lactobacilli* and *bifidobacteria*, can confer a health benefit to the host [60] and lead to the reduction of *S. mutans* count in saliva [49]. A reduction in initial caries was reported for schoolchildren after drinking milk containing *L. rhamuosus*. Ten randomized placebo-controlled probiotic trials were demonstrated to reverse root caries in preschool children, adolescents, and adults [26]. Probiotic therapy increased diversity of the salivary microbiome after short-term intake of fermented milk containing three probiotic strains. It increased the levels of common commensals, such as *S. oralis* and *S. mitis*, and reduced levels of *S. mutans*, *Fusobacterium* spp. and *Prevotella* spp. So far, there is insufficient evidence for general recommendations of probiotic therapy to modulate the oral microbiome [61]. Prebiotics inhibit the attachment of pathogenic bacteria and stimulate the immune system by altering the pH of the environment [42]. Prebiotics serve as nutrients for beneficial microbes that either inhibit acidogenic and aciduric microbes. The two main sources of alkali in the oral cavity are urea and arginine, which are catabolized by some of the oral bacteria (*S. gordonii* and *S. sanguinus*) to form ammonia, and thereby elevating the pH of the medium [62]. Arginine in combination with fluoride proved to be effective in arresting dental caries, where it enhanced the arginolytic potential and reduced acidogenicity on early coronal and root caries [63]. Incorporation of 1.5% arginine into toothpaste without fluoride resulted in increased arginine deiminase activity and growth of beneficial bacteria [64]. Numerous clinical studies revealed that CaviStat (arginine bicarbonate and calcium car-

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bonate toothpaste) was able to inhibit caries onset and caries progression in schoolchildren [65]. Subsequently, the Colgate company purchased the right to be the owner of using arginine bicarbonate/calcium carbonate in their toothpaste [24]. The company launched two products containing 1.5% arginine; the Pro-Argin toothpaste against dentin hypersensitivity and anticaries product [24]. All these strategies are extensively reviewed elsewhere [4,66,67].

Despite the significant progress made by preventive strategies to reduce and prevent dental caries, this disease is still considered the most prevalent dental disease in the world [68]. Eliminating carious lesions through restorative procedures in order to improve oral health may not always keep teeth functionality for life. The dental restorations are acceptable from both aesthetic and functional perspectives, although they remain inferior to the natural teeth. Dentures are not stable or comfortable and can affect speech and eating [24]. Preventive or non-operative actions should be used together with the restorative interventions in delivering adequate oral health care [69].

The burden of oral diseases, particularly untreated dental caries, represents a significant public health problem globally. As such, new oral health care products that can produce and ensure sustainable oral health effects are recommended [32]. Currently, there is growing interest in the use of nanomaterial-based systems for dental therapy. In addition to the therapeutic application of nanomaterials, they can also be used as drug carriers for targeted drug delivery, controlled/sustained drug release, improved drug stability and bioavailability [70]. Nanomaterials produced from organic sources are usually preferred over inorganic-based nanomaterials, due to their biocompatibility and biodegradable nature [71]. Some nanomaterials exist as nanoparticles (NPs), which is defined by the International Organization for Standardization as particles with a size range of 1 to 100 nm in one dimension. Organic-based nanomaterials have been used as hydrogels, films, hydrocolloids, and nano-/micro-particles for various biomedical applications [72]. These systems often suffer from low loading capacity and solubility. However, inorganic metallic NPs (MNPs) have an advantage of easily modifiable surfaces, and can easily penetrate the biofilm structure and release their conjugates to destroy the biofilm and inhibit microbial colonization.

1.3. Application of Nanomaterials in Dental Therapy

Nanotechnology has brought about a lot of exciting and novel applications in various fields, including medicine, through the use of nanomaterials. The use of NPs is now being considered for the treatment and prevention of dental infections and diseases [73]. Various types of NPs exist, and they are broadly classified as inorganic and organic NPs [74]. Inorganic NPs include semi-conductors (ZnO, ZnS, quantum dots), metallic NPs (gold, silver, copper, aluminium, platinum), and magnetic NPs (cobalt, iron, nickel, iron oxide); while organic NPs include carbon (fullerenes, carbon nanotubes) and polymeric (chitosan, liposomes) NPs [74,75]. All these nanomaterials have played a significant role in various experimental dental and oral applications, as shown in Figure 1. These nanomaterials could be incorporated into materials such as, resins, metals, ceramics, etc., used in restorative, prosthetic, endodontics, periodontal treatments, and implantations to prevent and treat oral diseases, including dental caries [76]. In the past few decades, substantial interest and research efforts were directed towards the biomedical applications of MNPs owing to their unique chemical, physical, and biological properties [2,77]. This review will focus on the dental applications of AgNPs synthesized from plant extracts, which are also considered to be green synthesized MNPs.

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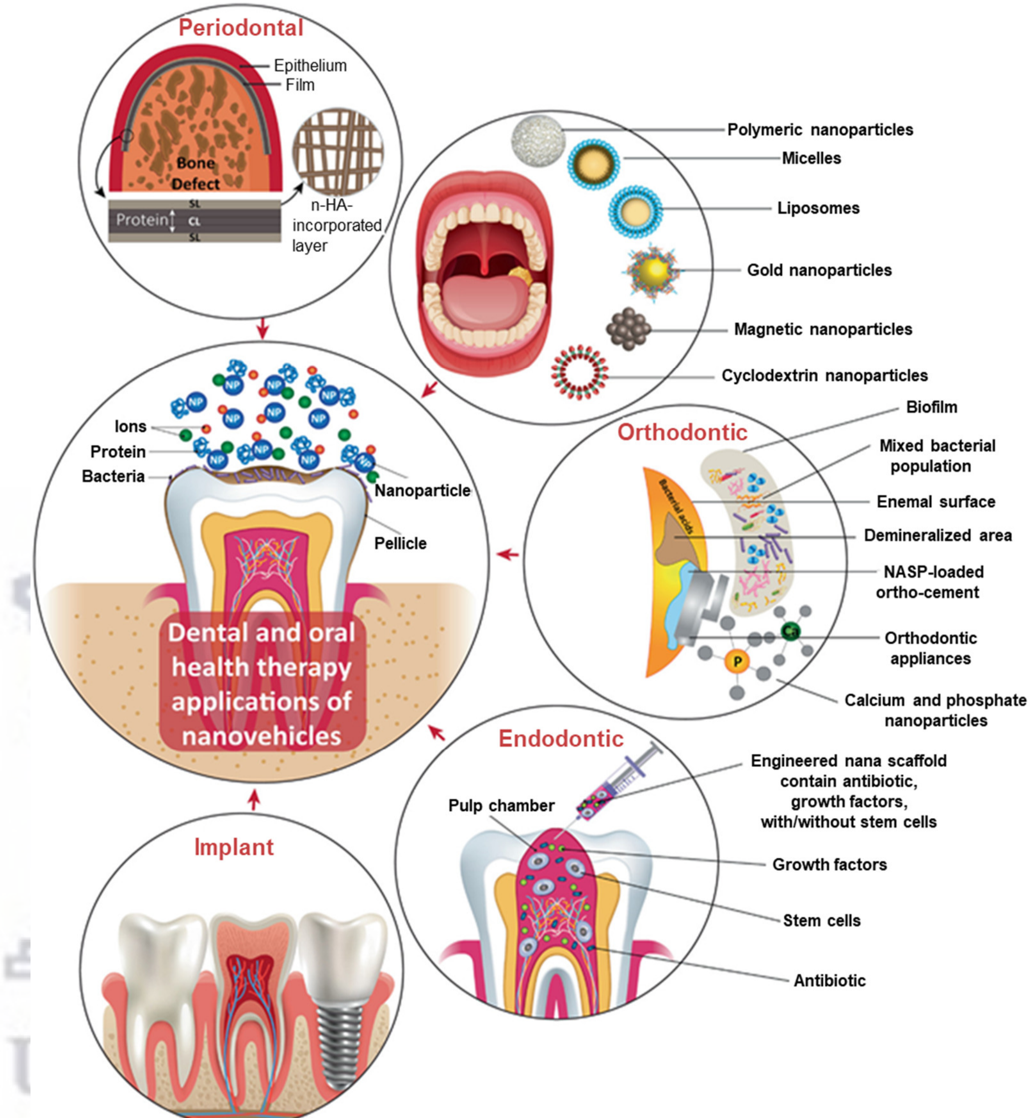

Figure 1. Applications of organic and inorganic nanomaterials in dental care and treatment. Abbreviations: HA: hydroxyapatite, NASP: nano-sized amorphous calcium phosphate. Reprinted with permission from Ref. [76], 2021, Wiley.

1.3.1. AgNPs in Dental Therapy

Among the available MNPs, much attention was given towards the exploitation of the biomedically-related effects of AgNPs, particularly its antimicrobial effects. Silver has a long history in dental application, initially in the form of ionic compounds [78], and recently, in NP formulation [79–81]. Many studies have demonstrated the antimicrobial effects of 38% silver diamine fluoride (SDF) in the prevention of caries, where the ionic silver and fluoride served as antimicrobial and remineralization agents, respectively [82,83]. However, the high concentration of Ag^+ that is required to prevent dental caries was found to have an unaesthetic appearance, due to the precipitation of black-coloured Ag on the surface of the tooth. Efforts have been made to overcome this problem in recent years; nanotechnology proved to be effective and showed that by reducing the size of bulk Ag, the surface area was considerably increased. In this way, bioactive molecules can be attached to improve the antimicrobial effects and prevent black staining in teeth [84]. Therefore, there is a growing enthusiasm for use of AgNPs as they provide superior bioactivity [75] due to their distinct physical and biochemical characteristics over Ag^+

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[11,85,86]. Several applications of AgNPs as antimicrobial agents [87] in diagnostics, optoelectronics, and water disinfection agents have been reported [36,88]. The use of AgNPs has been extended to dental applications as antimicrobial [89], anti-inflammatory and remineralization agents [90], as illustrated in Figure 2.

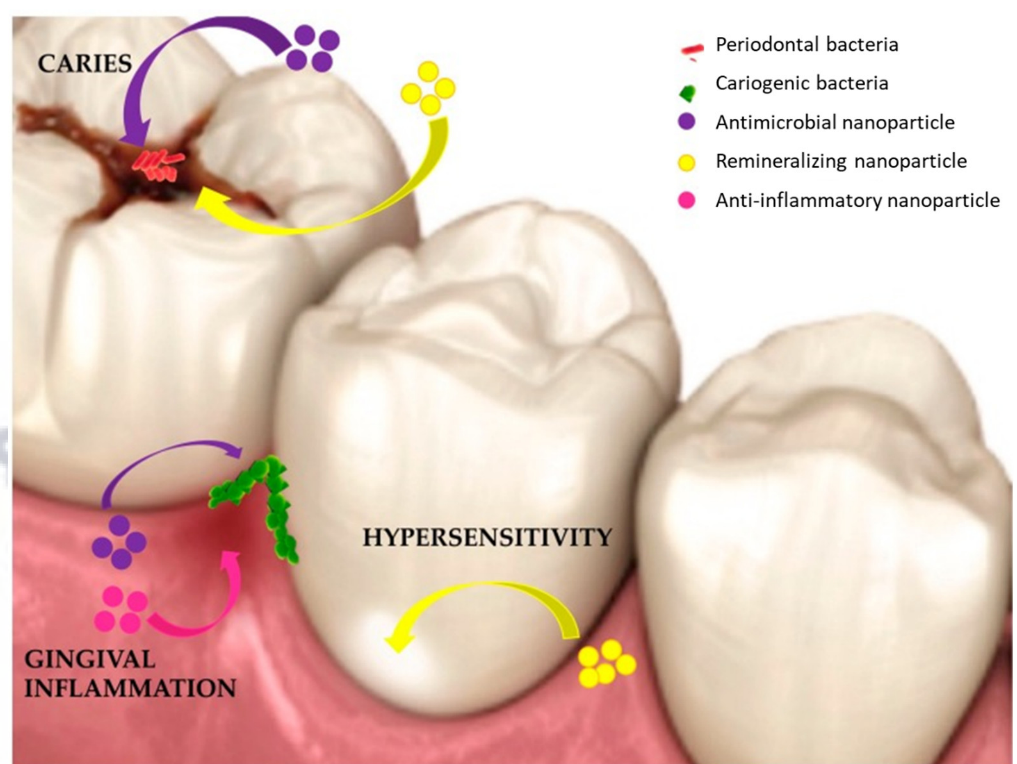

Figure 2. Application of nanoparticles (NPs) contained in oral care products. Reprinted with permission from Ref. [90], 2020, MDPI.

AgNPs have been used in various fields of dentistry (restorative, prosthetic, endodontics, implantology, and periodontology) to prevent caries and treatment of oral cancers. The incorporation of AgNPs into composite resins does not impact on its inherent mechanical and biological properties, but imparts notable antimicrobial properties to the composite material even when low concentrations of the AgNPs are used. It was also demonstrated that AgNPs can reduce the microleakage in the root canal system and can be used as a substitute for sodium hypochlorite (NaOCl) as a canal irrigant. AgNP-based irrigation solution had similar potency to NaOCl in elimination of *E. faecalis*. Although the two solutions were efficient in terms of their antibacterial activity, irrigants with AgNPs showed an added benefit of smear layer removal which may potentiate the ability of AgNPs to interact with the bacteria [91]. The incidence or recurrence of caries due to microleakage can thus be reduced by using composites containing AgNPs with antimicrobial properties and restorative agents [11].

In another study, spherical AgNPs containing polymethyl-methacrylate (PMMA) were able to reduce adherence of *C. albicans* on denture resins [92]. The PMMA-AgNPs were biocompatible and showed no cytotoxic or genotoxic effects on NIH-3T3 mouse fibroblasts and Jurkat cells. The study further indicated that the AgNPs could be added into acrylic resins as an antifungal agent, and possibly reduce the chances of denture stomatitis. Moreover, AgNPs can be favourably used to produce biocidal surface coating onto the titanium implants to prevent periimplantitis, and have also shown potential for osteogenic differentiation [93]. When immobilized on sand-blasted, large grit, and acid-etched titanium, AgNPs did not exhibit apparent cytotoxicity but inhibited the proliferation of *S. aureus* and *F. nucleatum*. These results suggested that by coating titanium implants with

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AgNPs, the implants can be endowed with balanced antibacterial and osteogenic functions, which bodes well for safe and prolonged clinical applications [94].

Periodontal diseases can also be prevented by AgNPs which can stimulate the regeneration of mammalian cells. The colonization and penetration of guided tissue regeneration (GTR) by *S. mutans, F. nucleatum, Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis* was studied using membranes impregnated with AgNPs (GTR-AgNPs), which was compared to GTR membrane impregnated with 25% doxycycline hydrochloride (GTR-DOX). The study showed that the bacterial adherence scores and colony forming units (CFUs) for GTR-AgNPs were significantly lower [95]. AgNPs have been shown to possess anticancer activity and prevent WSLs when incorporated into adhesive systems to treat orthodontic patients. The AgNPs prevented crack propagation and improved the fracture toughness within the dental ceramics, which negated the cracking of porcelain restorations with crowns, bridges, and veneers [11].

Chemically synthesized AgNP nanocomposites were also demonstrated to arrest progression of dental caries in 6–10-year-old children in clinical trials [81,82]. A single dose of either AgNP-chitosan-NaF nanocomposite [82] or 5% nano-silver incorporated sodium fluoride (NSSF) dental varnish was administered to school children, and the effects on their teeth were followed for a period of 12 months. The 5% NSSF was composed of polyvinyl pyridoline (PVP) as a dispersant and a commercial sodium fluoride varnish (FLUORITOP™-SR). The clinical cariostatic efficacy of 5% NSSF dental varnish compared to a commercial 38% SDF dental varnish (Saforide®, Toyo Seiyaku Kasei Ltd., Osaka, Japan) was evaluated in 6–10-year-old school children presenting carious lesions in primary molars. The active carious lesions were exposed to either 5% NSSF or 38% SDF, at 0, 1, 3, 6 and 12 months. Parameters such as caries activity, depth, size, colour, and presence or absence of pain were noted at the specified time points. Although there was no significant difference between the effects of the two treatments during the 12-month period, 5% NSSF performed similarly and in some cases better than the 38% SDF. In addition, the 5% NSSF did not cause dark staining of the dentinal tissue when compared to the treatment with 38% SDF. Of interest, the safety profile of the 5% NSSF was comparable to the commercial varnish in that no adverse effects were observed in the children subjected to these treatments. This further suggests that AgNP-based oral formulations can be safely used in children. Most importantly, these formulations are cheaper compared to the conventional systems, where 5% NSSF was projected to be eight times less than the cost of SDF [81]. Various AgNP-Fluoride nanocomposites are either recruiting or have completed clinical trials in children or adults, as antimicrobial, demineralization and remineralization agents. A total of 35 studies appear on the Clinicaltrials.gov website under the search for "silver nanoparticles"; some of these studies are summarized in Table 2.

Table 2. AgNP-based formulations registered for clinical trials.

AgNP Formulation	Study Title	Conditions	AgNP Activity	Status Online	NCT Identifier
AgNPs in vanishing cream	Topical silver nanoparticles for microbial activity	Foot fungal and bacterial infections (tinea pedis, capitis, versicolor)	Antimicrobial	Recruiting	NCT03752424
Innocuous containing gel AgNPs	Topical application of silver nanoparticles and oral pathogens in ill patients	Critical patients in ICU (coma or induced coma)	Antimicrobial	Completed	NCT02761525
AgNPs	Silver nanoparticles in multidrug resistant bacteria	Critically ill patients	Antimicrobial	Completed	NCT04431440
5% NSSF	Nano-silver fluoride to prevent dental biofilms growth	Dental caries	Antimicrobial	Completed	NCT01950546

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AgNPs incorporated into the primer orthodontic Transbond XT	Addition of silver nanoparticles to an orthodontic primer in preventing enamel demineralization adjacent brackets	Dental caries	Tooth demineralization	-	NCT02400957
Mouthwash and nose rinse with AgNPs	Evaluation of silver nanoparticles for the prevention of COVID-19	COVID-19	SARS-CoV-2 activity	Completed	NCT04894409
Colloidal silver	Colloidal silver, treatment of COVID-19	Severe Acute Respiratory Syndrome		Recruiting	NCT04978025
Fluor dental varnish with 25% AgNPs	Fluor varnish with silver nanoparticles for dental remineralization in patients with Trisomy 21	Dental caries in Trisomy 21 children	Remineralization	Active, not recruiting	NCT01975545
Nano-silver fluoride solution	Radiographic assessment of glass ionomer restorations with and without prior application of nano-silver fluoride in occlusal carious molars treated with partial caries removal technique	Partial dentin caries removal	Caries arrest prior to glass ionomer restoration	Completed	NCT03193606
Hydrogel/nano silver-based dressing	Evaluation of diabetic foot wound healing using hydrogel/nano-silver-based dressing vs. traditional dressing	Diabetic foot ulcer	Wound healing	Completed	NCT04834245
Central venous catheter impregnated with AgNPs (AgTive®)	Comparison of central venous catheters with silver nanoparticles vs. conventional catheters	Central venous catheter-related infections		Completed	NCT00337714
Silver colloid	The effectiveness of topical silver colloid in treating patients with recalcitrant chronic rhinosinusitis	Recalcitrant Chronic rhinosinusitis		Completed	NCT02403479
Nano-silver (SilvaSorb®) gel	Efficacy of silver nanoparticle gel vs. a common antibacterial hand gel		Antimicrobial	-	NCT00659204
Nano-silver Fluoride	Remineralization of dentine caries using two remineralizing agents which are nano-silver fluoride and casein phosphopeptides amorphous calcium phosphate	Dental caries	Remineralization	recruiting	NCT04930458
Nano-silver fluoride	Effect of using different varnishes on dentin hypersensitivity; Na fluoride and nano-silver fluoride	Dentin hypersensitivity	Tooth hypersensitivity	Not yet recruiting	NCT04731766

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Nano-silver fluoride varnish	P11-4 and nano-silver fluoride varnish in treatment of white spot carious lesions	WSL	Remineralization in permanent teeth	recruiting	NCT04929509
Nano silver fluoride solution	Antibacterial effect of nano silver fluoride vs. chlorhexidine on occlusal carious molars treated with partial caries removal technique	Dental caries	Antibacterial	Completed	NCT03186261
Nano silver fluoride	Clinical and radiographic evaluation of nano silver fluoride vs. calcium hydroxide in indirect pulp treatment of deep carious second primary molars, randomized clinical trial	Deep caries		-	NCT04005872
AgNPs vs. copper NPs	Effect of metallic nanoparticles on nosocomial bacteria	Nosocomial infections	Antibacterial	Recruiting	NCT04775238

The antimicrobial activities of AgNPs described above were for AgNPs that were synthesised using traditional chemical synthesis, which have shown superior effects against various oral pathogens when compared to other MNPs, such as gold (AuNPs) and zinc oxide (ZnO) NPs. This was demonstrated by Hernández-Sierra et al. who showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for these AgNP against *S. mutans* was of 4.86 µg/mL and 6.25 µg/mL, respectively [96]. In comparison, the MIC for AuNPs and ZnO NPs was significantly higher at 197 µg/mL and 500 µg/mL, respectively. AgNP-containing sodium dodecyl sulfate micelle aggregate assemblies were effective against various oral pathogens involved in development and progression of caries (*Pseudomonas aeruginosa*, *Streptococcus gordonii*), root canal infections (*Streptococci* spp.), and periodontal disease (*Enterococcus faecalis*) [86]. Commercially produced AgNPs (Nano-world Company, Calcutta, India) had a MIC between 2.82 and 90 µg/mL against *S. mutans*, *Streptococcus oralis*, *C. albicans*, *L. acidophilus*, and *Lactobacillus fermentum*. The amount of AgNPs required to inhibit bacterial growth was similar to that of conventional antimicrobial agents, such as CHX [97], SDF and 70% isopropanol [86]. Their effect was not diminished when formulated into dental products such as toothpaste. Toothpaste containing AgNP (TruCareNanosilver) had the highest antibacterial efficacy against *S. mutans* compared to those containing chitosan (Conybio Plus Chitosan Dental) and fluoride (Oral B Pro Health). This validates the effectiveness of AgNP-based dental formulations for plaque control and prevention of dental caries or infections [30], and it achieves important clinical effects with a reduced bystander toxicity. Although AgNPs demonstrated many dental health benefits, they can be limited by their instability in various media, and consequently, lose their bioactivities [98]. Their stability in solution can be improved by adding a variety of inorganic or organic [99], synthetic or natural [100], and biotic or abiotic materials as capping agents [101].

1.3.2. Plant-Synthesized AgNPs for Treatment of Dental Diseases

Several methodologies have been employed for the synthesis of MNPs with different sizes and shapes. Generally, MNP synthesis is achieved by various physical and chemical methods, such as laser ablation, lithography, chemical vapor deposition, sol-gel technique and electro-deposition. However, all these methods are not only costly, but were also reported to produce products that might be harmful to humans and the environment. The methods are discussed in detail elsewhere [74,77,102].

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With the challenges and concerns raised about MNPs synthesized using physical and chemical methods, many efforts have been geared towards the development of greener and cheaper methods for the synthesis of MNPs. The biosynthesis of MNPs has been shown to be more cost-effective and environmentally friendly compared to the chemical and physical methods [103], because they use natural products from microorganisms and plant extracts to reduce metal precursors, leading to the formation of MNPs [104]. However, while these are green synthesis methods, MNPs produced by microbiological procedures are time-consuming, and both the synthesis and recovery of the MNPs after synthesis can be costly due to the expenses incurred to maintain the organisms during synthesis. Green synthesis methods which involve first extracting natural compounds from microorganisms or plants and then using these extracts for the in vitro synthesis of the MNPs have also been developed, and those synthesized with plant extracts are rapid, use one step synthesis, and do not require sophisticated purification methods [105]. Plant extract-mediated MNP synthesis is more economical as plant extracts are readily available, and extracts from various plant material, such as leaves, roots, stems, barks, flowers, vegetables, fruits, etc., can be used in the synthesis [77,106,107]. In addition, plant-derived waste such as the peels of fruits can also be used to synthesise MNPs. These plant materials contain various phytochemicals (e.g., alkaloids, terpenoids, phenolic) and secondary metabolites (vitamins, amino acids, enzymes, proteins, polysaccharides, antioxidants) [108] that can act as reducing, capping and stabilizing agents during the synthesis of MNPs, either individually or collectively [109].

Synthesis of AgNPs Using Plant Extracts

Plant extract-mediated synthesis of MNPs, including AgNPs, have been widely explored and used for various applications [77]. Parameters such as metal precursor concentration, pH, phytochemical composition, reaction temperature and time largely influence the physicochemical properties of AgNPs, such as size, shape, surface charge and morphology [110,111], but can also influence the bioactivities of the AgNPs. Most studies have reported the synthesis of AgNPs in a basic pH medium which seems to produce NPs with a higher stability [112] and a more monodisperse nature. Other advantages reported for synthesis methods done using a basic pH is the rapid rate of synthesis [113] and enhanced reduction process. It was found that small and uniform-sized NPs were synthesized by increasing the pH of the reaction medium [114,115]. The nearly spherical AgNPs produced with the crude extracts were converted to spherical AgNPs by altering pH. However, synthesis in a very high pH (pH > 11) environment was associated with the formation of unstable AgNPs and NP agglomeration [116]. Additionally, the synthesis of AgNPs using plant extracts can be achieved at higher temperatures, including at boiling point (100 °C), while synthesis using mesophilic microorganism can only be performed at temperatures ≤ 40 °C. Mesophilic microorganism cannot survive at temperatures > 40 °C, and this might affect NP synthesis. At 30–90 °C range, AgNP synthesis occurs at higher rate and produce smaller sized AgNPs [117]. Overall, plant-mediated AgNPs have been synthesized at ambient temperatures (25 °C to 37 °C), which produce NPs that still have bioactivities [110]. Their antibacterial actions are usually attributed to various parameters, not limited to size, surface composition, charge and chemical reactivity [8,73]. The fact that these NPs can be synthesised at lower temperatures means that less energy is required to produce these nanomaterials, which does not only reduce the cost of synthesis, but these methods are also more energy efficient and therefore more environmentally friendly. Plant-extract-mediated synthesis of AgNPs have been achieved with various plants extracts; examples include extracts produced from fruit pulp and peels (pears [107]), vegetables (cauliflower [118]), and some of the plants used in traditional medicine (*Cotyledon orbiculata* [119], *Salvia africana-lutea* [120], *Terminalia Mantaly* [106]).

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Plant Extract-Synthesized AgNPs for Treatment of Dental Diseases

Green synthesis of AgNPs using plant extracts is one of the rapid, simple, and cost-effective approaches that produced stable and biocompatible NPs. Green synthesized AgNPs can be used as a substitute for chemically-synthesized AgNPs. Several in vitro studies that describe the antimicrobial activity of plant-mediated AgNPs against oral pathogens have been reported. AgNPs synthesized from the leaf extract of *Justicia glauca* showed antimicrobial activity alone and in combination with Azithromycin and Clarithromycin against *S. mutans, S. aureus, L. acidophilus*, *Micrococcus luteus, B. subtilis, E. coli, P. aeruginosa,* and *C. albicans*. These AgNPs were also effective against various microorganisms that are associated with dental caries and periodontal disease, with MIC values between 25–75 μg/mL [104]. In another study, biogenic AgNPs produced from plant extracts of *Azadirachta indica* (*A. indica*), *Ficus bengalensis* (*F. bengalensis*) and *Salvadora persica* (*S. persica*) showed antibacterial activity against *L. acidophilus*, *L. lactis*, and *S. mutans*. The *S. persica* AgNPs followed by *A. indica* were more effective against the oral pathogens than the *F. bengalensis* AgNPs [121]. *Haliclona exigua*-AgNPs showed potential to inhibit biofilms on some of the microbes involved in formation of oral biofilm, i.e., *S. oralis*, *S. salivarius* and *S. mitis* [122].

AgNPs synthesized from the aqueous extracts of three different parts of rice grain—viz., rice bran (RB), rice husk (RH), and rice germ (RG)—showed antimicrobial activity against *S. aureus, E. coli, S. mutans* and *C. albicans* [123]. The AgNPs inhibited the growth of all tested microorganisms [123]. One study showed that the biogenic AgNPs have improved activities compared to the chemically synthesized AgNPs and CHX. The chemical AgNPs were reduced by sodium borohydride ($NaBH_4$), and two green AgNPs were produced using extracts of *Heterotheca inuloides* (Hi) and *Camellia sinensis* (Cs) [124]. Hi produced smaller and more stable NPs when compared to Cs-AgNPs. The green AgNPs inhibited the growth of *S. mutans* and *L. casei* better than 2% CHX, while the smaller Hi-AgNPs had enhanced antibacterial activity compared to the Cs-AgNPs. [124].

Moreover, AgNPs synthesized from extracts obtained from different parts of the pomegranate fruit alone and with β-calcium glycerophosphate showed the highest antimicrobial and antibiofilm activity against *S. mutans* and *C. albicans* [125]. *Gum acacia*-AgNPs were also shown to have antibacterial action against *E. coli* and *M. Luteus*, while the *G. acacia* extracts showed no inhibition effects [126]. Plant extract-mediated AgNPs not only provide a simple one-pot method for in situ synthesis of AgNPs, but produce highly stable AgNPs as they act as both reducing and stabilizing agents. They can be incorporated in different formulations such as mouth rinse and toothpastes to improve their bioactivities [127]. Moreover, like the chemically synthesised AgNPs, the green AgNPs can be used alone or in combination with other dental agents for sustainable therapeutic effects. Thus, AgNPs produced by a green process may be a promising strategy to produce antimicrobial agents against oral pathogens.

1.4. Antimicrobial Mechanism of AgNPs

AgNPs have shown broad spectrum antibacterial effects against a number of microorganisms, including some drug resistant microorganisms. While most of the studies investigating the antibacterial mechanisms of AgNPs has been performed using chemically synthesised AgNPs, it can be expected that green synthesised AgNPs will function in the same way. The bactericidal properties of AgNPs were reported to be size and shape dependent [128], but they are also determined by the dissolution of AgNPs and the release of Ag^+ ions. The influence of size on the antimicrobial activity of chemically- and biologically-synthesized AgNPs was demonstrated in several studies. The antimicrobial activity of AgNPs was reported to be directly proportional to their concentration and inversely proportional to their size, with smaller AgNPs having a lower MIC. AgNPs in the size range of 1–10 nm were shown to have higher antimicrobial activity, as smaller NPs are able to translocate and penetrate into cells much faster than larger ones. Once inside the

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bacterial cells, smaller NPs can easily interact with various cellular components resulting in bacterial death [129]. AgNPs with a diameter of 5 and 15 nm presented a four-fold lower MIC against *S. mutans* (MIC = 50 μg/mL) when compared to 55 nm AgNPs (MIC = 200 μg/mL) [130]. An independent study corroborated the efficiency of smaller size AgNPs, where 9.3 and 21.3 nm AgNPs reduced *S. mutans* adherence on bovine enamel blocks more effectively than larger AgNPs (93 nm). The effects of the 9.3 and 21.3 nm AgNPs were similar to that of CHX [131]. A reduction of 2.3 log in the number of CFUs of *S. mutans* was also observed in biofilms exposed to 100 ppm of 9.5 nm AgNPs [129].

In addition to size, AgNP activity also depends on their shape [132], suggesting that the surface area and the reactivity of AgNP facets are responsible for their antibacterial activity. Silver nanowires showed the weakest antibacterial activity compared to silver nanocubes and nanospheres, while triangular shaped AgNPs were more effective than the nanospheres and rod-like shapes [133]. The triangular shaped AgNPs had a positive charge, which together with the active facets on a triangular-shaped particle was able to ensure a greater antimicrobial activity [134]. In addition to size and morphology of AgNPs, surface composition also plays a role in the activity of AgNPs. This was demonstrated by AgNPs stabilized with chitosan which had altered antibacterial activity against *S. mutans*. The AgNPs showed activity that was higher than the SDF and similar to CHX [134]. Furthermore, the antibacterial action of AgNPs is associated with its dissolution and release of Ag^+ ions. There are several physical and chemical conditions that can affect the dissolution process, such as temperature, metal ion concentration, surface composition, pH, medium, ionic strength, availability of the oxygen, shape, and size [99]. Morphological properties of AgNPs are indirect effectors that influence Ag+ ion release [135]. The antibacterial activity of AgNPs in some microbes is also associated with the AgNPs themselves, which have been shown to interact and penetrate the microorganisms more effectively than the release of Ag+ ions [80]. In essence, the antibacterial activity of AgNPs can be regulated by altering or modifying the above properties (i.e., size, shape, and (or) surface composition of NPs), to achieve a desired function [62].

A number of mechanisms pertaining to the antibacterial activities of AgNPs have been proposed, yet the exact modes of action are not thoroughly elucidated [11]. Figure 3 shows four of the common mechanisms described for AgNPs: (1) AgNPs can directly interact with the bacterial cell membrane and alter its permeability, causing cellular damage. They attach to bacterial cell walls by binding to sulfur-, nitrogen-, oxygen- or phosphorus-containing biomolecules, which changes the membrane permeability and consequently causes leakage of cellular contents. (2) When the AgNPs are exposed to the oxygen-rich environment, Ag^+ ions are released into the cytoplasmic matrix, which then promote microbial death [135]. The Ag^+ ions induce death by reducing the intracellular adenosine triphosphate (ATP) levels, by inhibition of the mitochondrial activity and protein denaturation [94]. (3) Interaction of the AgNPs with various cellular components induces cellular toxicity through the oxidation of lipids, proteins, and nucleic acids, which will affect the function of these biomolecules and ultimately disturb biochemical pathways. (4) Furthermore, AgNPs were reported to induce dephosphorylation of tyrosine phosphorylated proteins responsible for DNA replication and recombination, especially in Gram-negative bacteria [136]. These combined effects of AgNPs ultimately cause the leakage of cellular components, cell lysis, and death. These mechanisms are extensively reviewed elsewhere [137].

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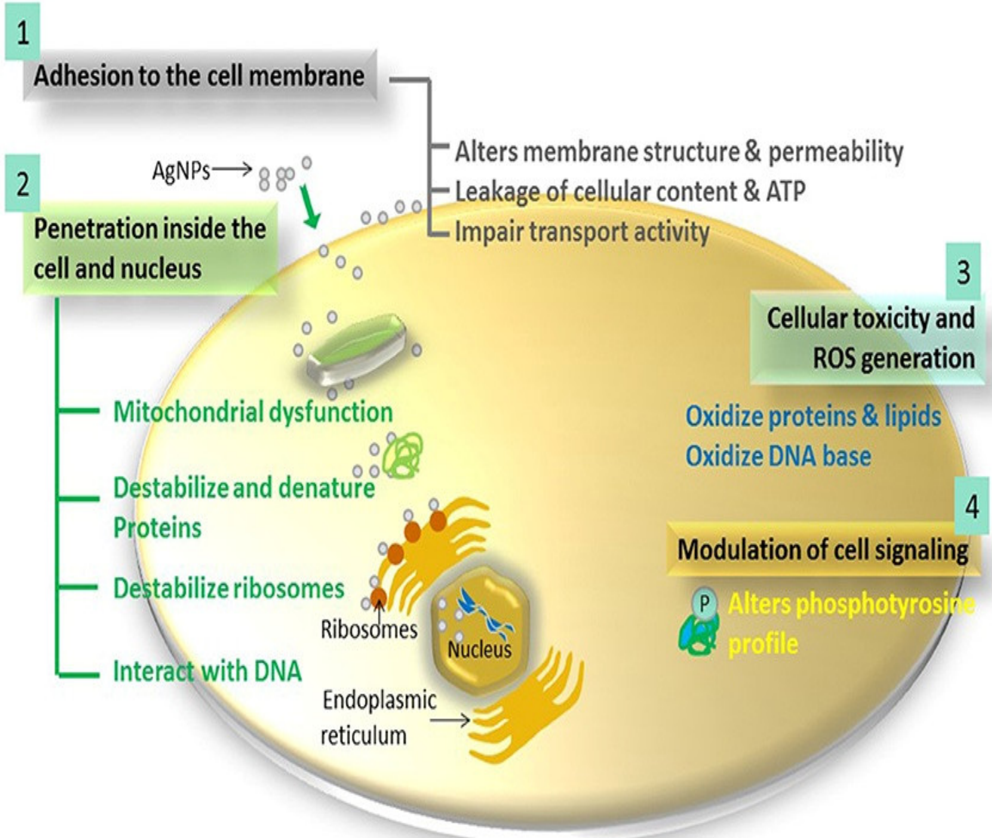

Figure 3. Antibacterial mechanisms of action for AgNPs. Interaction of the AgNPs with the bacterial cell membrane cause damage to the cell membrane and increases membrane permeability (**1**), AgNPs are internalized by the bacteria and interact with cellular organelles and biomolecules (**2**), AgNPs increases ROS production (**3**), and modulate cellular signals leading to cell death (**4**). Reprinted with permission from Ref. [137]. Copyright 2016, Frontiers.

1.5. Toxicity of AgNPs

Despite the advantages of AgNPs that recommend them for novel and improved activities in various biomedical applications, their potential toxicity towards humans and the environment is under scrutiny. Metals have been known for centuries to leach into the immediate environment it comes into contact with; this includes the biological environment after ingestion. The daily amount of silver derived from food and water ingested by humans' range between 0.4–30 µg [138]. With the increasing use of AgNPs in various consumer products, there are also increasing concerns about the possibility that increasing amounts of silver will accumulate in the environment and within humans. AgNPs intended for dental use will be in contact with all structures within the oral cavity, i.e., the teeth, oral cavity tissues, and cells. Thus, these AgNPs might induce serious adverse effects in the surrounding healthy tissues while fighting bacterial infections and oral diseases. There are many reports on the AgNP-induced cytotoxicity in various types of human cells and tissues, including the oral cavity [139–141], which makes the safety aspect of AgNPs questionable.

Several factors influence the biological effects of AgNPs; these include size, shape, surface charge and composition. Of clinical importance will be to investigate the relationship between the biological activity, pharmacological activity, and toxicity of AgNPs [142], before it is considered for human application. Studies so far report contradictory results with respect to the toxic effects exhibited by AgNPs within the biological system [143,144]. The toxicity of AgNPs is mainly associated with their dissolution into Ag^{+} ions

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[145], while their surface composition and charge affect their cellular uptake, translocation, and cytotoxicity. Positively charged AgNPs were more effective against *E. faecalis* and exhibited a high level of cytocompatibility when tested against L929 fibroblast cells [121].

The uptake of AgNPs by cells can induce cellular damage and consequently cell death by damaging vital organelles, such as the nucleus and mitochondria. Interaction of AgNPs with these organelles can cause oxidative stress, DNA damage, chromosomal abnormality, and cell death [146] via necrosis or apoptosis. These phenomena were demonstrated in various human cell lines (keratinocytes, [147], hepatic, neuronal, lung epithelial, and murine stem cells), as well as animal tissues and organs [147]. Repeated exposure to AgNPs can potentiate inflammatory responses, activate the innate immunity [148], and have been associated with renal- [149], hepatic- and genotoxicity [147]. Even at non-cytotoxic doses, repeated doses of AgNPs may be detrimental to vital organs, resulting in their dysfunction and possibly total organ failure [150].

Preclinical studies on rats showed increased accumulation of AgNPs, particularly in the liver, kidney, colon, and jejunum. The accumulation of AgNPs was found to be dose and size dependent [151]. Additionally, oral administration of 56 nm AgNPs in rats resulted in a dose-dependent accumulation of silver in all tissues examined. In this study, a no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) were determined at 30 mg/kg and 125 mg/kg, respectively. A higher incidence of bile-duct hyperplasia, with or without necrosis, fibrosis, and/or pigmentation, was observed in AgNP-treated animals. Accumulation of silver in tissues was gender-related, with a two-fold increase in the kidneys of females compared to males [152]. Although AgNPs were present in all the organs examined, higher concentrations were found in the liver and spleen. The high silver concentrations in these organs suggested that the AgNPs were able to penetrate the intestines of the rats. Although the silver content was cleared from most organs eight weeks after treatment, it was still retained in the brain and testicles [153]. Accumulation of the silver in these organs might result in morphological changes that will change the physiological functions of these organs overtime. For example, AgNPs that are taken-up through inhalation may accumulate in the alveolar regions, which might in turn cause lung injury, and the neighbouring organs or those involved in the reticuloendothelial system, such as the liver and kidneys [154,155]. Intranasal administration is convenient for targeted delivery of drugs to the brain, similarly AgNPs administered by this route can be transported to various organs via the neuronal and systemic pathways [154,156]. Although their repercussions are still largely unknown, the long-term retention of AgNPs can exacerbate organ and systemic disorders [154,155].

The antibacterial activity of AgNPs against oral pathogens are significant, but at the same time their potential toxicity cannot be ignored. Studies have shown that the toxicity of AgNPs can be tampered by modifying the surface composition. Surface functionalization of AgNPs improved their biocompatibility compared to the AgNPs without additional capping agents (AgNPs-UC). AgNPs functionalized with lipoic acid (AgNPs-LA), polyethylene glycol (AgNPs-PEG) or tannic acid (AgNPs-TA) induced cell death in a concentration dependent manner. The AgNPs-UC was not cytotoxic to human gingival fibroblast cells at concentrations ≤ 10 μg/mL; after surface modification, the toxicity of AgNPs -LA) and AgNPs-PEG was reduced to 20 μg/mL and 40 μg/mL, respectively. These were the concentrations required for AgNPs-LA to inhibit *Staphylococcus epidermidis and S. mutans* biofilm formation; at the same time, this was not toxic to the human gingival fibroblast cells. Conversely, the other NPs displayed effective antibacterial activities at concentrations that were toxic to the gingival fibroblast cells, i.e., 80 μg/mL for AgNPs-PEG against *S. epidermidis* biofilm formation, and 40 μg/mL for AgNPs-UC against *S. mutans* biofilm formation. The lowest cytotoxicity was observed for the AgNPs capped with LA, the differences in toxicity among the AgNPs clearly demonstrated that the capping agent influenced the AgNP toxicity [142].

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In vitro and in vivo toxicity evaluation of 5 nm colloidal AgNPs synthesized with ammonia and polyvinyl pyrrolidone did not induce production of inflammatory mediators (interleukin-1β and -6) at low concentration (≤ 25 μg/mL), when used in endodontic treatment [157]. When 35 nm AgNPs were used at 50 μg/mL as an irrigant to treat root canal space, they showed antibacterial activity against *E. faecalis*, but no cytotoxicity, whereas concentrations ≥ 80 μg/mL were cytotoxic [158]. Additionally, 10 nm spherical AgNPs were biocompatible in fibroblasts and keratinocytes [159]. The cytotoxicity of AgNPs is influenced by several physicochemical features, including dispersion rate, concentration, surface charge, size, morphology, and composition [160]. The physicochemical properties of nano-silver-based systems raise many toxicological concerns. The experimental results reported to date are insufficient regarding the accurate toxic effects of AgNPs and their related toxicity mechanisms [161]. There are few studies that have examined the cytotoxicity of green synthesized AgNPs to validate their presumed higher biocompatibility. Although studies reporting on AgNPs prepared using green methods are likely biased, these NPs can potentially have similar characteristics to chemically synthesize AgNPs. AgNPs synthesized from *Cotyledon orbiculata* were shown to reduce the viability of THP-1 differentiated macrophages at concentrations between 2.5 and 20 μg/mL [119]. AgNPs synthesized from red pear extracts were not toxic to RAW 264.7 cells at concentrations up to 500 μg/mL, while the AgNPs synthesized from green pear extracts reduced cell viability when concentrations were higher than 125 μg/mL. The fact that these AgNPs showed significant antibacterial effect at concentrations that were not toxic to mammalian cells means that these AgNPs can be considered as biocompatible and probably safe for applications at these doses [107]. Unfortunately, many studies lack appropriate normal cell controls to compare the effects of nanomaterials on normal and diseased cells. Nonetheless, green synthesized AgNPs have shown superior therapeutic activities. *Haliclona exigua*-AgNPs exhibited a dose-dependent cytotoxicity on the human oral cancer (KB) cell line with half the maximal inhibitory concentration (IC50) of 0.6 mg/mL [122]. In addition, AgNPs prepared with *Glycyrrhiza glabra* (*G. glabra*) and *Amphipterygium adstringens* extracts inhibited the bacterial growth of *E. faecalis* and the fungus *C. albicans*. Their antiproliferative activity was tested on human epithelial cells, and the results indicated that AgNPs synthesized with *A. adstringens* extract was more toxic to human cells compared to the nanoparticles synthetized with *G. glabra* extract [162]. Furthermore, AgNPs synthezied using natural black tea extract had higher cytotoxic activity against ovarian carcinoma when compared to the colorectal carcinoma cell line [154].

The benefits of AgNPs in dental therapy are indisputable; however, there are health and environmental concerns regarding the use of nanomaterials. Therefore, risk assessment measures must be put in place to ascertain and ensure the safety profile of the AgNPs before they are incorporated in consumer products. This will require strategies that will provide localized AgNP effects to reduce bystander cytotoxic effects [163]. Therefore, using natural products in dental formulations as alternatives to fluoride-based containing dentifrices will be more acceptable to consumers as it provides a safety aspect.

2. Conclusions

Plant products have been used traditionally for the treatment of many infectious and chronic diseases. The emergence of green nanotechnology has over the years proved that these effects can be enhanced or repurposed when the plant extracts are used as reducing and capping agents of MNPs, including AgNPs. Plant-mediated synthesis of AgNPs is an eco-friendly natural process, which is cost-effective, and renewable for producing innovative, bioactive and biocompatible AgNP-based products for human use. Thus, biogenesis of AgNPs has a potential application in dental therapy; they can be used as antimicrobial agents in various fields of dentistry. They can be incorporated in dental products that are currently used as canal irrigants, toothpastes, mouth wash, varnish, and also in dental restorative implants. Clinical trials of chemically-synthesized AgNPs are ongoing to evaluate their ability to prevent tooth demineralization, dental biofilms and treatment

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of dental caries. AgNP-based products are already incorporated in various consumer products, and expected to grow exponentially in the coming years. Therefore, it is pivotal that their biodistribution and toxicity is evaluated before their use in human products. Despite these concerns, plant-based AgNPs can be used for development of multifunctional dental care products that can aid in the prevention and treatment of dental infections and diseases.

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2.2.3.b: Peer review

Reviewer Comment	Responses
Metal nanoparticles instead of "metallic nanoparticles"	Changed
Platinum nanoparticles (PtNPs) instead of "platinum nanoparticles (NPs)" – in my opinion the authors should use each abbreviation for each type of described nanoparticles	Changed
The abbreviation MNPs was not explained but it is used in the text. I suppose that MNPs means metal nanoparticles, but MeNPs is a more common abbreviation. I suggest using it.	Changed
The reason for selection of green-prepared AgNPs in dental application should be explained in detail	It has been explained in section 1.4.2.1 Plant-synthesized AgNPs for treatment of dental diseases and enriched by adding synthesized AgNPs can then be used as a substitute for chemically synthesized AgNPs as it is eco-friendly, manageable, and easily scaled-up for large synthesis. Furthermore, in green synthesis there is no need for high temperature, pressure and the use of harmful and expensive chemicals
In my opinion the manuscript should be rearranged. The authors mixed general description regarding preparation of metal nanoparticles with the description of preparation of silver nanoparticles. I think that this induces a horrible mess. The authors should focus only on the description of preparation of silver nanoparticles because the title of the manuscript suggests that the text will be devoted to this type of metal nanoparticles.	It has been addressed in section 1.4.1 as the general description regarding the preparation of metal nanoparticles is an introduction to the preparation of silver nanoparticles which the review is specifically talking about.
The section "1.4.1.1. Synthesis of AgNPs using plant extracts" must be rewritten. From a chemical point of view this part is really poor. It describes randomly collected data. I suggest describing examples of green methods that	It has been combined with section 1.4.1 as the review is not concentrated in describing the size, shape, surface charge and chemical structure of AgNPs but is

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enable the preparation silver nanoparticles of controlled: a) size, b) shape, c) surface charge (positively and negatively charged), d) chemical structure.	concentrated in dental application of AgNPs.
- I strongly suggest developing a description of green-methods of silver nanoparticles synthesis. The authors should describe types of plants (extract of plants) or other microorganisms which were used for the synthesis of silver nanoparticles which then were applied in dental application. This issue is poorly exposed.	The type of plants used for the synthesis of AgNPs which have been applied in dental application were described in section 1.4.2.1 plant synthesized AgNPs for treatment of dental disease, which include examples from the literature.
The authors collected in Table 2 diverse examples of use of silver nanoparticles in clinical trials. Nevertheless, I did not find information that the silver components collected in this Table were synthesized by green-methods. My feeling is that the title of the manuscript does not fit well with the main text of the work. The authors should change the title and remove the description "green-synthesized" or they should develop the manuscript describing extensively green-procedures and examples regarding green-prepared silver nanoparticles.	Table (2) which is sketching the chemical clinical trials pointing out the void in green synthesis clinical trials. This could be becuase of the novelty of green synthesis.
The advantages of using silver nanoparticles in dental treatments were described correctly. However, I recommend considering disadvantages and likely undesirable effects from the use of silver nanoparticles. Please notice that silver nanoparticles prepared using green methods can be highly toxic for tumor and normal cells. In the manuscript the authors selected and described only promising examples of the use of silver however they also should consider the problems related to long application of silver, acquiring resistance to silver by bacteria and pathogenic fungi ect.	The last comment has been changed by the addition of examples related to green AgNPs in the cytotoxicity section.

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Editors Plagiarism Comments	Texts in question were paraphrased
Dental caries, a biofilm related disease, is a chronic disease that results when microbial biofilm (plaque) formed on the tooth surface converts the free sugars contained in foods and drinks into acids that dissolve the tooth enamel and dentine over time. With continued high intake of free sugars without regular microbial biofilm removal, the tooth structures are destroyed, resulting in development of cavities and pain.	Dental caries is a chronic disease that results from the accumulation of microbial biofilm on the tooth surface that converts the free sugars contained in foods and drinks into acids that destroy the tooth structure over time leading to demineralization and subsequently the development of caries.
At physiological pH, saliva is supersaturated with calcium and phosphate ions, ensuring that the ions remain bioavailable to diffuse into mineral deficient lesions. However, longitudinal studies that followed the natural progress of WSLs found that although some lesions get smaller, the majority are largely unaffected.	Saliva is normally concentrated with calcium and phosphate ions, which spread into the mineral deficient lesions to prevent the demineralization process. However, some studies demonstrated that these lesions remain largely unaffected, while few lesions get smaller.
Fluoride toothpaste supplemented with lysosome, lactoferrin and proteins generating hydrogen peroxide and hypothiocyanite can modify the composition of saliva by increasing the proportion of bacteria associated with gum health and decreasing the bacteria associated with disease.	Fluoride toothpaste may contain some components such as lysosome, and proteins which produces hydrogen peroxide and hypothiocyanite facilitating the growth of beneficial bacteria associated with gum health while inhibiting the growth of cariogenic bacteria
As reported by the 23rd WHO Expert Committee on the selection and use of essential medicines' Executive Summary, "the burden of oral diseases, particularly untreated dental caries, represents a significant public health problem globally". The establishment of a new section for dental preparations is a key development to tackle the burden of dental caries and oral health inequalities, ensuring oral health promotion and care are part of efforts to increase access to essential medical products.	The burden of oral diseases, particularly untreated dental caries, represents a significant public health problem globally, therefore, the WHO recommends the development of a new section for dental preparations as essential medical products to promote oral health and care.
Temperatures up to 100˚C were used by many researchers for AgNP synthesis using biopolymers and plant extracts, whereas the use of mesophilic microorganism restricted 295 the reaction temperature to 40˚C. At higher temperatures the mesophilic microorganism dies due to the inactivation of their vital enzymes. The temperature increase (30˚C - 90˚C) resulted in increased rate of AgNPs synthesis and also promoted	Temperatures used for the synthesis of AgNPs from biopolymers and plant extracts can be up to 100˚C, while AgNPs synthesized from mesophilic microorganism uses 40˚C, as they can die at higher temperatures. Overall, plant mediated AgNPs have been synthesized at ambient temperatures (25˚C to 37˚C) and still have bioactivities. However, increasing the tempreture (30˚C - 90˚C) resulted in the

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the synthesis of smaller size AgNPs. Overall, plant mediated AgNPs have been synthesized at ambient temperatures (25˚C to 37˚C) and still have bioactivities [86]. Their antibacterial actions are usual attributed to their unique physicochemical properties such as small size, large surface-area-to-volume ratio and increased chemical reactivity. The surface charge of the AgNPs enables them to interact with the negatively charged surface of bacterial cells to a greater extent resulting in enhanced antimicrobial activity	synthesis of smaller size AgNPs as well as it promoted the rate of AgNPs synthesis.
AgNPs against the selected dental caries and periodontal disease-causing microorganisms was noticeable between 25–75 μg/mL. In another study, biogenic AgNPs produced from plant chewing sticks of Azadirachta indica, Ficus bengalensis and Salvadora persica had antibacterial activity on L. acidophilus, L. lactis, and S. mutans. The S. persica AgNPs were extremely effective against oral pathogens, followed by A. indica and F. bengalensis AgNPs. Flower-like silver nanocolloids biosynthesized using a marine sponge (Haliclona exigua) extracts had antimicrobial and antiproliferative activities against important primary colonizers of oral biofilm (S. oralis, S. salivarius and S. mitis) and oral cancer cell lines, respectively.	The MICs value of the *Justicia glauca* AgNPs were noticeable between 25–75 μg/mL (Emmanuel *et al.*, 2015). In another study, biogenic AgNPs synthesized from plant chewing sticks of *Azadirachta indica*, *Ficus bengalensis* and *Salvadora persica* was found to have antibacterial activity on *L. acidophilus*, *L. lactis*, and *S. mutans*. The *S. persica* AgNPs had more antibacterial activity against the oral pathogens tested, followed by *A. indica* and *F. bengalensis* AgNPs.
An independent study also showed that smaller size AgNPs (9.3 and 21.3 nm) were more efficient than larger AgNPs (93 nm) in reducing S. mutans adherence on the bovine enamel blocks in vitro. The smaller AgNPs had similar effects to CHX, a gold standard for antimicrobial activity against oral microbes.	An independent study corroborated the efficiency of smaller size AgNPs , and the smaller size (9.3 and 21.3nm) reduced S.mutans adherence on bovine enamel blocks more than the larger AgNPs (93nm).
Even though the antibacterial activity of AgNPs is mainly associated with the release of Ag+ ions, in some bacterial species its antimicrobial 490 activity is also attributed to the AgNPs, which are taken up by the bacteria more effectively than Ag+ ions. AgNP morphological properties are indirect effectors that influence Ag+ ion release . Therefore, the antibacterial activity of AgNPs could also be controlled by modulating Ag+ ion release, possibly	In some bacterial species the mechanism of action is attributed to the uptake of AgNPs which is more efficient than Ag+ions. Additionally, AgNP structural properties can influence Ag+ ion release. Therefore, the microbicidal activity of AgNPs could also be controlled by modifying the Ag+ ion release through modulating the particle size, shape and surface composition.

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through manipulation of oxygen availa- 494 bility, particle size, shape, and (or) surface composition	
Exposure to AgNPs were reported to increase oxidative stress, genotoxicity, and 541 apoptosis levels in-vitro. AgNPs may cause significant oxidative damage to the cel- 542 lular membrane and organelles such as the nucleus, mitochondria, and lysosomes, leading 543 to cell death via necrotic or apoptotic phenomena. Mitochondrial dysfunction induced by AgNPs was demonstrated in hepatic, neuronal murine stem, and lung epithelial cells. The oxidative stress caused by AgNPs can result in inflammatory responses, activa tion of innate immunity and increased cell permeability. Even at non-cytotoxic doses, AgNPs may cause chromosomal abnormality, DNA damage, and possible mutagenicity	Chemical synthesis of AgNPs were reported to cause cell necrosis and eventually cell death by damaging the cellular membrane and organelles such as nucleus and mitochondria. Impairment of cell mitochondria will have negative effect in hepatic, neuronal, murine stem, and lung epithelial cells. Furthermore, AgNPs may result in chromosomal abnormality, and carcinogenicity even at non-cytotoxic doses. *In-vitro* and *in-vivo* toxicity evaluation of colloidal AgNPs (5nm) synthesized with ammonia and polyvinyl pyrrolidone when used in endodontic treatment did not induce production of inflammatory mediators (interleukin-1β and -6) at low concentration (≤25 µg/mL). When 35 nm AgNPs were used as irrigant to treat root canal space, at 50 µg/mL they showed antibacterial activity against *E. faecalis* without any cytotoxicity, whereas at ≥ 80 µg/mL they were cytotoxicity. Furthermore, the inhalation of AgNPs can lead to serious organs damage. For example, inhaled AgNPs can result in lung injuries, alterations in the liver, kidneys and nervous system, and if it affects the trachea it can result in ischemic injury
AgNPs have shown antibacterial activity against a panel of oral pathogenic bacteria and bacterial biofilms, which was accompanied by potential cytotoxic effects on human gingival fibroblasts. Uncapped AgNPs (AgNPs-UC) as well as AgNPs surface-functional-sized with lipoic acid (AgNPs-LA), polyethylene glycol (AgNPs-PEG) or tannic acid (AgNPs-TA) induced cell death in a concentration dependent (5, 10, 20, 40, 60, 100 µg/mL) manner after 24 h treatment. AgNPs-UC did not cause any cytotoxicity at concentrations 573 ≤ 10 µg/mL; AgNPs-LA ≤ 40 µg/mL; AgNPs-PEG ≤ 20 µg/mL; and AgNPs-TA ≤ 10 µg/mL. 574 AgNPs-LA eradicated Staphylococcus epidermidis and S. mutans biofilm at 20 µg/mL and 40 µg/mL, respectively; and these concentrations were nontoxic to human gingival fibroblast cells.	In a study by Niska et al, they tested the antimicrobial activity of different commercial types of AgNPs-Uncapped AgNPs (AgNPs-UC) as well as AgNPs surface-functionalized with lipoic acid (AgNPs-LA), polyethylene glycol (AgNPs-PEG) or tannic acid (AgNPs-TA) against a group of oral pathogenic bacteria, and subsequently they evaluated the cytotoxic effect of these AgNPs on human gingival fibroblast cells. The results indicated that the cytotoxicity was concentration dependent (5, 10, 20, 40, 60, 100 µg/mL). AgNPs-LA inhibited *Staphylococcus epidermidis and S. mutans* biofilm at 20 µg/mL and 40 µg/mL, respectively; and these concentrations were nontoxic to human gingival fibroblast cells. While the others were effective at concentrations that were toxic to the gingival fibroblast cells, i.e. AgNPs-PEG at 80

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	µg/mL against *S. epidermidis* biofilm and AgNPs-UC at 40 µg/mL against *S. mutans* biofilm. The lowest cytotoxicity was observed for the AgNPs capped with LA, this variation in the cytotoxicity among the AgNPs clearly demonstrated that the capping agent influenced the AgNP toxicity.
Although there is a general concern that some nanomaterials may be potentially harmful to people and the environment, it is necessary to conduct a hazard assessment associated with exposure to nanomaterials and support sustainable nanotechnologies. By using an ideal matrix that ensure the local activity of the antimicrobial agent with low release to the environment, it should be possible to use the NPs without inducing by-stander cytotoxic effects. Furthermore, even though many studies have shown the antimicrobial effect of AgNPs against certain oral microbes, most of the studies have been limited to exploring the effects of AgNPs on monoculture biofilms, and it is not possible to extrapolate how such AgNP-systems might perform when treating the complex multi-species biofilms associated with dental caries in the oral cavity. Therefore, using natural product will find greater acceptability among the general public compared to fluoride-based containing dentifrices.	Although it is known that some NPs may have detrimental effect to people and to the environment, it is mandatory to carry a risk assessment associated with exposure to nanoparticles and to ensure their local antimicrobial activity with low diffusion to the environment to use the NPs without including bystander cytotoxic effect. Depending on the above-mentioned studies, the cytotoxicity of AgNPs is influenced by several physicochemical features, including dispersion rate, concentration, surface charge, size, morphology, composition, and agglomeration of AgNPs. Up to date, the experimental results reported are insufficient regarding the accurate toxic effects of AgNPs and their related toxicity mechanisms

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2.3. *In vitro* laboratory tests

This section describes the detailed methodologies of independent *in vitro* experiments, whose results are statistically analyzed for inference and therefore each individual experiment leads to the subsequent one. There were 3 levels of experiments, listed under 2.3.1, 2.3.2 and 2.3.3.

The GA-AgNPs are synthesized and characterized in the first experiment (2.3.1). Gram positive and Gram negative bacteria, including *S. aureus*, MRSA, *S. epidermidis*, and *S. pyogenes*, were employed to test the antibacterial potency of the produced GA-AgNPs. Additionally, the cytotoxicity of the produced nanoparticles was assessed on three human cell lines: HT-29, Caco-2 cancer cells, and KMST-6 normal skin fibroblasts.

STATUS: This study has been published in the International Journal of Molecular Science. Manuscript ID: 23031799.

In the second experiment (2.3.2) these nanoparticles were tested on selected oral microbes for their efficacy in controlling these microbes. Furthermore, the attachment of GA-AgNPs to the tooth enamel was evaluated.

STATUS: This study has been published in Journal of Bioinorganic Chemistry and Applications, Manuscript ID: 9602325.

The third experiment (2.3.3) includes testing the cytotoxicity of the synthesized GA-AgNPs on the buccal mucosa fibroblast cells and the KMST-6 cells.

STATUS: The paper of this study is being prepared for submission to an appropriate journal.

2.3.1. Broad spectrum anti-bacterial activity and non-selective toxicity of gum Arabic silver nanoparticles

2.3.1.a Executive summary

This publication manipulates objective (b) and answered the research question (b). It explains the rationale of choosing and the use of gum Arabic as a reducing agent to synthesize AgNPs. wide-spectrum antibacterial activity against both Gram-positive and -negative species. GA-

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AgNPs had nonselective toxicity to the selected cell lines. However, in combination with sodium borohydride ($NaBH_4$) it showed selective toxicity**.**

STATUS: This paper has been published in the International Journal of Molecular Sciences. Manuscript ID: ijms-1555642.

2.3.1.b. Publication

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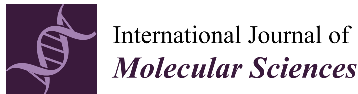
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Article

Broad Spectrum Anti-Bacterial Activity and Non-Selective Toxicity of Gum Arabic Silver Nanoparticles

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Abstract: Silver nanoparticles (AgNPs) are the most commercialized nanomaterials and presumed to be biocompatible based on the biological effects of the bulk material. However, their physico-chemical properties differ significantly to the bulk materials and are associated with unique biological properties. The study investigated the antimicrobial and cytotoxicity effects of AgNPs synthesized using gum arabic (GA), sodium borohydride ($NaBH_4$), and their combination as reducing agents. The AgNPs were characterized using ultraviolet-visible spectrophotometry (UV-Vis), dynamic light scattering (DLS), transmission electron microscopy (TEM), and Fourier-transform infrared spectroscopy (FT-IR). The anti-bacterial activity was assessed using agar well diffusion and microdilution assays, and the cytotoxicity effects on Caco-2, HT-29 and KMST-6 cells using MTT assay. The GA-synthesized AgNPs (GA-AgNPs) demonstrated higher bactericidal activity against all bacteria, and non-selective cytotoxicity towards normal and cancer cells. AgNPs reduced by $NaBH_4$ (C-AgNPs) and the combination of GA and $NaBH_4$ (GAC-AgNPs) had insignificant anti-bacterial activity and cytotoxicity at $\geq$50 µg/mL. The study showed that despite the notion that AgNPs are safe and biocompatible, their toxicity cannot be overruled and that their toxicity can be channeled by using biocompatible polymers, thereby providing a therapeutic window at concentrations that are least harmful to mammalian cells but toxic to bacteria.

Keywords: anti-bacteria; cytotoxicity; green synthesis; gum arabic; silver nanoparticles

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1. Introduction

In the past, silver-based compounds were used as antimicrobial agents due to their microbicidal activities [1]. Their biomedical application was encouraged by the fact that silver ions (Ag^+) and their related compounds are less toxic towards mammalian cells while being highly toxic to microorganisms, such as bacteria and fungi [2,3]. Recent advances in the field of nanotechnology have influenced and increased the use of silver-based compounds at a nanometer size. Several physical and chemical methods have been reported for the synthesis of AgNPs [4,5], however, AgNPs produced by these methods lead to the production of noxious compounds that are toxic to cells and the environment. To overcome these toxic effects, green synthesis methods, using natural products as reducing and stabilizing agents, were developed [6]. Green synthesis methods produce nanoparticles (NPs) using eco-friendly and non-toxic biological agents, such as microorganisms (e.g., bacteria, yeasts, fungi, and algae) and plant extracts as reducing and stabilizing agents [1,7,8]. Plant-extract mediated green synthesis of NPs is often preferred over the microbial-mediated synthesis

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method due to the biohazards and laborious process associated with the latter [9,10]. The use of plant extracts in green synthesis is easier, more efficient, eco-friendly and incurs low cost in comparison with the chemical or microbial mediated synthesis methods. Plant materials are cost-effective as plants are renewable, readily available, and contain antioxidant-rich phytochemicals [4] that can play a major role in the reduction and stabilization of Ag^+ into bioactive AgNPs. The availability of plants makes the green method amenable to large-scale production of NPs. Over the last few years, there has been an upsurge in the application of plant-extract-reduced AgNPs on account of their immense antimicrobial efficacy, and they are perceived as future-generation therapeutic agents against drug-resistant microbes. Examples of plant-based AgNPs that have demonstrated good anti-bacterial properties and potential anticancer effects include those synthesized using *Chrysanthemum indicum L* [11], *Acacia leucophloea* [12] and *Ganoderma neojaponicum Imazeki* [13] extracts.

AgNPs have distinct and superior properties compared to their bulk materials, and this has afforded their integration into numerous consumer (e.g., cosmetic and household) and health products to prevent microbial infestation and growth. AgNPs are now present in commercial products used daily, such as toothpaste, sunburn lotions, food packaging, medical devices, and clothing [14,15]. In addition to the antimicrobial effects of AgNPs against infectious microbes [16], they are used in catalysis [17], disease treatment [18,19], and as additives in polymerizable dental material [20–22].

Polysaccharides have played a huge role in the application of nanomaterials, especially in biomedical applications. Polysaccharides derived from algae (*Pterocladia capillacae*, *Jania rubins*, *Ulva faciata*, and *Colpmenia sinusa*) [23] and plants (gum arabic, GA [24]) alike, were previously used as stabilizers and capping agents for nanomaterials, both chemical and green synthesized NPs, to enhance their biocompatibility and biosafety. The most widely explored polysaccharide-rich compounds are chitosan [25] and GA [24]. GA is a natural plant-based gum composed of a complex mixture of glycoproteins and polysaccharides, in addition to being a historical source of monosaccharides, arabinose and ribose. GA is considered a safe additive with no adverse effects [26] and has wide applications in the food (e.g., stabilizer, thickening agent and hydrocolloid emulsifier), textile (e.g., pottery, lithography, and cosmetics) and pharmaceutical industries [27]. In the field of nanotechnology, GA has been employed because of its biocompatibility and stabilization effects for nanomaterials [28,29], such as iron oxide NPs [30–32], gold nanoparticles (AuNPs) [33–35], carbon nanotubes [36], quantum dots [37], AgNPs [24], and chitosan NPs (CT-NPs). Cross-linking the carboxylic groups of GA with CT produced CTGA-NPs that had improved mechanical properties, and which consequently found application as a bone graft substitute for bone regeneration [38]. GA has also been used as a reducing agent for the synthesis of GA-AgNPs [6,39]. GA-AgNPs showed potential as promising candidates in the development of antioxidant, anti-inflammatory, antimicrobial [6] and anticorrosive agents [40]. This study demonstrated the anti-bacterial and cytotoxicity effects of AgNPs green-synthesized using GA.

2. Results and Discussion

GA is a non-toxic glycoprotein polymer commonly used as a stabilizer in the food and pharmaceutical industries. It has various pharmacological properties; apart from being used as an emulsifying agent, it has antioxidant, anti-diabetic, and anti-lipid peroxidation properties, among others [41,42]. The chemical composition of GA is complex and varies among species, where all have high levels of carbohydrates and very low protein content [43].

The GA species used in the current study (*Acacia senegal*) had negligible flavonols, flavanols, TPC, with no antioxidant, radical scavenging or reducing abilities at 4 mg/mL, as shown in Table 1. As such, GAE on its own was incapable of reducing a metal precursor into metallic NPs at temperatures $\leq$100 °C. Due to its high sugar content, solubility and binding capacity, GA has been used as a stabilizer for AuNPs [24,30]. It stabilizes NPs by binding to other biomolecules on their surface through its abundant carboxyl groups [30].

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Table 1. Phytochemical analysis and antioxidant capacity of GAE.

Phytochemical Content	4 mg/mL GAE
Flavanols (mg/g)	0.0187
Flavonols (mg/g)	0.0019
TPC (mgGAE/g)	0.0003
DPPH (μmolTE/g)	0.0000
ORAC (μmolTE/g)	0.0000
FRAP (μmolAAE/g)	0.0000

2.1. Synthesis of GA-AgNPs

Synthesis of GA-AgNPs was first attempted at R_T and by boiling (~100 °C) solutions that contained 4 mg/mL GAE and various $AgNO_3$ concentrations (1–5 mM). No GA-AgNPs were formed at all the tested concentrations; there was no color change in the solution at R_T and a pinkish color was observed after boiling the solution (data not shown). The negligible phytochemical and lack of antioxidant contents reported for GAE in Table 1 provides a clear indication that the GAE at the concentration used in the current study was incapable of reducing $AgNO_3$ to form GA-AgNPs. Other studies attempted to synthesize GA-AgNPs by devising methods to potentiate the reducing abilities of GAE, by changing the GAE pH [44], and by using honey as a reducing agent, while using GA as a stabilizer for the AgNPs [40].

A novel, greener approach, using an autoclave method, was established for other gum species to produce sterile AgNPs without additional reducing agents or change of pH. This method was successful in the reduction of $AgNO_3$ by gum acacia [39], gum tragacant [45] and *piyar* gum [46]. The same method was adapted for the synthesis of GA-AgNPs in the current study and was optimized by first varying the concentrations of $AgNO_3$ (0.1–0.5 g/40 mL) then the GAE concentrations (2–6 mg/mL/40 mL). The optimized conditions (i.e., concentrations of GAE and $AgNO_3$) were further used in the combined approach, with $NaBH_4$ as an additional reducing agent, to synthesize GAC-AgNPs.

Using the green synthesis approach, the solution containing GAE and $AgNO_3$ was colorless before autoclaving and changed to brown after autoclaving (Figure 1A). The color intensity increased with increasing concentrations of $AgNO_3$ and GAE. In the combined approach, the samples turned yellow immediately after adding ice cold $AgNO_3$, then to a grayish green color for the C-AgNPs, and brown for the GAC-AgNPs (Figure 1B) after autoclaving. Based on the colors, the GAC-AgNPs were more stable than the C-AgNPs.

The color change was a first indication of formation of the AgNPs, which are reported to have yellow, orange or brown colors [46,47]. Thus, the brown color indicated that GAE at high temperature (120 °C) and pressure (15 psi) was able to reduce Ag^+ into Ag^0, and form GA-AgNPs and GAC-AgNPs. The GAE in the green synthesis approach acted as both a reducing and capping agent for the GA-AgNPs. It is very common in green synthesis, especially for plant-derived NPs, for the biomolecules found in the extracts to serve as reducing, capping and stabilizing agents [47,48]. Plants contain a lot of phytochemicals (e.g., alkaloids, flavonoids, terpenoids, etc.), enzymes/proteins, amino acids, polysaccharides, and vitamins, that can aid in the reduction of metal salts in a rapid and environmentally benign process. Green synthesis is quite advantageous, as it is cost-effective and can be easily scaled up to produce biocompatible AgNPs. Moreover, the medicinal efficacy of the extracts will be a valuable addition to the NPs and enhance their pharmacological activities [40,47,49].

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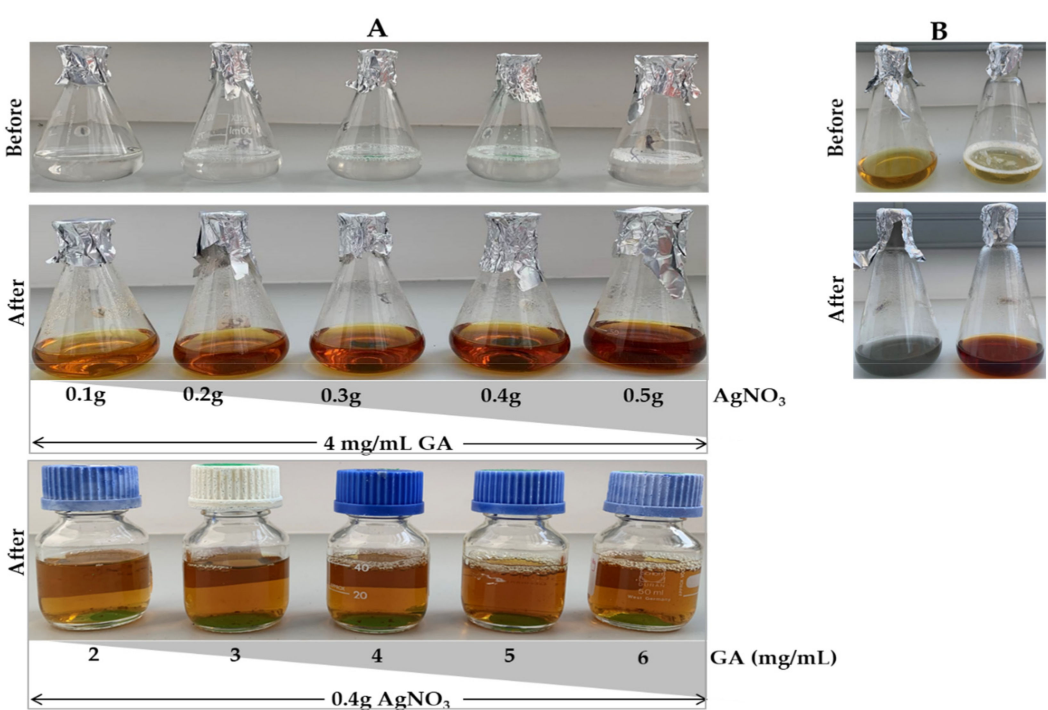

Figure 1. Synthesis of GA-AgNPs using green (**A**), chemical and combined (**B**) approaches.

2.2. Characterization of the AgNPs

2.2.1. Optical Properties of the AgNPs

UV-Vis spectrophotometry was used to confirm the formation of the AgNPs, which have a characteristic SPR around 400 nm [47,49]. Figure 2 shows the absorption spectra for the AgNPs produced via the green (GA-AgNPs), chemical (C-AgNPs) and combined (GAC-AgNPs) approaches. All the concentrations of GAE and $AgNO_3$ were able to synthesize AgNPs, which was confirmed by a characteristic SPR for AgNPs at ~400 nm. The peak intensity of the GA-AgNPs synthesized with 0.4 g $AgNO_3$ was higher than all the other concentrations (Figure 2A), which suggested that more AgNPs were formed at this concentration [48]. An amount of 0.4 g $AgNO_3$ was selected as an optimum concentration and used to optimize the concentration of GAE (2–6 mg/mL). The optimum GAE concentration was 4 mg/mL; both 4 and 5 mg/mL of GAE gave a similar spectral profile, indicating that GA-AgNPs of the same yield, size and shape were produced by the two concentrations (Figure 2B). Although 6 mg/mL showed higher biomass compared to all the GAE concentrations, there were some black precipitates after autoclaving the sample. The precipitates might have contained excess GAE and indicated that the extract concentration might be too high.

In the combined approach, $AgNO_3$ was reduced in the presence of GAE and a chemical reducing agent ($NaBH_4$) to produce GAC-AgNPs (Figure 2C). There are two assumptions as to how the GAC-AgNPs were produced, the first involves $NaBH_4$ acting as a reducing agent to form C-AgNPs (before autoclaving) which are then capped/stabilized by GAE to form GAC-AgNPs during the autoclave process. The second assumption is that GAE and $NaBH_4$ might have acted synergistically as reducing agents. The differences in the spectral profiles of the C-AgNPs$_A$ and GAC-AgNPs (Figure 2C) might have occurred as a result of the instability of C-AgNPs when exposed to high temperatures. The C-AgNPs synthesized at 70 °C were used in further studies (Figure 2D).

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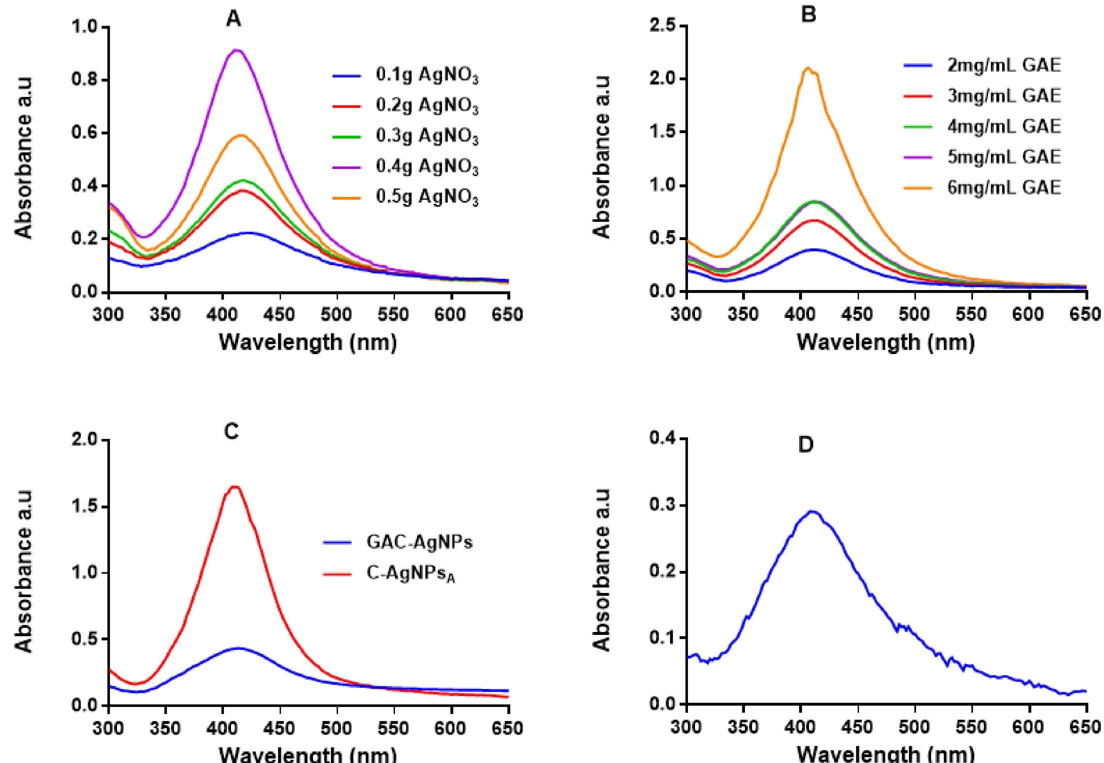

Figure 2. UV-Vis analysis of the AgNPs. The synthesis of GA-AgNPs was optimized using varying concentrations of $AgNO_3$ and 4 mg/mL GAE (**A**), varying GAE concentration and 0.4 g $AgNO_3$ (**B**), and by using $NaBH_4$ alone and in combination with 4 mg/mL GAE (**C**); all these solutions were autoclaved at 121 °C. C-AgNPs were synthesized at 70 °C (**D**).

2.2.2. Morphology and Size Distribution of the AgNPs

The morphology and core size of the AgNPs were analyzed by HRTEM. As shown in Figure 3A, the majority of the AgNPs were spherical in shape; their core size distribution varied from 1–30 nm.

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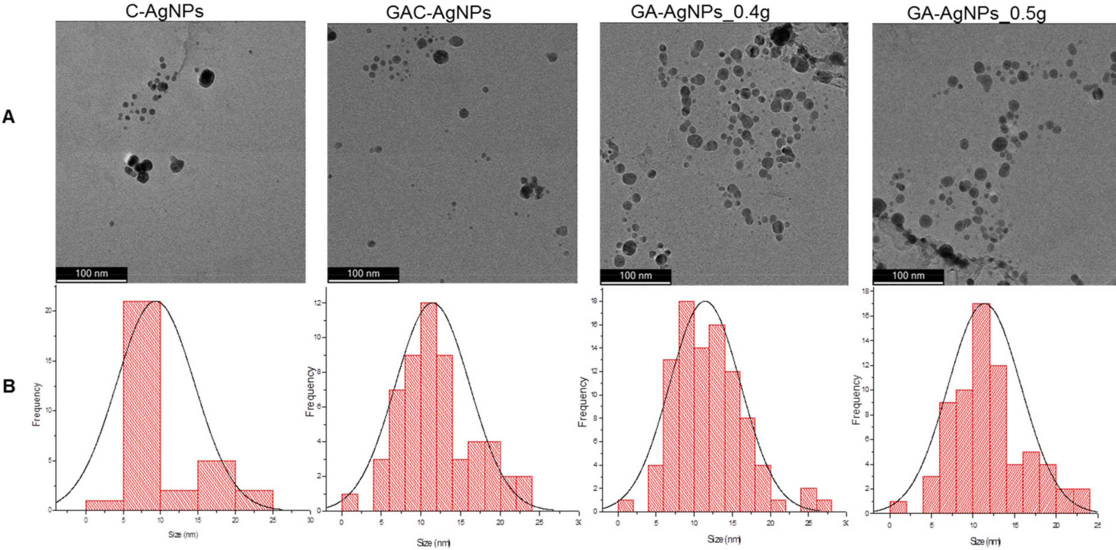

Figure 3. HRTEM micrographs of the AgNPs (**A**) and the AgNP core size distribution (**B**).

DLS analysis revealed a hydrodynamic diameter range from 87.22 nm for the C-AgNPs to 94.62 nm for the GA-AgNPs to 144.39 nm for the GAC-AgNPs (Table 2). These sizes

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vary from those obtained from the HRTEM as they account for both the core size and the molecules on the surface of the AgNPs [47]. The C-AgNPs had a smaller hydrodynamic size, followed by the GA-AgNPs, while the GAC-AgNPs were the largest in size. This indicates that the GAE played a crucial role in the synthesis of the GA-AgNPs as both reducing and capping agents.

Table 2. Physicochemical properties of the AgNPs.

AgNPs	λmax/SPR (nm)	Core Size (nm)	Hydrodynamic Size (nm)	ζ-Potential (mV)	Pdi
C-AgNPs	408	10 ± 1.69	87.22 ± 5.94	-30.50 ± 4.63	0.30 ± 0.03
GAC-AgNPs	414	12 ± 0.61	144.39 ± 4.99	$+9.33 \pm 17.23$	0.55 ± 0.01
GA-AgNPs_0.4g	416	12 ± 0.47	76.21 ± 6.35	-29.60 ± 1.90	0.28 ± 0.03
GA-AgNPs_0.5g	414	12 ± 0.25	94.62 ± 10.06	-27.07 ± 3.71	0.23 ± 0.06

All the AgNPs had a negative zeta (ζ) potential, except for the GAC-AgNPs (9.33 mV). The polydispersity index (Pdi) indicated that GA-AgNPs, followed by C-AgNPs, were the most stable. Pdi serves as an indicator for the dispersity and stability of NPs, thus, NPs with a Pdi that is $\leq$0.05 are regarded as stable and monodispersed. Materials with a Pdi of $\geq$0.7 are classified as polydispersed, with broad size distribution and being less stable in suspension [47]. The GA-AgNPs_0.4g and GA-AgNPs_0.5g demonstrated similar physicochemical properties (Table 2), and the two were investigated further to determine if they have similar bioactivities as well.

2.2.3. FT-IR Analysis of GAE and AgNPs

FT-IR was used to identify the functional groups in GAE and those that were involved in the intermolecular interactions between the precursor ($AgNO_3$) and reducing agents ($NaBH_4$ and GAE). The intermolecular interactions between the samples occurs via hydrogen bonding or dipole–dipole interactions during synthesis and cause shifts in the frequency or absorption of the functional groups [50] that can be assigned to a particular biomolecule.

The dominant absorption bands at 3306–3321, 2139–2161, 1635–1636 and 695–667 cm^{-1} were identified in the FT-IR spectrum of all the AgNPs (Figure 4). These bands were associated with the alkyne C-H stretch (3320–3310), terminal alkyne monosubstituted (2140–2100), C≡C stretch (2260–2100), alkenyl C=C stretch (1680–1620), amide (1680–1630), secondary amine NH bend (1650–1550), alkyne C-H bend (680–610), organic nitrates (1640–1620), and aromatic C-H out-of-plane bend (900–670) [51].

The GAE FT-IR spectra had five major absorption peaks at 3514 cm^{-1} (3570–3200 cm^{-1} OH stretch), 2978 (CH_2 group in aliphatic chains), 2315, 1628 cm^{-1} (1650–1550 cm^{-1} secondary amine NH bend), and 1371 cm^{-1} (1380–1350 aliphatic nitro compounds) and 1065 cm^{-1}. The presence of different functional groups was a reflection of the phytochemical composition of the GAE; the OH bonds are attributed to alcohols or phenols and the N–H bond to amides which might be from the carbohydrates and proteins in GAE. The GAE FT-IR peaks showed similarity to those of other GA species, such as *Acacia senegal* and *Acacia seyal* [40,50,52]. Thus, the carbohydrates and proteins in GAE were responsible for the reduction, capping and stabilization of the GA-AgNPs and possibly the GAC-AgNPs.

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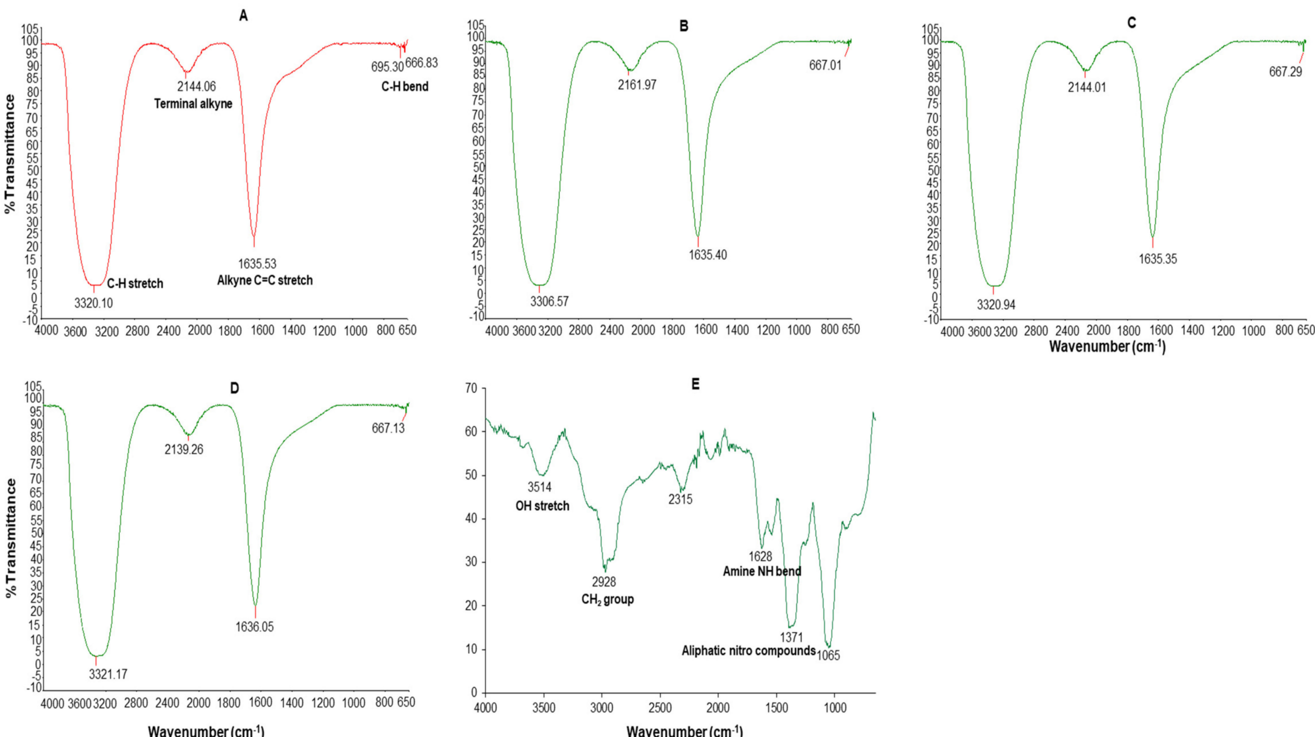

Figure 4. FT-IR spectra of C-AgNPs (**A**), GA-AgNPs_0.4g (**B**), GA-AgNPs_0.5g (**C**), GAC-AgNPs (**D**), and GAE (**E**).

2.3. Stability of GA-AgNPs

Stability of NPs in solutions other than water is crucial for bio-applications and requires NPs that can retain their physical characteristics when introduced into a biological environment. AgNPs are usually very stable in water; however, water is hypotonic and not a suitable vehicle for bioassays [53]. In addition to Pdi, the stability of AgNPs in suspension can also be predicted by their UV-Vis spectral profiles with a characteristic SPR at 400 nm [48]. AgNPs that are not stable will be recognized by aggregation or precipitation out of solution, and if the AgNPs precipitate they will not be useful as antimicrobial agents, as Ag^{+} are known for this effect and have been used for the same purposes [54]. Stability of the AgNPs was assessed at hourly intervals for 6 hr after incubation at 37 °C, as shown in Figure 5A–C; the AgNPs were relatively stable in water, DPBS, and Mueller–Hinton broth (MHB). Cellular uptake of AgNPs is time and size dependent, where uptake and internalization of AgNPs by mammalian cells can occur within 0.5 h [55]. Following the growth kinetics of *Burkholderia pseudomallei*, the interaction and uptake of AgNPs by the bacterial species could be rapid, as the bacteria were killed within 5 min [56]. Biological assays, such as bacteria and cell culture, are performed at 37 °C and AgNPs can be used in culture media for bioassays without aggregation [57]. The components in the media can interact with the AgNPs and change their physicochemical properties and activity; hence, the AgNP-media interactions must be assessed to confirm NP stability before evaluating their activity [58]. Subjecting AgNPs to solutions with higher salt (NaCl) concentration, not only causes NP aggregation, but also a change in size and biological activity [59]. To improve on AgNP stability, biopolymers, such as GA and chitosan, were used as stabilizing agents; this led to plant-mediated synthesis of NPs with enhanced stability, biocompatibility and biological activity.

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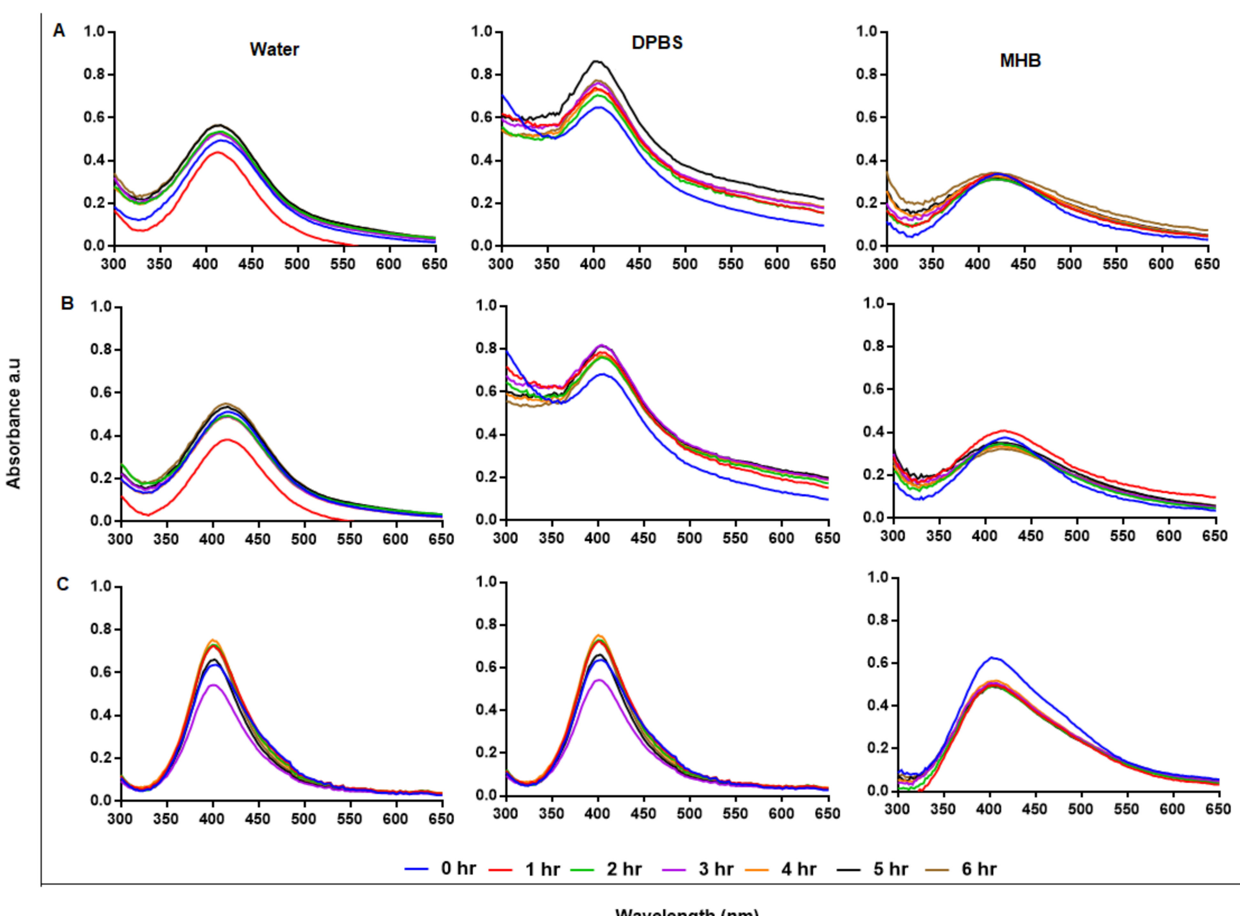

Figure 5. Assessment of AgNP stability in solution by UV-Vis. The GA-AgNPs_0.4g (**A**), GA-AgNPs_0.5g (**B**) and GAC-AgNPs (**C**) were diluted in water, DPBS and MHB and incubated at 37 °C for 1–6 h.

2.4. Anti-Bacterial Activity

Microbial resistance is among the leading factors responsible for death worldwide, due to the overwhelming abuse and misprescription of antibiotics [60,61]. Over the years, alternative antimicrobial agents effective against resistant strains have continually been sought [62,63]. Among others, AgNPs have displayed broad spectrum antimicrobial effects, even against multi-drug-resistant microbes. Of interest are the AgNPs produced through green synthesis, which are presumed to be biocompatible since they are reduced and coated by natural products [47–49,62,64].

The anti-bacterial effects of GAE and AgNPs were evaluated on Gram-positive (*S. aureus*, *MRSA*, *S. epidermidis*, *S. pyogenes*) and Gram-negative (*K. pneumoniae, E. coli*) bacteria. The susceptibility of the bacteria to the treatments was assessed through agar well diffusion and broth microdilution methods. Agar well diffusion demonstrated a lack of clearing zones (zone of inhibitions, ZOIs) in the bacteria that were exposed to MHB (negative control), GAE and GAC-AgNPs, indicating lack of anti-bacterial activity (Table 3) at the concentrations used in this test. Anti-bacterial activity of GAE was reported at concentrations ≥40 mg/mL for various GA species [65], while organic solvent GA extracts were effective from 0.25 to 2 mg/mL [66]. The GA-AgNPs and the C-AgNPs showed potency against the selected bacteria, both Gram-positive and Gram-negative strains; the highest anti-bacterial activity was observed with the GA-AgNPs when compared to the C-AgNPs. The two GA-AgNPs exhibited similar activity against the test bacteria. Similar effects were reported for GA-AgNPs synthesized using other GA species; the GA-AgNPs were potent against oral (*Streptococcus mutans*) [67] and fish (*Aeromonas hydrophila* and *P. aeruginosa*) [68] pathogens.

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The activity of the GA-AgNPs in these pathogens was size, as well as concentration, dependent. C-AgNPs capped with citrate were reported to show size-dependent activity against *E. coli* and *S. aureus* [69].

Table 3. Anti-bacterial activity of the synthesized AgNPs.

Treatments	ZOI (mm)				
	S. aureus	*MRSA*	*S. epidermidis*	*K. pneumoniae*	*E. coli*
MHB	0	0	0	0	0
GAE	0	0	0	0	0
C-AgNPs	9.8	9.8	8.4	11	6.2
GAC-AgNPs	0	0	0	0	0
GA-AgNPs_0.4g	14.2	13.8	20	13.6	11.2
GA-AgNPs_0.5g	13	9.8	19	14.6	10.2

The MICs of the treatments were visually evaluated on the bacteria following microdilution assay. After 24 h treatment, GAE, C-AgNPs and GAC-AgNPs were unable to inhibit growth at all tested concentrations (6.25–100 µg/mL), as shown in Table 4. Bacterial growth inhibition was observed at 6.25–100 µg/mL for the two GA-AgNPs for all strains, with an MIC of 6.25 µg/mL, except for GA-AgNPs_0.4g effect in *E. coli* which had an MIC of 25 µg/mL. The MIC values were consistent with the GA-AgNPs reported by other studies; the NPs had an MIC of 10 µg/mL in *S. mutans* [67], 11–45 µg/mL in *P. aeruginosa* [44], 1.625 and 3.25 µg/mL for *A. hydrophila* and *P. aeruginosa*, respectively [68]. The results in the current study were further confirmed by the Alamar Blue assay, which quantifies the metabolic activity of cells. Only live bacteria can convert the blue resazurin dye into a pink and fluorescent resorufin. The color/fluorescent intensity is directly proportional to live bacteria [62,70].

Table 4. MIC of the AgNPs on test bacteria.

Treatments	MIC (µg/mL)					
	S. aureus	*MRSA*	*S. epidermidis*	*S. pyogenes*	*K. pneumoniae*	*E. coli*
GAE	>100	>100	>100	>100	>100	>100
C-AgNPs	>100	>100	>100	>100	>100	>100
GAC-AgNPs	>100	>100	>100	>100	>100	>100
GA-AgNPs_0.4g	6.25	6.25	6.25	6.25	6.25	25
GA-AgNPs_0.5g	6.25	6.25	6.25	6.25	6.25	6.25

Alamar Blue assay demonstrated reduction in bacterial growth with all treatments (Figure 6), including those that did not show ZOIs or MICs (i.e., GAE, C-AgNPs and GAC-AgNPs). The GAE showed stronger activity against Gram-positive bacteria, *S. pyogenes*, *MRSA* and *S. aureus* (Figure 6A). In contrast, *S. epidermidis* and the Gram-negative bacteria displayed some resistance towards these treatments. The GA-AgNPs were consistent in their activity, with significant effects being observed against all the strains above 6.25 µg/mL (Figure 6D,E).

The effects of GAE, C-AgNPs and GAC-AgNPs were not bactericidal, and their MBC values were undetermined. The GA-AgNPs had bactericidal effects on >60% of the selected strains, with MBCs ranging between 12.5 and 100 µg/mL (Table 5). The GA-AgNPs were active against the Gram-positive and Gram-negative bacteria and demonstrated similar trends in both antibiotic susceptible and resistant strains. This is a desirable property and implies that these NPs can be used as broad-spectrum anti-bacterial agents.

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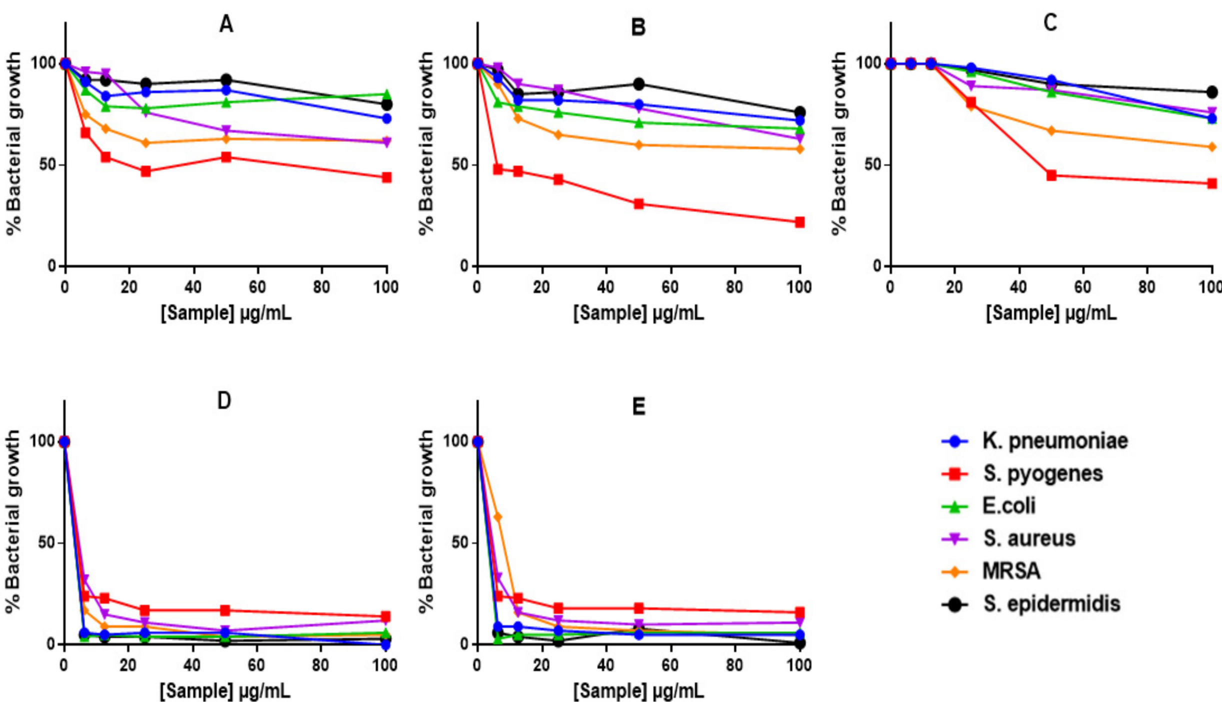

Figure 6. The anti-bacterial effects of GAE and AgNPs using Alamar Blue assay. Bacteria were treated with GAE (**A**), GAC-AgNPs (**B**), C-AgNPs (**C**), GA-AgNPs_0.4g (**D**), and GA-AgNPs_0.5g (**E**).

Table 5. MBC of the AgNPs on test bacteria.

Treatments	MBC (µg/mL)					
	S. aureus	*MRSA*	*S. epidermidis*	*S. pyogenes*	*K. pneumoniae*	*E. coli*
GAE	>100	>100	>100	>100	>100	>100
C-AgNPs	>100	>100	>100	>100	>100	>100
GAC-AgNPs	>100	>100	>100	>100	>100	>100
GA-AgNPs_0.4g	>100	12.5	100	>100	25	12.5
GA-AgNPs_0.5g	100	12.5	25	>100	12.5	12.5

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2.5. In Vitro Cytotoxicity of GA-AgNPs

AgNPs have demonstrated unique properties compared to their bulk counterparts, and these have raised many concerns for biomedical application due to their ability to cross all cellular barriers and interact with important cellular organelles, such as the mitochondria and nucleus [7]. When inside cells, AgNPs can react with biomolecules, such as nucleic acids, proteins, enzymes, etc., resulting in dissolution and release of Ag^+. The Ag^+ are presumed to be responsible for the toxicity of the AgNPs [3].

The cytotoxicity of the AgNPs was investigated *in vitro* in two colon cancer (Caco-2 and HT-29) and non-cancerous (KMST-6) cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay quantifies live cells by evaluating their mitochondrial metabolic activity, where live cells are able to reduce the MTT salt into the water-insoluble purple formazan. The color intensity of the dimethyl sulfoxide (DMSO)-dissolved formazan, which is measured by a spectrophotometer, is directly proportional to the amount of live cells [71]. As shown in Figure 7, GAE exhibited insignificant effects on the three cell lines. Of the four AgNPs, GAC-AgNPs were least toxic and showed selective effects to the non-cancer cells. Significant effects of GAC-AgNPs were observed on cancer cells at ≥50 µg/mL. The C-AgNPs were toxic to all cells at ≥50 µg/mL. GA-AgNPs were non-selective and were toxic against both cancer and non-cancer cells, with <15% viable cells at all concentrations.

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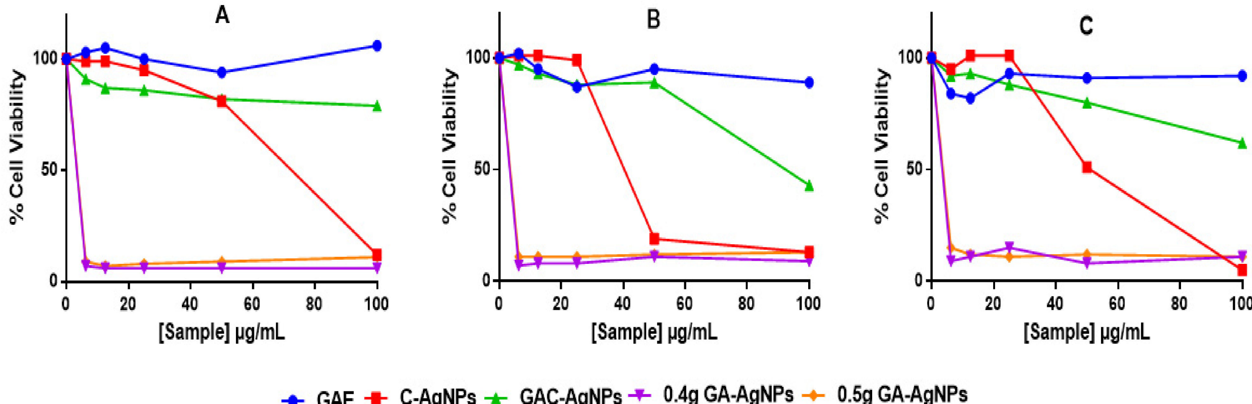

Figure 7. Cytotoxicity of the GAE, C-AgNPs, GAC-AgNPs and GA-AgNPs on KMST-6 (**A**), Caco-2 (**B**) and HT-29 (**C**) cells.

Therapeutic agents are deemed biocompatible when they have selective toxicity towards diseased cells or are at least cytotoxic at concentrations that are not toxic to normal cells. However, this was not the case with the GA-AgNPs, as these NPs were extremely toxic and nonspecific. The GA-AgNPs had an IC_{50} (Table 6) that was >5-fold lower than their MIC and MBC. Their toxicity was even higher to the non-cancer cells than the cancer cells, with the IC_{50} values of 0.67 µg/mL on the KMST-6 cells and 0.82–1.16 µg/mL on the colon cancer cells.

Table 6. The IC_{50} values of treatments against different cell lines.

Cell-Lines	IC_{50} (µg/mL)				
	GAE	C-AgNPs	GAC-AgNPs	GA-AgNPs_0.4g	GA-AgNPs_0.5g
KMST-6	>100	87.40	>100	0.67	0.90
Caco-2	>100	41.67	92.00	0.82	1.26
HT-29	>100	50.54	>100	1.16	1.55

The anti-bacterial effects of AgNPs have led to their use in several consumer and medical products. With an increased exposure rate to consumers who use, handle or manufacture these products, AgNPs can easily accumulate in human organs via inhalation, transdermal absorption, and ingestion [72]. Although it is known that over exposure to silver salts cases argyria [73], the chronic effects of AgNPs are still elusive and still under investigation. Based on their physicochemical properties, the biological effects of AgNPs can vary. Many studies have reported the biocompatibility, as well as toxicity, of AgNPs in *in vitro* and *in vivo* models [15,16,74]. The toxicity of AgNPs, which is often attributed to the leaching of Ag^+ [75], has been demonstrated to be size and cell-specific [76]. Green synthesized *Annona muricata*-AgNPs were only toxic to acute monocytic leukemia (THP-1) and breast cancer (AMJ-13) cells, while sparing the normal breast epithelial (HBL) cells [74]. Poly(*N*-vinyl pyrrolidone)-coated AgNPs were not toxic to T cells at concentrations up to 50 ppm, but induced cell death of human mesenchymal stem cells (hMSCs) and monocytes at 30 and 50 ppm, respectively [3]. The anti-cancer properties of AgNPs were shown in several cancer cell lines; however, their toxicity towards both healthy and diseased cells is a huge concern for human health, as these NPs accumulate in biologically important organs, such as the liver, spleen, lung, kidney, and brain. Moreover, smaller size AgNPs are more toxic than larger sizes and surface coatings can be used to defer or control their activity [77].

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3. Materials and Methods

3.1. Preparation of the GA Extracts

The GA extract (GAE) was prepared by dissolving a required amount of GA obtained from *Acacia senegal* (North Kordofan, Sudan) in hot water and filtering through 0.45 µm filters. The GAE was prepared fresh before use.

Phytochemical Analysis and Antioxidant Capacity

The amount of flavanols, flavonols, total polyphenolic content (TPC) and antioxidant capacity, was assessed using the ferric reducing antioxidant power (FRAP) assay kit (Sigma, St. Louis, MO, USA). An oxygen radical absorbance capacity (ORAC, Sigma) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma) assay of 4 mg/mL GAE was quantified following standard biochemical methods as previously described [78].

3.2. Synthesis of AgNPs

The AgNPs were synthesized through: (a) chemical synthesis (C-AgNPs), (b) green synthesis (GA-AgNPs), and (c) a combined approach (GAC-AgNPs). All solutions were prepared in double-distilled water.

3.2.1. Chemical Synthesis

C-AgNPs were prepared following a previously described method with few modification [79]. Briefly, 10 mL of 2 mM $NaBH_4$ (Sigma) was added to 30 mL of double-distilled water and heated to 70 °C on a heating mantle while stirring. The solution was stirred vigorously at 250 rpm. Subsequently, 20 mL of 1 mM ice-cold silver nitrate ($AgNO_3$, Sigma) was added dropwise into $NaBH_4$ solution. The solution was removed from the heating mantle after a color change to yellow/brown and cooled to room temperature (R_T, ~25 °C).

3.2.2. Green Synthesis

GA-AgNP synthesis was adapted from a method by Venkatesham et al. [39]. A fixed concentration of GAE (4 mg/mL) was used to prepare GA-AgNPs by varying concentrations (0.1–0.5 g) of $AgNO_3$ in a final volume of 40 mL double-distilled water. The method was repeated, keeping the $AgNO_3$ (0.4 g) constant and varying the concentration of GAE (2–6 mg/mL). The samples were autoclaved at 121 °C and 15 psi for 20 min and removed after 60 min when the pressure had reduced to 0 psi.

3.2.3. Combined Approach

GAC-AgNPs were synthesized following the green synthesis method (Section 3.2.2) in a reaction mixture comprised of 20 mL of 1 mM silver $AgNO_3$, 4 mg/mL GAE and 10 mL of 2 mM $NaBH_4$ in a final volume of 40 mL. The synthesis was carried in the autoclave, as described in Section 3.2.2.

All the AgNPs (C-AgNPs, GA-AgNPs, and GAC-AgNPs) were washed twice and harvested by centrifugation at 9000 rpm for 30 min. The pellets were resuspended in double-distilled water and stored in amber bottles at R_T in the dark.

3.3. Characterization of the AgNPs

3.3.1. UV-Visible Spectrophotometer

The formation of AgNPs was monitored by measuring the UV-Vis spectrum of the reaction medium in the wavelength range from 300 to 650 nm using a POLARstar Omega plate reader (BMG LABTECH, Offenburg, Germany).

3.3.2. Dynamic Light Scattering (DLS)

The hydrodynamic size, surface charge, and Pdi of the AgNPs were analyzed by a Malvern NanoZS90 Zetasizer (Malvern Panalytical Ltd., Enigma Business Park, UK). The synthesized AgNPs were diluted 5-fold with double-distilled water; 1 mL aliquots were sampled in DLS cuvettes or DS1070 zeta cells (Malvern Panalytical Ltd.) and examined for

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size distribution and zeta potential, respectively. The particle diameters were assessed at a scattering angle of 90 °C at R_T. The data were represented as mean particle diameter of three measurements.

3.3.3. FT-IR

The infrared spectra of absorption or emission of the AgNPs and GAE in solution were identified using a Perkin Elmer Spectrum Two FT-IR spectrophotometer (Waltham, MA, USA) in the wavelength range 4000–500 cm^{-1}. The baseline corrections were performed for all spectra.

3.3.4. HRTEM

HRTEM analysis was performed by the addition of a drop of each AgNP solution on carbon-coated copper grids, then left to dry under ambient conditions. The shape and size of AgNPs were analyzed using TecnaiF20 HRTEM (FEI Company, Hillsboro, OR, USA) with an accelerating voltage of 300 kV at the Electron Microscope Unit (University of Cape Town, South Africa). In addition, the core size distribution of the AgNPs was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3.4. Assessment of the Stability of AgNPs

The stability of AgNPs over time was evaluated following a previous method [80], by measuring the UV–Vis profile hourly for 0–6 hr in water, Dulbecco's phosphate-buffered saline (DPBS; Lonza, Walkersville, MD, USA), and MHB (Sigma). The AgNPs were washed as before, and the pellets were resuspended in the test solutions and then incubated at 37 °C. Their UV–Vis profile (300–650 nm) was measured using a POLARstar Omega plate reader.

3.5. Anti-Bacterial Activity of the AgNPs

The anti-bacterial activity of the AgNPs was evaluated using Gram-negative and Gram-positive bacterial strains; i.e., *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Methicillin-resistant Staphylococcus aureus* (*MRSA*), *Staphylococcus epidermidis* (*S. epidermidis*), and *Streptococcus pyogenes* (*S. pyogenes*). All the bacterial strains were purchased from American Type Culture Collection (ATCC, Manassas, USA). The anti-bacterial activity was determined by agar well diffusion and microdilution assays according to the standards set by the Clinical and Laboratory Standard Institute with few modifications [81].

Bacterial colonies were cultured in MHB while shaking at 120 rpm overnight at 37 °C, then diluted at 1:100 in fresh MHB and cultured until reaching a 0.5 McFarland turbidity standard prior to experiments. The bacteria were used for anti-bacterial tests at 1.5×10^6 CFU/mL by diluting the 0.5 McFarland turbid suspensions to 1:150.

3.5.1. Agar Diffusion Assay

The bacterial cultures were streaked on Mueller–Hinton Agar (MHA; Sigma) plates at 1.5×10^6 CFU/mL using sterile cotton swabs. Wells of 6 mm diameter were made on the MHA plates, to which 20 µL of the AgNPs were added. The MHA plates were then incubated overnight at 37 °C. Ciprofloxacin (10 µg/mL; Sigma) was used as a positive control. The anti-bacterial activity of the AgNPs was determined by the presence of clear zones surrounding the wells. The diameter of the clear zones was measured using calipers after 24 hr.

3.5.2. Microdilution Assay

Microdilution assay was used to determine the minimum inhibitory concentration (MIC) [47,49] and minimum bactericidal concentration (MBC) [82] of the AgNPs following previously described protocols.

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Minimum Inhibitory Concentration (MIC)

The MIC of the AgNPs required to inhibit the visual growth of the bacteria was determined according to the microdilution method [47,49]. The bacteria (1.5×10^6 CFU/mL) were exposed to different concentrations (0–100 µg/mL) of the AgNPs and incubated at 37 °C for 24 hr. The MIC values were visually observed, followed by measuring the optical density (OD) of the bacterial culture at 600 nm using a POLARstar plate reader. Bacterial growth was further evaluated by Alamar Blue colorimetric assay (Invitrogen, Eugene, Oregon, USA), where 10 µL of the dye was added to each well and incubated for 3 hr. The blue color was converted to a pink-purplish color by live bacteria that was quantifiable by measuring absorbance at 570 nm and a reference wavelength at 700 nm [83].

Minimum Bactericidal Concentration (MBC)

The MBC of the AgNPs was determined in the bacteria used for MIC, where a loopful of broth from the wells was spotted onto fresh MHA and incubated at 37 °C for 24 hr. The lowest concentration that exhibited no growth on the MHA was considered as the MBC.

3.6. Cytotoxicity Assay of the AgNPs

The effect of the AgNPs was evaluated by MTT assay, as previous described, on the human cell lines, KMST-6 normal skin fibroblasts, HT-29 and CaCo-2 colon carcinoma cells [80]. The cells were purchased from ATCC, and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (Gibco, Waltham, Massachusetts, USA) and 1% penicillin-streptomycin cocktail (Lonza) and incubated at 37 °C. The cells were then seeded in 96 well plates at 1×10^5 cell/mL density, 100 µL in each well and incubated for 24 hr. The cells were treated with 0–500 µg/mL of the AgNPs and extracted in triplicates. The cell viability was assessed by adding 10 µL of 5 mg/mL of MTT (Sigma) solution to each well and incubated for 3 hr. Later, the MTT solution was discarded and 100 µL of DMSO was added to each well. The absorbance of the formazan product was measured at 570 nm with a reference at 700 nm using a POLARstar Omega plate reader. The concentration that inhibited 50% cell growth (IC_{50}) was further analyzed by Graphpad Prism 6.0.

3.7. Statistical Analysis

All the experiments were carried out in triplicate and the results were analysed using Graphpad Prism 6.0. The data are presented as means ± SD according to one-way ANOVA test followed by a post hoc, multiple comparisons (Tukey's) test. A p-value of <0.05 was considered statistically significant.

4. Conclusions

The growing interest in medical application of AgNPs has warranted greener methods for their synthesis to prevent toxicity and improve biocompatibility. Synthesis of AgNPs using plant extracts presents not only a greener method but a sustainable, reproducible and upscalable approach. However, for biomedical applications, the safety of plant-synthesized AgNPs must be authenticated. The current study demonstrated that GAE alone, and in the presence of a chemical reducing agent, produced AgNPs with distinct bioactivities. The GA-AgNPs demonstrated broad spectrum anti-bacterial effects on both Gram-positive and Gram-negative bacteria, and non-selective cytotoxicity on normal and colon cancer cells in the same concentration range. Interestingly, these effects were reduced in the GAC-AgNPs, suggesting that surface coating can be used to channel the effects of AgNPs. The selective and reduced cytotoxicity demonstrated by GAC-AgNPs towards colon cancer cells demonstrated that surface composition can be used to control the biodistribution, uptake and efficacy of AgNPs. AgNPs represent the next generation of antimicrobial agents, and have potential to help solve the antimicrobial resistance problem. Their biocompatibility can be enhanced by modifying the surface of AgNPs with targeting molecules or biocompatible molecules, such as PEG, for medical application.

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2.3.2 Antimicrobial effects of gum Arabic-silver nanoparticles against oral pathogens

2.3.2.a. Executive summary

Following the previous chapter's demonstration of antibacterial action against Gram positive and negative microorganisms, the antimicrobial effect of GA-AgNPs against four distinct oral pathogens in humans was examined. In this publication, objective (c) and (d) were covered and the answers for the research questions (c) and (d) were stated. Two fractions of GA-AgNPs (0.1 and 0.4g) were assayed for antimicrobial activity against selected oral microbes. The latter fraction showed higher antimicrobial activity and exhibited attachment to the tooth enamel suggesting a potential for dental application. This paper has been published in Bioinorganic Chemistry and Applications. Manuscript ID: 9602325.

2.3.2.b. Publication

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Research Article

Antimicrobial Effects of Gum Arabic-Silver Nanoparticles against Oral Pathogens

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Dental caries is considered one of the most prevalent oral diseases worldwide, with a high rate of morbidity among populations. It is a chronic infectious disease with a multifactorial etiology that leads to the destruction of the dental tissues. Due to their antimicrobial, anti-inflammatory, antifungal, and antioxidant properties; silver nanoparticles (AgNPs) are incorporated in dental products to help prevent infectious oral diseases. In this study, the antimicrobial effects of AgNPs synthesized using Gum Arabic extracts (GAE) were examined. The GA-AgNPs were synthesized and characterized using ultraviolet-visible (UV-Vis) spectrophotometer, dynamic light scattering (DLS), transmission electron microscopy (TEM), and Fourier transform infrared (FTIR) spectroscopy. The antimicrobial activity of the GA-AgNPs was evaluated on *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus mutans* (*S. mutans*), *Lactobacillus acidophilus* (*L. acidophilus*), and *Candida albicans* (*C. albicans*) using agar disc diffusion and microdilution assays. The antibiofilm of GA-AgNPs was evaluated on the surface of human tooth enamel that had been exposed to *S. mutans* with and without the GA-AgNPs using scanning electron microscopy (SEM). GA-AgNPs were spherical in shape with a particle size distribution between 4 and 26 nm. The GA-AgNPs exhibited antimicrobial activity against all the tested oral microbes, with GA-AgNPs_0.4g having higher antimicrobial activity. The GA-AgNPs_0.4g inhibited *S. mutans* adhesion and biofilm formation on the surface of the tooth enamel. Therefore, this study supports the prospective implementation of the plant extract-mediated AgNPs in dental healthcare.

1. Introduction

Phytotherapy, i.e. the use of herbs or plant extracts to manage health, has played an important role in medicine for centuries. In fact, a significant number of drugs in clinical use are of plant origin or are inspired by plant-based products [1, 2]. Examples include cancer chemotherapeutic drugs such as paclitaxel, a dipertene isolated from the *Taxus brevifolia* Nutt bark extracts [2]; and camptothecin from the *Camptotheca acuminate* stem [3]. Similarly, products for oral health and hygiene were developed from the use of plant products [4–6], where chewing sticks resulted in toothbrushes [5], plant-based decoctions into mouthwash, varnish, and remineralization agents. These traditional practices are still used in lower income areas to date. In addition, some of the herbal remedies such as propolis, and clove are incorporated into commercial oral health-promoting products such as toothpaste, varnish, and irrigants [4, 7]. Over the years, several plant species with oral health promoting properties have been identified. These include plant products made from medicinal plants (aloe vera), vegetables (garlic), herbs/spices (turmeric), fruits (pomegranate), and so on. [8]. Their application in dentistry is encouraged for their varied biological benefits, particularly, their anti-inflammatory, analgesic, antimicrobial, antiplaque, antigingivitis, and

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antioxidant properties, all of which are attributed to the various phytochemicals present in those plant extracts [4, 5, 7–10].

There has been renewed interest in plant extracts as a source of bioactive agents [11] for the treatment of diseases, including oral infections. Plant products have demonstrated low side effects compared to commercial chemical agents [12]. In search of novel antimicrobial agents against certain pathogenic species causing plaque formation and tooth decay, GA has shown promising results [13]. GA is an exudate acquired from the stem and branches of Acacia tree species [14]. It contains minerals such as calcium, magnesium, and potassium [15]. Therefore, GA is considered a prebiotic agent, promoting the growth or activity of microbes that support the health of the host [16]. GA has been proven to alleviate digestive discomfort, reduce inflammation in the intestinal mucosa [17], and has been implicated in the treatment of both chronic renal failure and diabetes [18, 19]. As an oral hygiene agent, GA may enhance remineralization of caries due to its high concentration of calcium [13] and inhibits early deposition of dental plaque [20]. Moreover, GA inhibits growth of certain pathogenic periodontal species such as *Porphyromonas gingivalis* and *Prevotella intermedia* [21], as well as cariogenic pathogens such as *Streptococcus mutans* (*S. mutans*) [13]. Adding GA (specifically, Acacia Arabica) to toothpaste has been shown to reduce plaque build-up and gingival inflammation [9], this suggested that GA could be used for oral health and maintenance due to its antimicrobial [10, 22, 23], anti-inflammatory [10, 22], biofungicidal, and anticoagulant properties [20].

Oral infections (i.e. dental caries, periodontitis, pulpal, apical, peri-implant diseases, and candidiasis) are a public health problem, with dental caries and periodontitis among the most prevalent diseases globally [6]. The current chemical plaque control strategies have certain limitations, as they may lead to mucosal desquamation, and tooth staining, thus impacting on compliance and efficiency, and causing safety concerns [24]. Hence, natural products are sought as effective alternative antimicrobial agents for plaque control with minimal side effects. The use or incorporation of natural products in these agents is attractive, and could lead to innovative treatment agents in the fight against dental plaque and its consequences [7]. Even more interesting is phytonanotherapy, or the use of nanotechnology to enhance the efficacy and bioavailability of the phytochemicals, which are used as reducing and stabilizing agents in the synthesis of nanoparticles [25]. Nanoparticles (NPs) at a size range between 1 and 100 nm, possess unique physical and chemical properties that found applications in many fields. In medicine, they are used as drug delivery, diagnostic and therapeutic agents [26]. Plant extract-synthesized AgNPs have the potential to prevent and treat dental infections [6]. AgNPs are receiving attention in dentistry due to their antimicrobial activity [6, 27]. Plants are readily available, renewable, safer than the chemical reducing agents in NP synthesis [6, 28, 29], and are potentially less toxic and more environmentally friendly [30, 31]. The broad-spectrum antimicrobial activity of GA-AgNPs [23] encouraged its application in dental therapy. GA-AgNPs have been shown to have activity against pathogenic oral microbes, such as *Escherichia coli* (*E. coli*) and *Micrococcus luteus* (*M. luteus*) [33], *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae* (K. *pneumoniae*) [34, 35], and *S. mutans*[36]. While *S. mutans* is fundamental in the induction of dental caries [32], oral infections can be caused by multiple pathogens such as nonstreptococcal bacteria (e.g. *Bifidobacterium* spp., *Scardovia* spp., and *Actinomyces* spp.) and fungi (e.g. *C. albicans*) [37]. The aim of the current study was to investigate the antimicrobial effects of GA-AgNPs against 4 different human oral pathogens.

2. Materials and Methods

2.1. Synthesis and Characterization of Gum Arabic (GA)-AgNPs

2.1.1. Synthesis of GA-AgNPs. GA-AgNPs were synthesized as previously reported [23]. Briefly, 4 mg/mL GAE and two concentrations of $AgNO_3$ (0.1 g and 0.4 g) were used to produce GA-AgNPs_0.1g and GA-AgNPs_0.4g, respectively. All reactions were performed in an autoclave at 15 psi at 120°C for 20 min [23].

The GA-AgNPs were centrifuged at 9000 rpm for 45 min and resuspended in sterile deionized water. The AgNPs were stored at room temperature until further characterization. The dry mass was determined by freeze-drying 10 mL of GA-AgNPs on the Virtis Freezer dryer (SP Scientific, Gardiner, NY, USA) and used to calculate their concentrations.

2.1.2. Characterization of GA-AgNPs. The GA-AgNPs were characterized by UV-Vis, DLS, FTIR, and high resolution TEM (HRTEM), as previously described [23, 29]. The GA-AgNPs were diluted 1 : 10 in distilled water and used for analyses.

(1) UV-Vis Analysis. The GA-AgNPs were added into a 96 well plate (100 μL), their UV-Vis spectra were measured at 300–650 nm using a POLARstar Omega microplate reader (BMG Labotech, Offenburg, Germany).

(2) DLS Analysis. The hydrodynamic diameter, polydispersity index (PDI), and zeta potential of the GA-AgNPs were determined by DLS using a Zetasizer NanoZS90 (Malvern Panalytical Ltd., Malven, UK). The size distribution and PDI of each sample were measured on a cuvette (Malvern Panalytical Ltd), and the zeta potential was measured using a DTS1070 folded capillary cuvette (Malvern Panalytical Ltd). The data were measured in triplicate and represented as the mean particle diameter of the three measurements. The data was analyzed by Zetasizer software version 7.11.

(3) FTIR Analysis. FTIR analysis of GA-AgNPs and GA powder were analyzed as previously described [29, 38] using Perkin Elmer Spectrum Two Fourier transform infrared (FTIR) spectrophotometer (Waltham, MA, USA) at the School of Pharmacy (UWC).

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(4) TEM Analysis. The morphology and core size distribution of GA-AgNPs were examined using TecnaiF20 HR-TEM (FEI Company, Hillsboro, OR, USA) at the Electron Microscope Unit (University of Cape Town, South Africa). One drop of the GA-AgNPs solution was placed onto a carbon coated copper grid and left to dry for a few minutes under a Xenon lamp, as previously described. The core size of the GA-AgNPs was measured from the TEM micrographs by using ImageJ software (http://www.imagej.nih.gov/ij) [39].

2.2. Stability Evaluation of the GA-AgNPs. The stability of GA-AgNPs was assessed in water, phosphate buffered saline (PBS), and Müeller–Hinton broth (MHB; Sigma–Aldrich, St Louis, USA) following a previous protocol [40]. Briefly, 200 μL of the GA-AgNPs were mixed with 800 μL of each of the solutions in separate tubes and incubated at 37°C. The stability of the GA-AgNPs was monitored by the UV-Vis spectra of the samples at 1 hour intervals for the first 6 hours, and again at 24, 48, and 72 hours.

2.3. Antimicrobial Activity of the GA-AgNPs. The antimicrobial activity of the GA-AgNPs against three bacterial strains: *S. sanguinis* (NCTC 7865), *S. mutans* (NCTC 10449), *L. acidophilus* (ATCC 314), and one fungal strain *C. albicans* (ATCC 10231) was determined using the agar disc diffusion and the microdilution assays. The *S. sanguinis* and *S. mutans* were purchased from Davies Diagnostics (Randburg, Johannesburg, South Africa); and *L. acidophilus* and *C. albicans* were purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, USA).

All the microbes were cultured on Brain Heart Infusion (BHI) Broth (Sigma–Aldrich), and single colonies were subcultured in BHI agar (Sigma–Aldrich) for all the bacterial strains and Sabouraud dextrose agar for *C. albicans* at 37°C for 24 hours. Following the overnight incubation, the microbes were adjusted to the 0.5 McFarland standard (Mcf) using DensiCHEK Plus standards (BioMérieux, Inc., Durham, NC, USA).

2.3.1. Agar Disc Diffusion Method. Sterile 6 mm filter paper discs (Lasec, Cape Town, South Africa) were aseptically infused with 50 μL of each of the treatments: GA-AgNPs at 100 μg/mL, GAE at 4 mg/mL, 0.2% chlorhexidine (CHX), and 5000 units of nystatin. The discs were placed in sterile Petri-dishes and left to air-dry overnight in a laminar flow class 2 cabinets. CHX and nystatin were used as positive controls for bacteria and fungi, respectively; and a sterile disc infused with distilled water was used as a negative control. The microbes at 0.5 Mcf (100 μL of each standardized inoculum) were spread evenly onto the Müeller–Hinton agar (MHA; Sigma–Aldrich), and treatment and control discs were placed on the plate. The plates were incubated for 24 hours at 37°C. Antimicrobial activity was determined by measuring the diameters of the zones of inhibition (ZOI) formed around the paper disc in millimeters using a vernier caliper [23, 41]. The assay was carried out in triplicate for all organisms tested with all treatments on the same plate and repeated three times.

2.3.2. Biofilm Inhibition by Microdilution Method. The microdilution assay was used to determine the minimum inhibitory concentration (MIC), following the M07 guidelines [42] set by the Clinical Laboratory Standards Institute (CLSI). In a 96 well plate, 100 μL of MHB was pipetted into all wells, and 100 μL of the 0.5 Mcf was added in all wells with the exception of the blank well. Following a 24-hour incubation period, the plates were rinsed with PBS three times, and 100 μL of MHB was pipetted into all wells except the blank well. The biofilms were treated with increasing concentrations of GA-AgNPs (1.5625–100 μg/mL), 0.2% CHX, and 5000 units of nystatin. All experiments were conducted in triplicate, and the plates were incubated at 37°C for 24 hours. After treatments, the XTT reduction method was used according to the manufacturer's instructions to measure the biofilm activity, as previously described [43]. The plates were read at 450 nm and 620 nm (reference wavelength) using the SpectroStar Nano microplate reader (BMG Labotech). The $MIC_{50/90}$ were further determined by subculturing 5 μL of each sample on the agar plates and incubated at 37°C for 24 hours following a previous protocol [44]. The total number of colonies appearing on the agar plates was used to determine the $MIC_{50/90}$ for the various treatments.

2.4. Adhesion of S. mutans to Tooth Enamel. Fifty extracted human molar teeth (protocol approved by the Biomedical Research Ethics Committee of the University of the Western Cape, reference number: BM20/1/7) were scaled, cleaned, sonicated, and stored in saline solution at 4°C. The crowns of each extracted tooth were cut into enamel blocks of 5 mm × 5 mm (25 mm^2) using a diamond disc with irrigation at 10,000 rpm. The enamel blocks received two coats of nail varnish, then were placed inside a 2 mL Eppendorf tube and sterilized in an autoclave for 15 min. The enamel blocks were randomly divided into 5 groups (10 specimens in each group) and incubated with: Group 1: *S. mutans* (100 μL Mcf); Group 2: *S. mutans* (100 μL Mcf) + GA-AgNPs_0.4g (825 μL); Group 3: GA-AgNPs_0.4g (825 μL); Group 4: *S. mutans* (100 μL Mcf) + 0.12% CHX (125 μL); and Group 5: enamel blocks. Then, 1 mL of MHB was added into each tube, and incubated for 24 hours at 37°C. After incubation, the enamel blocks were transferred into tubes containing 1 mL of saline solution.

To determine the surface adhesion of *S. mutans* after treatments, the enamel blocks were prepared for scanning electron microscopy (SEM) following a previous protocol [45]. The enamel blocks were fixed in a 0.1% glutaraldehyde solution for 5 min, washed three times with the saline solution and immersed in ethanol (50, 60, 70, 90, 95, and 100%) for 20 min each at room temperature. Aluminium stubs were used to mount the samples. Carbon tabs were placed on the stubs to keep the sample in place. Finally, the enamel blocks were gold coated with a sputtering coater technique (Q 150T ES) for 60 seconds. SEM images were

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obtained using a field emission SEM (SmartSEM, Zeiss, Germany) operating at 5 kV and 10 μA at the Electron Microscope Unit (University of Cape Town).

2.5. Statistical Analysis. The data are presented as the mean ± standard error of the mean of the three independent experiments, which were carried out in triplicate. Statistical analysis was performed by one-way and two-way ANOVA using GraphPad Prism version 6, a value of $p < 0.05$ was considered statistically significant. Finally, post hoc pairwise testing was carried out to elucidate the statistical differences between two sets of data.

3. Results and Discussion

Many regimens for the prevention of dental caries have demonstrated short-lived successes due to their drawbacks, such as mucosal damage and tooth staining [46]. AgNPs have demonstrated antimicrobial activity against several pathogens, including oral microflora, and are now being investigated in the prevention of oral diseases, including dental caries [47]. Their broad spectrum antimicrobial properties, including the fight against drug resistant microbes, has aroused interest in their use in the treatment and prevention of dental caries [46]. In this study, AgNPs synthesized using readily available, affordable, and environmentally friendly GAE were investigated for their antimicrobial activities against oral pathogens.

3.1. GA-AgNPs Synthesis and Characterization. GA-AgNPs were synthesized following our optimized protocol as previously reported by Fadaka et al. [23]. The color of the solution was clear upon the addition of GAE into the aqueous solution of $AgNO_3$, and changed to dark brown after autoclaving, as shown in Figure 1. The color change was an indication of the formation of GA-AgNPs. This has been reported for GA-AgNPs synthesized using *Acacia Senegal* (L) wild [36], *Gum Acacia* [33], and *Acacia Senegal* [23], the latter being the same species that was used in this study. This confirmed that phytochemicals present in the GAE were able to reduce and stabilize the GA-AgNPs, either individually or collectively [48, 49]. Similar findings were reported for other plant-synthesized AgNPs using plant extracts from *Cotyledon orbiculata* [50], *Salvia Africana Lutea*, *Sutherlandia frutescens* [38], *Justicia glauca* [31], and *Terminalia mantaly* [51], as well as pear fruit extracts [29].

3.1.1. UV-Vis Spectra for GA-AgNPs. UV-Vis spectra confirmed the presence of GA-AgNPs. This method has been one of the most important tools for the characterization of metal nanoparticles (MNPs). It is based on the absorption of light by a sample as a result of the excitation of the surface plasmon vibration in the MNPs [33]. In this study, a UV-Vis spectrum with a surface plasmon resonance (SPR) or maximum absorbance (λ_{max}) around 400 nm (Figure 2) was indicative of GA-AgNPs formation, which is within the characteristic SPR range for AgNPs [23, 31, 51]. The SPR values were 450 and 425 nm for GA-AgNPs-0.1g and GA-AgNPs-0.4g, respectively. The peak intensity of the GA-AgNPs synthesized with 0.4 g $AgNO_3$ was slightly higher than those synthesized with 0.1 g $AgNO_3$, which suggested that more AgNPs were formed at this concentration since absorbance is related to the concentration of NPs [51]. The GA-AgNPs_0.1g spectra were broad compared to those of GA-AgNPs_0.4g, which implied that the GA-AgNPs_0.1g were polydispersed [51].

3.1.2. Size Distribution of GA-AgNPs. The hydrodynamic diameter of the GA-AgNPs was 226.4 nm and 220 nm for GA-AgNPs_0.1g and GA-AgNPs_0.4g, respectively (Table 1). The zeta potential of GA-AgNPs_0.1g was −22.9 mV and −24.6 mV for GA-AgNPs_0.4g. Zeta potential is an important parameter that is used to determine the surface charge and the stability of NPs. The zeta potential within the range of +30 mV to −30 mV is considered to be stable, while those outside this range will coalesce due to interparticle van der Waal's attractions. The negative zeta potential of the GA-AgNPs indicated strong repulsion forces between the AgNPs in suspension and will thus prohibit the agglomeration of the AgNPs in solution [52]. The PDI values were 0.060 and 0.156 for GA-AgNPs_0.1g and GA-AgNPs_0.4g, respectively. This confirmed that the AgNPs were uniform and monodispersed, as the PDI values >0.7 suggest that the NPs have a very broad size distribution, while PDI values ≤0.5 are likely to be monodispersed [51].

The HRTEM micrographs in Figure 3 demonstrated that the majority of GA-AgNPs were spherical in shape, with core sizes between 4 and 26 nm. These sizes were smaller than their hydrodynamic sizes, as the latter accounts for both the core size and the molecules that are adsorbed on the surface of the GA-AgNPs, while the HRTEM only represents the core size [51]. Increasing scientific evidence has demonstrated that AgNPs activity depends strongly on their shape and size [53, 54], with the shape being the most relevant physicochemical parameter influencing their bioactivities, including their antimicrobial properties [53]. This was confirmed by the weakest antibacterial activity demonstrated by the silver nanowires when compared with silver nanocubes and nanospheres [54]. In addition, the size of the AgNPs plays an important role in their antimicrobial activity, with smaller sizes showing higher activity than larger AgNPs [54]. This was in line with the current study, where the GA-AgNPs were spherical and had superior antimicrobial activity.

3.1.3. FTIR Analysis of GA-AgNPs. The FTIR spectra of GAE and the GA-AgNPs were compared in order to identify the types of phytochemicals that were involved in the synthesis of the GA-AgNPs. Figure 4 outlines the similarities between the FTIR spectra of GAE and GA-AgNPs. The GAE showed noticeable peaks at 3514, 2978, 2315, 1628, 1371, and 1065 cm^{-1}; the GA-AgNPs_0.4g spectrum showed peaks at 2929, 1615, 1345, and 1077 cm^{-1}, while the GA-AgNPs_0.1g showed peaks at 2966, 1638, 1358, and 1041 cm^{-1} (Figure 3). The GAE spectral distribution observed at 3514 cm^{-1}

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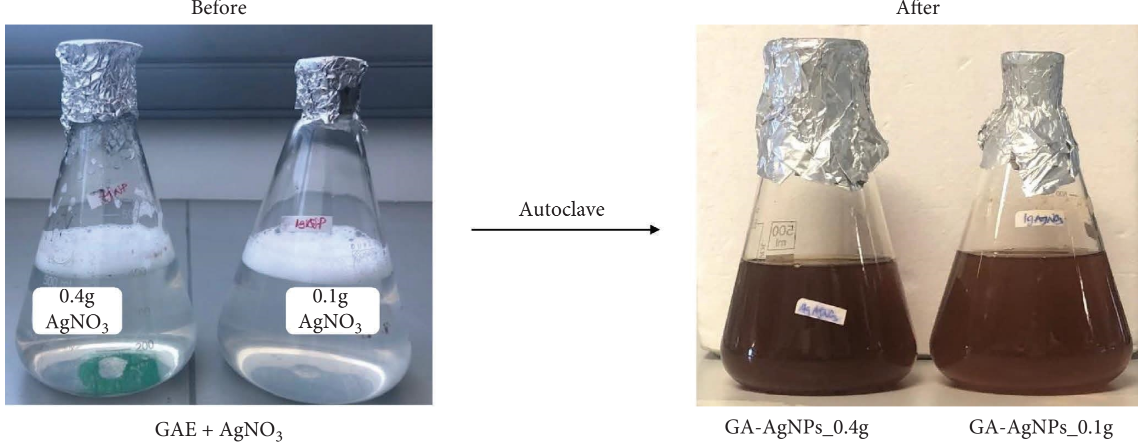

FIGURE 1: Synthesis of GA-AgNPs using an autoclave method. The solutions containing $AgNO_3$ and GAE for the synthesis of GA-AgNPs_0.1g and GA-AgNPs_0.4g, before and after autoclaving for 20 min.

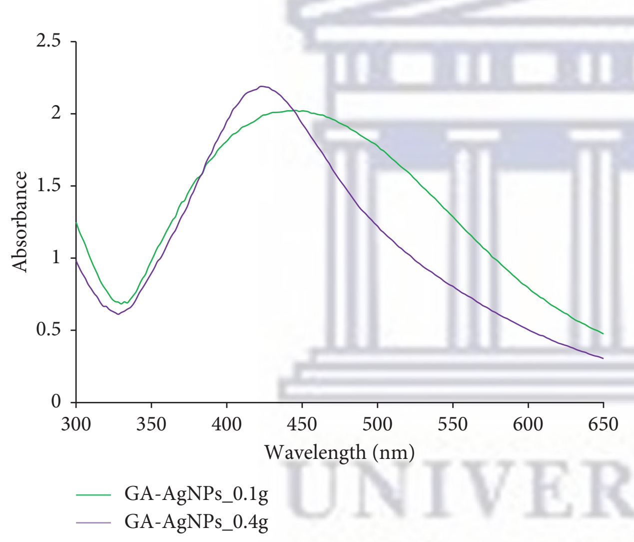

FIGURE 2: UV-vis analysis for GA-AgNPs.

TABLE 1: DLS analysis of GA-AgNPs.

Samples	Hydrodynamic size (nm)	PDI	Zeta potential (mV)
GA-AgNPs_0.1g	226.4	0.060	−22.9
GA-AgNPs_0.4g	220	0.156	−24.6

demonstrated the presence of OH stretch; while the 2978 cm^{-1} for GAE, 2929 cm^{-1} for GA-AgNPs_0.4g and 2966 for GA-AgNPs_0.1g indicated the presence of alkanes with C-H bond stretch. The sharp spectral peak at 1628 cm^{-1} for GAE, 1615 cm^{-1} for GA-Ag NPs_0.4g, and 1638 cm^{-1} for GA-AgNPs_0.1g signified the presence of secondary amine NH bend. The spectral peak at the wavelength of 1371 cm^{-1} for GAE, 1345 cm^{-1} for GA-AgNPs_0.4g and 1358 cm^{-1} for GA-AgNPs_0.1g implied organic nitrates. Moreover, another set of peaks representing S = O stretching for sulfoxide in GAE, GA-AgNPs_0.4g, and GA-AgNPs_0.1g were observed at 1065 cm^{-1}, 1077 cm^{-1}, 1041 cm^{-1}, respectively. The variations or shifts in the peak positions of the GAE and GA-AgNPs were observed due to the GAE contribution toward the reduction and stabilization process. The presence of phenols, alcohols, amides, sulfoxide, flavonoids, and steroids was also revealed in other studies using GAE in the synthesis of GA-AgNPs [33, 36].

3.2. Stability of GA-AgNPs. The stability of GA-AgNPs was tested in water, PBS, and MHB and measured by their UV-Vis spectral profiles. As shown in Figure 5, the GA-AgNPs were stable in water, PBS, and MHB, as indicated by no changes in the UV-Vis spectra for up to 72 hours. The GA-AgNPs_0.1g was only stable in water and MHB, and revealed signs of instability when subjected to PBS. The GA-AgNPs_0.4g was relatively stable in water, PBS, and MHB.

3.3. Antimicrobial Effects of GA-AgNPs on Oral Microbes. In recent years, there has been a growing interest in the use of natural products to fight against drug resistant microbes. This is fueled by the successful use of plant extracts in traditional medicine as a source of antimicrobial agents. GA in particular, has been effective against various periodontal [21] and cariogenic pathogens [13]. In fact, when mixed in water, GA was used as a tooth paste formulation in ancient times, long before commercial toothpastes arrived on the market. Due to its high concentration of calcium and other cations, GA also possesses remineralization effects and prevents caries in enamel lesions [55]. Its use in oral hygiene and health [17] is motivated by its antimicrobial, antioxidant, and anti-inflammatory properties [10, 22], biofilm inhibition and biofungicide activities [20]. These activities are attributed to the presence of flavonoids, chalcones, tannins, phenolic acid, alkaloids, and terpenes in GA [35], and reported to have enhanced activities when used in the bioreduction and synthesis of AgNPs. The NPs penetrate the biofilm structure and release metal ions that destroy the biofilm and inhibit microbial colonization [56].

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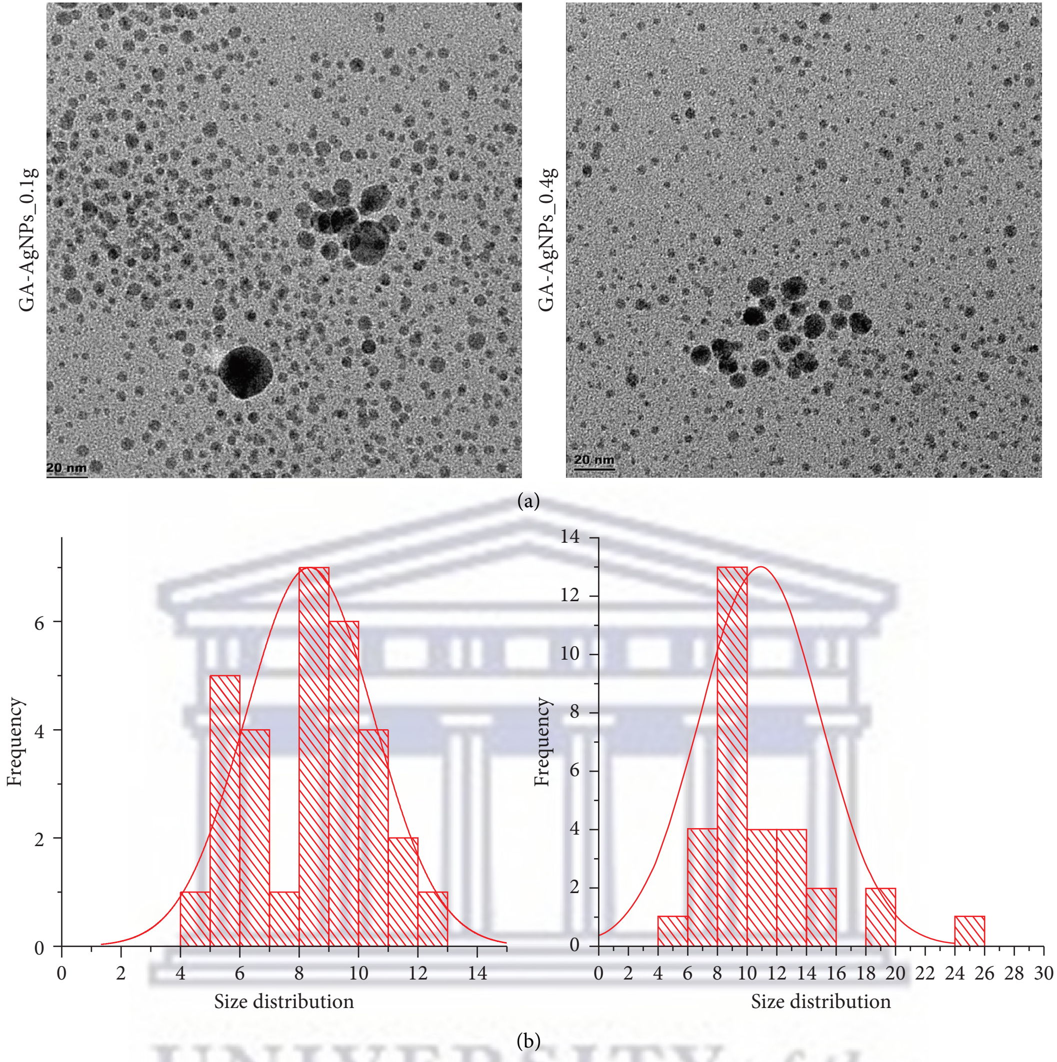

Figure 3: (a) HRTEM micrographs and (b) core size distribution of the GA-AgNPs.

The antibacterial and antifungal activities of GA-AgNPs and GAE were investigated against three dominant oral pathogens i.e., *S. mutans*, *L. acidophilus,* and *C. albicans*; and one oral commensal species (*S. sanguinis*). Table 2 shows that there were statistical differences in the zones of inhibition (ZOIs) of bacteria exposed to GA-AgNPs_0.1g/0.4g and the positive controls (CHX and nystatin) compared to GAE. There were no ZOIs around the microbes treated with 4 mg/ml GAE and the negative control, indicating that the GAE at the tested concentration had no antimicrobial activity against all microbes tested. This finding was similar to the study conducted by Venkatesham et al. where the GAE from *Gum Acacia* had no antimicrobial activity against *E. coli* and *M. luteus* [33]. GAE from *Acacia Senegal* showed time and dose-dependent effects against the *S. aureus* and *E. coli* at 5–40 mg/ml [34]. GAE from *Gum Acacia* and *Acacia Senegal* were also reported to have antibacterial activity against *S. aureus*, *E. coli*, and *K. pneumoniae* at 0.25–2 mg/ml [35] and at 5–40 mg/ml [34], respectively.

The antimicrobial effects of GA-AgNPs_0.1g, GA-AgNPs_0.4g, and CHX were also observed in the nonpathogenic strain (*S. sanguinis*), with stronger effects in the *S. mutans*, *L. acidophilus* and *C. albicans*. Generally, the GA-AgNPs_0.4g yielded larger ZOIs compared to GA-AgNPs_0.1g, suggesting that GA-AgNPs_0.4g was more potent. The antimicrobial activity of GA-AgNPs was previously reported against *S. mutans,* and the ZOIs at 25, 50, 100, and 200 μg/ml were 14.1 ± 0.7 mm, 15.5 ± 0.8 mm, 16.3 ± 1.0 mm and 18.3 ± 0.5 mm, respectively [36]. In the present study, ZOIs for *S. mutans* at 100 μg/ml for GA-AgNPs_0.1g and GA-AgNPs_0.4g were 10.5 ± 0.04 mm and 16.6 ± 0.34 mm, respectively. Studies have reported that the bactericidal properties of AgNPs are size and shape-dependent [57], where the smaller size AgNPs presents a larger surface area which is ideal for interaction with the bacterial cell wall [58].

The MIC_{50} and MIC_{90} for the two GA-AgNPs were determined from the microdilution assay, and by subculturing a sample from each well. MIC_{50} and MIC_{90} were defined as the lowest concentration, which inhibited 50% and 90% of the growth when compared with the untreated microbes, respectively [59]. The MIC_{50} and MIC_{90} for GA-

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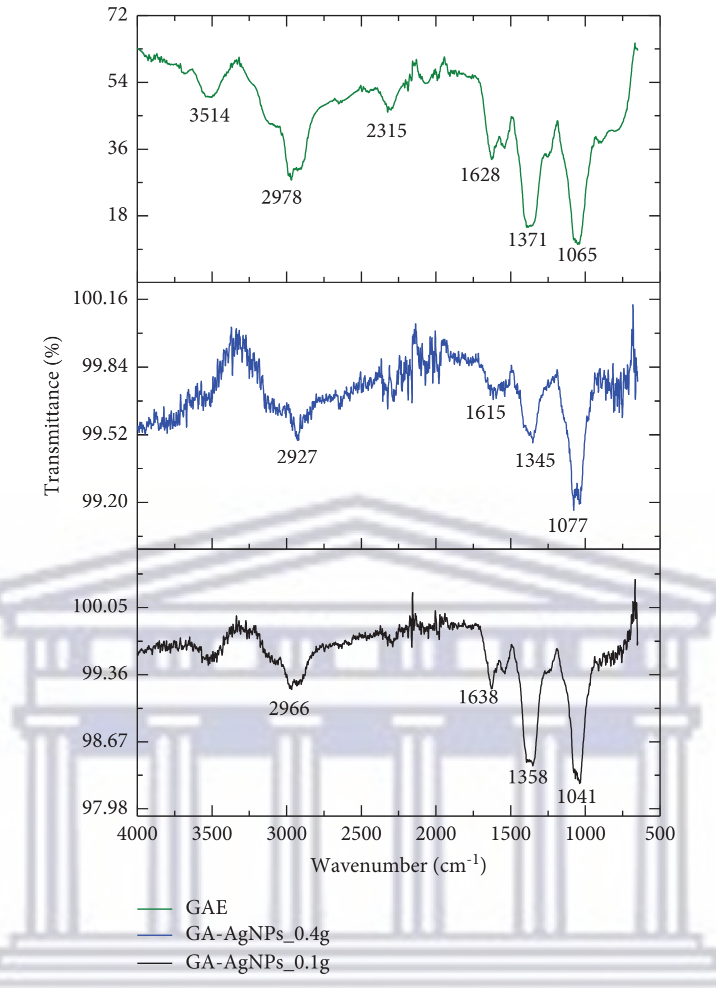

FIGURE 4: FTIR images of GAE, GA-AgNPs_0.4g, and GA-AgNPs_0.1g.

AgNPs_0.4g against all microbes (*S. sanguinis*, *S. mutans*, *L. acidophilus*, and *C. albicans*) were 8-fold lower than that of GA-AgNPs_0.1g; at 3.125 and 12.5 μg/ml, respectively (Table 3). The GA-AgNPs_0.4g had higher ZOIs and lower MICs than GA-AgNPs_0.1g across the tested microbes. This indicated that GA-AgNPs_0.4g were more effective than the GA-AgNPs_0.1g. This effect can be correlated with their small size, as illustrated in Figure 3. The smaller AgNPs had higher antimicrobial activity than the larger particles as corroborated by Lu et al. [54]. In our previous study, we demonstrated the broad spectrum antibacterial activity of GA-AgNPs_0.4g with a MIC of 6.25–25 μg/ml against a number of human pathogens, namely, *S. aureus*, MRSA, *S. epidermidis*, *S. pyogenes*, *K. pneumoniae,* and *E. coli* [23]. Independent studies also reported the potency of GA-AgNPs against an oral pathogen (*S. mutans*) with MIC of 10.0 μg/mL [36], and between 1.625 and 3.25 μg/mL against the fish bacterial pathogens (*Aeromonas hydrophila* and *Pseudomonas aeruginosa*) [60].

3.4. GA-AgNPs Prevents S. mutans Adhesion Biofilm Formation on Tooth Enamel. For dental application, AgNPs must have the ability to attach themselves to the enamel surfaces for extended activity. In this work, we evaluated the adherence capacity of *S. mutans* on the healthy human dental enamel when it was first exposed to the GA-AgNPs_0.4g. The surface of the enamel was colonized by the *S. mutans* biofilm when no treatment was added (Figure 6(a)). The SEM micrographs indicated that the application of GA-AgNPs_0.4g made the enamel appear smoother, and prevent bacterial colonization (Figure 6(b)). There was no bacterial growth in Figures 6(b)–6(d), the texture of the enamel exposed to GA-AGNPs (Figures 6(b) and 6(c)) appeared to be smoother compared to the CHX-treated enamel (Figure 6(d)) and the vehicle enamel (Figure 6(e)). These results imply that the GA-AgNPs_0.4g may have the ability to prevent attachment of *S. mutans* on the teeth. Similarly, adding AgNPs to the commercial adhesive systems changed the texture of the enamel [61], which might also prevent colonization of the enamel by bacteria. In other studies, *S. mutans* biofilm treated with chemically synthesized AgNPs presented apparent structural destruction, suggesting that biofilm formation was inhibited [45]. The GA-AgNPs presented similar effects to that of CHX, and prevented the attachment of *S. mutans* biofilm, which suggested that AgNPs can be added to dental care products to prevent infections.

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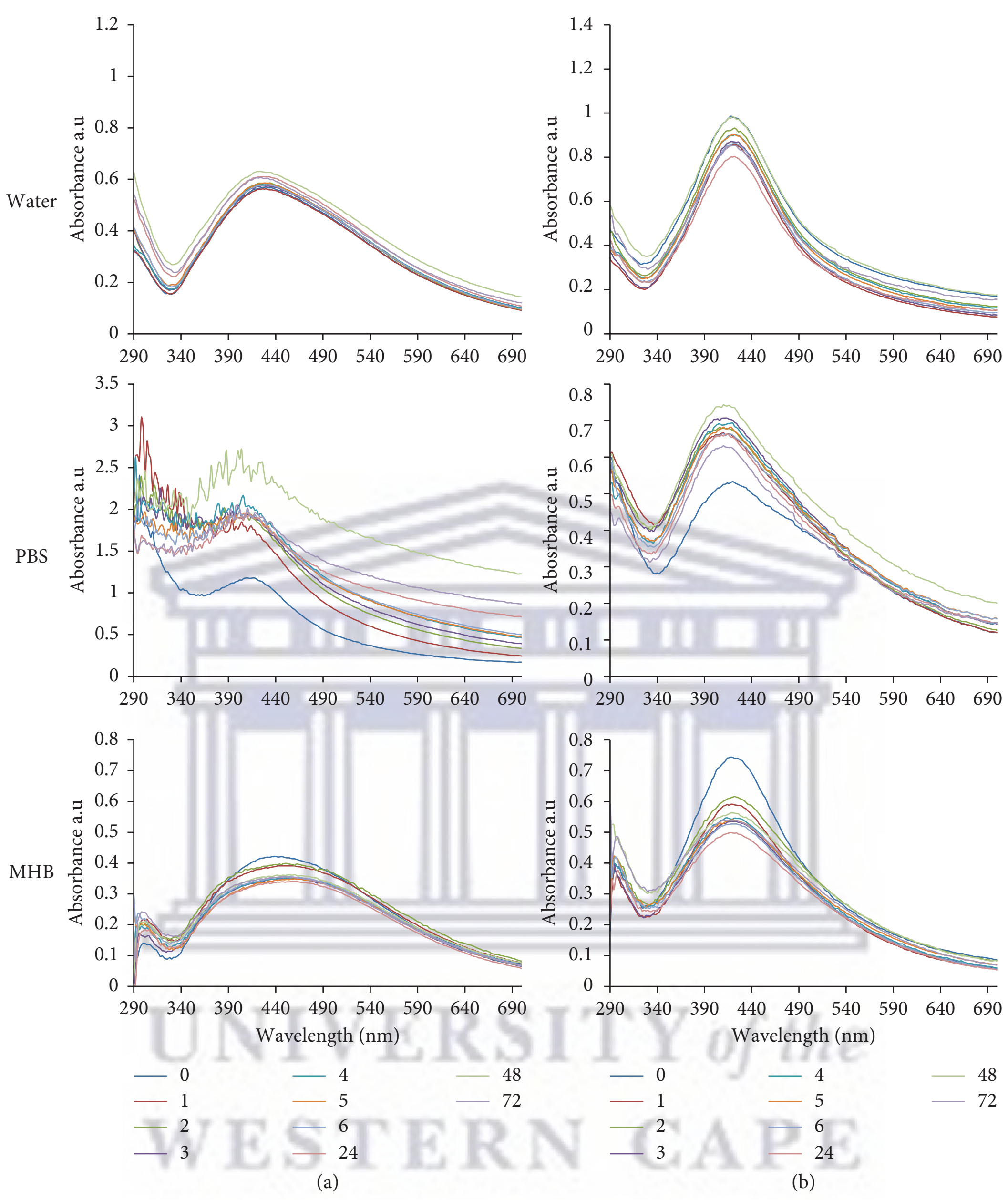

FIGURE 5: UV-vis spectra of (a) GA-AgNPs_0.1g; and (b) GA-AgNPs_0.4g in water, PBS, and MHB measured over a 72 hour period.

TABLE 2: ZOIs as a measure of antimicrobial activity of GA-AgNPs.

Microbes	ZOIs (mm)				
	GAE	GA-AgNPs_0.1g	GA-AgNPs_0.4g	0.2% CHX	Nystatin
S. sanguinis	0	9.96 ± 0.09	13.38 ± 0.10	19.74 ± 0.08	
S. mutans	0	10.48 ± 0.04	16.62 ± 0.34	22.65 ± 0.05	
L. acidophilus	0	9.77 ± 0.02	13.92 ± 0.20	18.53 ± 0.10	
C. albicans	0	12.44 ± 0.28	17.97 ± 0.41		22.86 ± 0.01

TABLE 3: MICs values for the GA-AgNPs.

Microbes	MIC_{50} (μg/ml)		MIC_{90} (μg/ml)	
	GA-AgNPs_0.1g	GA-AgNPs_0.4g	GA-AgNPs_0.1g	GA-AgNPs_0.4g
S. sanguinis	25	3.125	100	12.5
S. mutans	25	3.125	100	12.5
L. acidophilus	50	3.125	100	12.5
C. albicans	50	3.125	100	12.5

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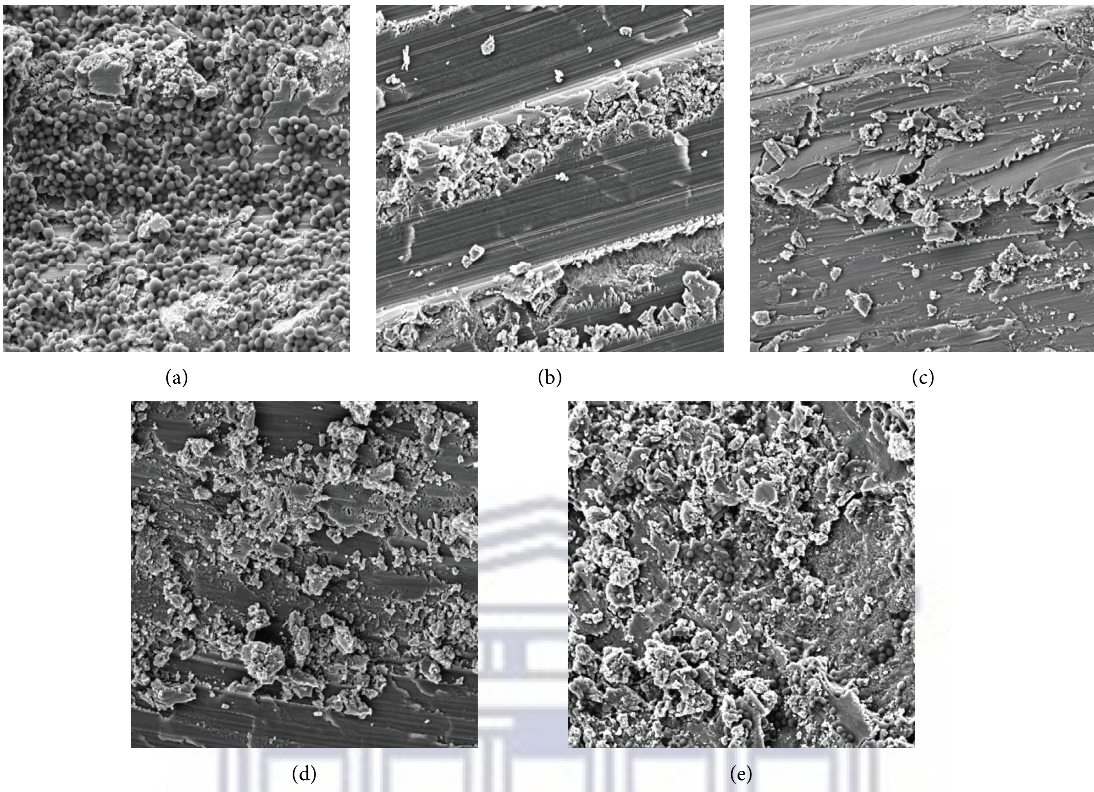

Figure 6: Exposure of GA-AgNPs_0.4g to *S. mutans* inhibits bacterial adhesion on the enamel surfaces. (a) Tooth enamel with *S. mutans* without treatment, (b) with GA-AgNPs_0.4g with *S. mutans*, (c) GA-AgNPs_0.4g without bacteria, (d) *S. mutans* and CHX D), (e) tooth enamel without bacteria and treatment.

4. Conclusions

The plant extract-mediated synthesis of AgNPs has emerged as a new avenue to produce biocompatible AgNPs. The findings in this study suggest that GA-AgNPs are promising antimicrobial agents against oral microbes. The GA-AgNPs_0.4g had enhanced antimicrobial activity compared to GA-AgNPs_0.1g. Therefore, the GA-AgNPs can be used as an additive to dental products, particularly because it can attach itself to enamel and prevent bacterial biofilm formation on the teeth.

Data Availability

The data are presented as tables and figures in the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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2.3.2.c Peer review

Reviewer Comment	Responses
Authors must explain how GA-Ag Nanoparticles synthesis and characteristics differ from samples of cited previous work. What is the substantial novelty?	We are from the same university. This work is a collaboration between our department (Restorative Dentistry) and the department of the previously cited work (Biotechnology). However, the primary paper is a preliminary study towards general application of AgNPs, while the current study evaluated the effects of green synthesized AgNPs in oral microbes. This is because oral microbes are the first microflora to be exposed to the external environment leading to internal infections.
The manuscript requires an English editing	This has been reviewed and changed
Clarify the method used to calculate the size of AgNPs in DLS characterization	The size of GA-AgNPs were determined by DLS using Zetasizer NanoZS90 (Malvern Panalytical Ltd., Malven, UK) which automatically analyzed and calculate the particle size.

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2.3.3 An *in-vitro* study evaluating gum Arabic-silver nanoparticles cytotoxicity on selected cells

2.3.3.a. Executive summary

In this paper, the biosafety of the synthesized GA-AgNPs is assessed with the aim of future incorporation in any topical dental applications. Thus, this section deals with objective (e) and reported on the research question (e). Both fractions of GA-AgNPs (0.1g/0.4g) were cytotoxic to KMST-6 and BMF cells, with GA-AgNPs_0.4g having the highest toxicity. Due to the greater antimicrobial activity of GA-AgNPs_0.4g, it was advised to add other compounds to reduce this toxicity, as shown in Chapter 2.4 (A). This paper is prepared and due for submission to an appropriate journal.

2.3.3.b. Manuscript

Abstract

Biological synthesis of silver nanoparticles (AgNPs) by using plants provides an ecological alternative to chemical synthesis. However, little data on their safety in biomedical applications. This study evaluated the cytotoxicity of AgNPs synthesized using gum Arabic (GA) as a reducing agent. The cytotoxicity of gum Arabic-silver nanoparticles (GA-AgNPs) was assessed on buccal mucosa fibroblasts (BMF) and the noncancerous (KMST-6) cells using an MTT assay. Although the synthesis of AgNPs from plants is considered safe and biocompatible, the findings of this investigation showed that GA-AgNPs' cytotoxicity was concentration-dependent; and toxicity can be masked by using molecular additives, therefore providing a concentration that is toxic to microbes but less deleterious to mammalian cells.

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1. Introduction

Metal nanoparticles are commonly used in the food and medical industry, diagnostics, and electronics due to their unique physical and chemical properties (Ghabban *et al.*, 2022). Particularly in medicine, the interest in and production of metal nanoparticles have grown (Wang, Hu and Shao, 2017). Silver nanoparticles (AgNPs) have been developed as a potent product among the different existing metal nanoparticles, as they possess good conductivity, chemical stability, antimicrobial, antioxidant, anti-cancer, and anti-inflammatory properties (Srikar *et al.*, 2016; El-Naggar, Hussein and El-Sawah, 2017; Belle Ebanda Kedi *et al.*, 2018; Huq, 2020). Additionally, it has been claimed that they may serve as antibacterial agents against a variety of pathogenic microorganisms (Salem and Fouda, 2011; Pérez-Díaz *et al.*,

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2015; Rafique *et al.*, 2017; Akter and Huq, 2020). As a result of their significant antimicrobial activity, they have been successfully applied in several biomedical fields (Burdușel *et al.*, 2018; Lee and Jun, 2019). In dentistry, they have been applied as disinfectants and prophylactics, in the inhibition and control of oral infections (Noronha *et al.*, 2017; Bapat *et al.*, 2018; Şuhani *et al.*, 2018; Yin *et al.*, 2020; Fernandez *et al.*, 2021).

With the extensive production and use of AgNPs, concerns regarding their toxicity to the environment and mammalian cells are gaining attention. Silver nanoparticles used in dental settings can accumulate in the oral cavity, i.e. teeth and oral mucosa, which can result in adverse effects (Ahmed *et al.*, 2022). AgNPs must therefore be tested for safety before being considered for oral use. Numerous studies have confirmed that the cytotoxicity of AgNPs is influenced by the activity of liberated free Ag+ ions in the medium, and that this relationship is dependent on the particles' size, composition, coating agent, surface charge, and form (Butler *et al.*, 2015; Dayem *et al.*, 2017; Noronha *et al.*, 2017; Ahmed *et al.*, 2022). Silver nanoparticles can enter the body through ingestion, inhalation, skin contact, and intraperitoneal or intravenous injection directly into the systemic circulation (De Matteis, 2017). In the case of chronic exposure to AgNPs, skin and eye darkening (argyrosis) are both possible (Panyala, Peña-Méndez and Havel, 2008; Ferdous and Nemmar, 2020). In addition, it can cause damage in various organs of the body such as the liver, kidney (Nosrati *et al.*, 2021), respiratory and digestive system and can lead to alterations in the blood cells (Panyala, Peña-Méndez and Havel, 2008; Gaillet and Rouanet, 2015; Noronha *et al.*, 2017; Bapat *et al.*, 2018). Cells that have absorbed AgNP may experience oxidative stress, DNA damage, chromosomal abnormalities, and even death as well as harm to essential organelles like the nucleus and mitochondria (El Mahdy *et al.*, 2015; Ferdous and Nemmar, 2020). This has been demonstrated in several different human cell lines, including macrophages (RAW 264.7), bronchial epithelial cells (BEAS-2B), alveolar epithelial cells (A549), hepatocytes (C3A, HepG2), colon cells (Caco2), skin keratinocytes (HaCaT), human epidermal keratinocytes (HEKs) (Dayem *et al.*, 2017; Akter *et al.*, 2018; Jiang *et al.*, 2018; Ferdous and Nemmar, 2020).

There have been reports of various restrictions in the production of AgNPs utilizing chemical and physical methods since they are frequently expensive, and they release toxic or cariogenic residues which are harmful to the environment and cells (Iravani *et al.*, 2014; Srikar *et al.*, 2016; Ghabban *et al.*, 2022; Padilla-Camberos *et al.*, 2022). Today, the use of plants as a renewable, biodegradable, and inexpensive source for the synthesis of AgNPs has been welcomed due to its economic and environmentally friendly characteristics (Venkatesham *et*

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al., 2012; Iravani, 2020; Al-Ansari, Al-Dahmash and Ranjitsingh, 2021; Ahmed *et al.*, 2022; Fadaka *et al.*, 2022). Furthermore, the existence of reducing agents such as alkaloids, tannins, phenols, and terpenoids in the plant extract provide an additional source for the synthesize of AgNPs (Venkatesham *et al.*, 2012; Fadaka *et al.*, 2022). The use of gum Arabic as a non-toxic, edible glycoprotein polymer seems promising for the synthesis of AgNPs (Venkatesham *et al.*, 2012; Al-Ansari, Al-Dahmash and Ranjitsingh, 2021; Fadaka *et al.*, 2022). Gum Arabic (GA) is a plant exudate that may be extracted from the Acacia tree's stem and branches. It is composed of a mixture of glycoproteins and polysaccharides mainly consisting of galactose and arabinose which are effective reducing agents (Musa *et al.*, 2018; Barak, Mudgil and Taneja, 2020; Verma and Quraishi, 2021). Gum Arabic exhibits excellent anti-bacterial, anti-inflammatory, immunostimulatory, and antioxidant properties that allow them to be used as a stabilizer in the pharmaceutical and food sectors (Patel and Goyal, 2015; Jaafar, 2019).
In this paper, we report on the possible *in vitro* cytotoxic effect at cellular level of GA-AgNPs against buccal mucosa fibroblast cells (BMF) and non-cancerous KMST-6 cells.

2. Materials and methods

2.1 Synthesis and characterization of GA-AgNPs

GA-AgNPs were created and characterized in accordance with the procedure previously outlined by Fadaka et al. (Fadaka et al., 2022). In a nutshell, the GA extract (GAE) was made by melting 4 mg/ml GAE in 100 ml of hot water and filtering through 0.45 m filters. It was made from Acacia senegal (North Kordofan, Sudan). To create GA-AgNPs_0.1 g and GA-AgNPs_0.4 g, respectively, two concentrations of AgNO3 (0.1 g and 0.4 g) were added, and the volume was adjusted to 400 ml of deionized water. All reactions were conducted for 20 minutes at 15 psi and 120 °C in an autoclave (Fadaka et al., 2022). Both GA-AgNPs were centrifuged before being further characterized using a dynamic light scattering (DLS), transmission electron microscopy (TEM), and fourier-transform infrared (FTIR) spectrophotometer.

2.2 Cytotoxicity assay of the GA-AgNPs

The cytotoxicity of each GA-AgNPs_0.1g/0.4g was tested on both buccal mucosa fibroblast and KMST-6 cell lines. Using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test, the viability of both cells was assessed. The MTT assay is a sensitive and precise colorimetric assay that assesses the viability and proliferation of cells based on how well living

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cells convert MTT reagent into formazan crystals, which reveals mitochondrial activity (Meerloo, Kaspers and Cloos, 2011).

As these fibroblasts were ideally adapted for modeling the oral environment, the first cell line employed was a Human Oral Fibroblast cell line developed at the Oral and Dental Research Institute, University of the Western Cape. These cells were preserved in liquid nitrogen storage and later extracted for usage. Standard conditions were used to maintain and culture the cells. The second cell line used was the KMST-6 normal skin fibroblast cells which were purchased from ATCC. These cells were found on the buccal mucosa and the labial mucosa which were also suitable for representing the oral environment.

Thereafter the cells were first grown to near confluency and afterwards they were seeded in 96-well microtitre plates at a density of 2x 10^4 cells per well. The cells were kept alive in a 37 °C humidified incubator with 5% CO2 saturation using Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) from Hyclone Laboratories in South Logan, Utah, and 1% penicillin-streptomycin cocktail (penstrep) from Gibco in Germany. In a humidified CO2 incubator set to 37°C, the plates were incubated. After 24 hours, a fresh medium containing one of each GA-AgNP was added to the culture media. Different concentrations of the GA-AgNPs were determined from their UV-Vis spectra at increasing concentrations of 0.3 to 100 g/ml in order to examine the quantities that are hazardous to the microorganisms and less poisonous to the cells. Cells were given 50.0 M C2-Ceramide (Sigma), a known inducer of apoptotic cell death, as a positive control. Cells that weren't treated served as a negative control. There were three duplicates of each therapy. 24 hours later, the GA-AgNPs agents were detached, and the wells were thoroughly cleaned with phosphate buffer saline (PBS) (Elbagory *et al.*, 2017).

Subsequently, each well received 100 l of the MTT reagent, which was produced from a 5.0 mg/ml stock solution and diluted with DMEM medium using a dilution factor of 1:10 (Sigma). The plates underwent a second incubation at 37 °C for four hours. The purple formazan crystals were subsequently dissolved using 100 l of alkaline DMSO in place of the MTT reagent. Using the RT-2100C microplate reader, the samples' absorbance at 540 nm was determined during a 15-minute incubation period at 37 °C (Rayto Life and Analytical Sciences Co., Shanghai, China). multiplate reader (Kayto RT2100C, Shanghai international group, Germany). A 630

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nm absorbance was used as a reference wavelength (Elbagory *et al.*, 2017). The following equation was used to compute the percentage of viable cells:

$$\% \, Cell \, Viability = sample \, absorbance \; \div control \, absorbance$$

2.3 Statistical analysis

The results of the three separate trials, each of which was carried out in triplicate, were presented as mean standard deviation. GraphPad Prism version 6 was utilized for the statistical analysis, which employed one-way and two-way ANOVA. Statistics were judged significant at p 0.05. A post hoc pair-wise test was also run to clarify the distinctions between two sets of data.

3.Results

In the present study, the GAE does not demonstrate any cytotoxicity at all concentrations on the two cell lines tested. The cytotoxicity of GA-AgNPs was found to be concentration dependent. The GA-AgNPs_0.1g was least toxic compared to GA-AgNPs_0.4g and shown to be non-toxic at concentrations below 12.5 µg/ml, for both cell lines with ±80% cell survival (Figure 1 A, B). The GA-AgNPs_0.4g were toxic to the KMST-6 cells at all concentrations tested, whereas on the BMF cells it exhibited non-toxic effects at concentrations at 1.56 µg/ml with ±60% cell survival below 6.25 µg/ml (Figure 1B).

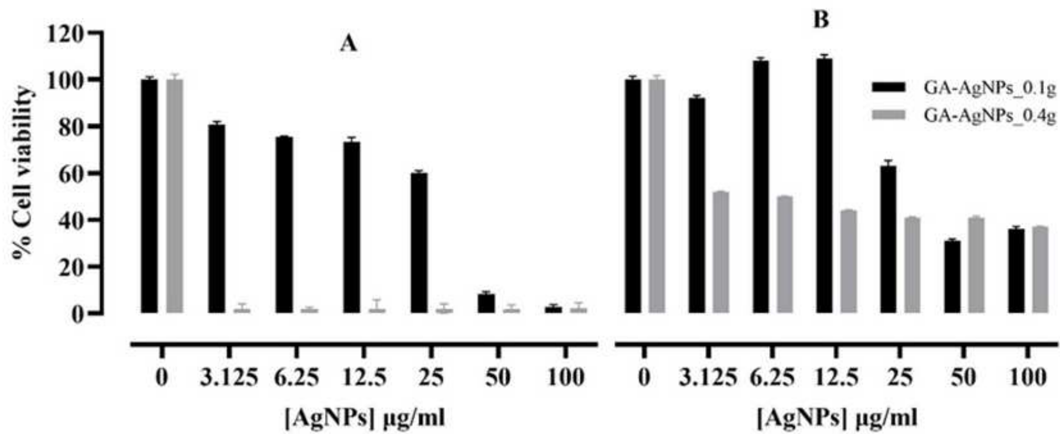

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Figure (1): Cytotoxicity of the GA-AgNPs_0.1/0.4g on KMST-6 (A) and BMF (B) cells assessed using the MTT assay after 24 hrs exposure to different concentrations.

4. Discussion

Due to their high antimicrobial activity, the use of AgNPs in commercial products has been increasing (Burdușel *et al.*, 2018; Panja *et al.*, 2021). Strategies to ensure their biocompatibility have been devised, such as surface modification with biopolymers and use of natural products for their synthesis (Idrees *et al.*, 2020; Huq *et al.*, 2022). However, these strategies are not always successful as the non-biodegradable nature of the AgNPs core remains a major concern for any application concerning humans (Jaswal and Gupta, 2021). Although green-synthesized AgNPs have been reported to have lower toxicity in contrast to the chemically synthesized AgNPs (Torres Rheder *et al.*, 2018; Ahmed *et al.*, 2022), emerging evidence disputes these claims. It is now understood that the activity of biogenic AgNPs depends on their physicochemical properties, among others their size, shape, capping agents, concentration, etc. This has been confirmed in mammalian cells where the toxicity of biogenic AgNPs was reduced (Jaswal and Gupta, 2021). The findings of this investigation supported those of earlier studies, further demonstrating that smaller-sized AgNPs, whether produced chemically or biologically, are more harmful than larger AgNPs. This could be due to that they can easily interact with and penetrate cells (Haase *et al.*, 2011; Pratsinis *et al.*, 2013; Liao, Li and Tjong, 2019). Furthermore, the findings in this study were similar to a prior study that reported non-selective cytotoxicity of GA-AgNPs to skin fibroblasts and colon cancer cells (Fadaka *et al.*, 2022). In addition, these results were comparable to a study by Taleghani *et al*., (2014), which reported that AgNPs exhibit toxic effects on human gingival epithelial cells but the toxicity was time and dosage dependent (Taleghani *et al.*, 2014). Interestingly, AgNPs stabilized with GA demonstrated no cytotoxicity against mouse embryonic fibroblast cells (Eghbalifam *et al.*, 2020), and Sprague Dawley rats (Maziero *et al.*, 2020). The outcomes in this study indicated that the GA-AgNPs toxicity was non-selective to cells and the mechanism of their toxicity has to be further investigated. AgNPs can penetrate the cell membrane, disrupt the respiratory chain of the mitochondria and produce reactive oxidative species that cause DNA damage and eventually cell death (Panyala, Peña-Méndez and Havel, 2008; Marambio-Jones and Hoek, 2010).

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In as much as the GA-AgNPs demonstrated cytotoxicity to these cells, they can be applied to the oral cavity due to the rapid regeneration of the KMST-6 and BMF cells if they are not ingested (Rippa, Kalabusheva and Vorotelyak, 2019). For instance, toxic effects in the oral mucosa of humans are reversible upon the termination of exposure (Tang *et al.*, 2017). Moreover, the cytotoxicity may be masked using additives or mucoadhesive polymers (Garg *et al.*, 2018), and limited use of formulations in the oral enviroment. Given that the exposure in this study was 24 hours, reducing exposure time of GA-AgNPs to fibroblast cells (e.g 2 minutes, recommended for use of oral hygiene products) may increase cell survival to above 80%.

5. Conclusion

GA-AgNPs at lower concentrations have potential for use in the oral cavity especially for limited time. The cytotoxicity of GA-AgNPs_0.4g is higher than GA-AgNPs_0.1g. Therefore, the use of biopolymers to mask cytotoxicity at higher concentrations is advisable for them to be used as effective antimicrobial agents against oral microbes with less cytotoxicity to the KMST-6 and BMF cells.

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2.4. Prospects of using gum Arabic-silver nanoparticles in toothpaste to prevent dental caries

2.4.a. Executive summary

After testing antimicrobial and cytotoxicity activities as described in the previous sections, GA-AgNPs_0.4g were selected to be integrated into a commercial toothpaste with low antimicrobial activity, hence objective (f) was discussed and research question (f) was rejoined. The GA-AgNPs_0.4g toothpaste (GA-AgNPs_TP) demonstrated an increase in the antimicrobial activity, however with lowered cytotoxicity compared to the GA-AgNPs_0.4g in its native form. It was suggested that the inclusion of polymers like chitosan could further reduce the cytotoxicity of GA-AgNPs_TP.

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2.4.b. Publication

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Article

Prospects of Using Gum Arabic Silver Nanoparticles in Toothpaste to Prevent Dental Caries

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Abstract: There is growing interest in the use of green synthesized silver nanoparticles (AgNPs) to control and prevent dental diseases. The incorporation of green synthesized AgNPs into dentifrices to reduce pathogenic oral microbes is motivated by their presumed biocompatibility and broad-spectrum antimicrobial activity. In the present study, gum arabic AgNPs (GA-AgNPs) were formulated into a toothpaste (TP) using a commercial TP at a non-active concentration, to produce GA-AgNPs_TP. The TP was selected after evaluating the antimicrobial activity of four commercial TPs 1-4 on selected oral microbes using agar disc diffusion and microdilution assays. The less active TP-1 was then used in the formulation of GA-AgNPs_TP-1; thereafter, the antimicrobial activity of GA-AgNPs_0.4g was compared to GA-AgNPs_TP-1. The cytotoxicity of GA-AgNPs_0.4g and GA-AgNPs_TP-1 was also assessed on the buccal mucosa fibroblast (BMF) cells using the MTT assay. The study demonstrated that antimicrobial activity of GA-AgNPs_0.4g was retained after being combined with a sub-lethal or inactive concentration of TP-1. The non-selective antimicrobial activity and cytotoxicity of both GA-AgNPs_0.4g and GA-AgNPs_TP-1 was demonstrated to be time and concentration dependent. These activities were instant, reducing microbial and BMF cell growth in less than one hour of exposure. However, the use of dentifrice commonly takes 2 min and rinsed off thereafter, which could prevent damage to the oral mucosa. Although, GA-AgNPs_TP-1 has a good prospect as a TP or oral healthcare product, more studies are required to further improve the biocompatibility of this formulation.

Keywords: green synthesis; gum arabic; silver nanoparticles; toothpaste; antimicrobial; cytotoxicity

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1. Introduction

The oral cavity is the second most complex microbiota in the human body after the colon and contains a diverse microbial community [1]. An imbalance in the microbial composition, which can be caused by certain local factors including the consumption of carbohydrates, plaque accumulation, pathogenic oral microbes, and poor oral hygiene, can lead to the development of oral diseases such as dental caries and periodontitis [2,3]. Preventing these diseases through regular brushing and the use of antimicrobial agents added to oral hygiene products assists in the removal of pathogenic microbes from the enamel surfaces and presents a simpler and more economical approach than a later treatment [4].

The use of oral care products such as TPs and mouth rinse is a fundamental means for the control and prevention of oral diseases [5]. The majority of the TPs are composed of three major chemicals, i.e., fluoride (an antimicrobial and dental enamel remineralizing agent), triclosan (an antibacterial agent), and sodium lauryl sulfate (an anionic surfactant with detergent action) [6,7]. In addition, there are other components such as humectants, abrasive agents, and flavoring agents [5]. Although there are many TP formulations

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with antimicrobial activities, unfortunately, the emergence of antimicrobial-resistant oral microbes necessitates the development of alternative antimicrobial agents that are safe and relatively economical [8]. There is a growing interest in the use of natural products to combat microbial infections [9]. Studies have shown that natural products can be used as reducing agents in the synthesis of metal nanoparticles (MNPs) with enhanced antimicrobial activities [10]. The incorporation of these green synthesized NPs in oral hygiene products was demonstrated to improve their efficacy and expand their field of application. Nanomaterials, or NPs, are defined as materials with one or more dimensions at a size range between 1 and 100 nm and exhibit different properties as compared to their precursors or bulk materials [11,12]. The morphology and size distribution of the NPs are key factors for their inventive bioapplications [13–15], including in oral care products [16–19].

AgNPs are among the MNPs that have received special attention in recent years, especially in biomedicine, due to their immense antibacterial, anti-inflammatory, antiviral, and antifungal activities [13,17,18,20–23]. They have been synthesized from synthetic and natural compounds, although the biosynthesis route is preferred to reduce the toxicity associated with the chemical synthesis method [10,24]. The synthesis of bioinspired AgNPs involves using microbial (bacteria, fungi) and plant extracts as reducing and stabilizing agents [10,25,26]. Plants have many advantages over microorganisms, as they are economical and easy to process [27]. Bioactive AgNPs have been synthesized using various plant extracts from *Punica granatum* [28], *Justica glauca* leaf [29], *Plectranthus ambionicus* [30], Rice [31], and Gum Arabic [18,32], and have been shown to have broad-spectrum antimicrobial activity against various oral microbes including *Streptococcus mutans* (*S. mutans*), *Candida albicans* (*C. albicans*), *Lactobacillus acidophilus* (*L. acidophilus*), and *Enterococcus faecalis* (*E. faecalis*). Their antimicrobial activity against Gram-positive, Gram-negative bacteria, as well as drug resistant microbes [33], suggests that AgNPs can potentially be used as antimicrobial agents. The bioactivity of AgNPs is influenced by several factors, including, among others, their size, shape, concentration, and surface composition [34–37].

Due to their potent antimicrobial activity, AgNPs have been incorporated into TPs, toothbrushes, and mouthwashes to combat cariogenic bacteria [38]. AgNPs in dentifrices demonstrated superior activity in reducing the oral microbes associated with dental caries and periodontal diseases [39], while 1.5–2 g of TP is required to inhibit the growth of oral cavity microbes [40]. The antimicrobial activity of a commercial nanosilver-containing TP (TruCare Nano Silver TP) against *S. mutans* was compared to fluoride-based TP (Oral B Pro Health) and chitosan TP (Conybio Plus Chitosan Dental TP) and showed favorable and superior effects [4]. Previously, a commercial TP (Royal denta) containing AgNPs (R.D. Silver) was reported to be most effective against *Staphylococcus aureus* (*S. aureus*) and *E. faecalis*, while neither R.D. Silver nor "Royal denta" TP containing gold nanoparticles as well as tangerine and orange oils (R.D. Orange and Gold) had effect on *Escherichia coli* (*E. coli*) or *Proteus mirabilis* (*P. mirabilis*) [40].

The bioactivity of AgNPs has been hampered by contradictory reports regarding their toxic effects to human cells and warrants further studies on their mechanism of action as it is still not clear. Therefore, it is imperative to know their adverse effects in humans and to understand how they behave within biological systems before their clinical application [41]. It was indicated that AgNPs, after oral exposure, could be responsible for inflammation of the gastrointestinal tract, weight loss, disruption of blood biochemistry, and dysfunction of liver enzymes [42]. In an in vitro study by Tang et al., AgNPs were toxic to human gingival epithelial cells and significantly reduced cell viability at concentrations $\geq$20 µg/mL [43]. Since TPs have been suggested for topical application and not for systematic use, it is unlikely that harmful effects would be observed [41]. The aim of the present study was to evaluate whether the antimicrobial activity of green synthesized GA-AgNPs can be altered when incorporated into a TP formulation.

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2. Materials and Methods

2.1. Antimicrobial Activity of the Commercial TPs

2.1.1. Preparation of the Commercial TPs

Four commercial TPs (TP1-4) were purchased from a local pharmacy in Cape Town (South Africa). Three of the TPs (TP 1-3) were fluoride-based TPs, and one (TP-4), was a charcoal-based TP. The TPs were prepared as a slurry following a previous protocol [44], where 5 g of the TPs was dissolved in 10 mL of Mueller-Hinton Broth (MHB; Sigma Aldrich, St Louis, MO, USA) and vortexed until completely resuspended in solution. The concentration of each TP in solution was 500 μg/mL and considered as 100%.

2.1.2. Microorganisms and Culture Conditions

The antimicrobial activity of the TP1-4 was investigated by agar disc diffusion and microdilution assays in one fungal and three bacterial strains: *Streptococcus sanguinis* - *S. sanguinis* (NCTC 7865; Davies Diagnostics, Randburg, South Africa), *S. mutans* (NCTC 10449; Davies Diagnostics), *L. acidophilus* (ATCC 314; American Type Culture Collection (ATCC) Manassas, VA, USA) and *C. albicans* (ATCC 10231). A single colony was sub-cultured in Brain Heart Infusion Agar (Sigma Aldrich) for all the bacterial strains and Sabouraud dextrose agar for *C. albicans* at 37 °C for 24 h. Following the overnight culture, the microbes were adjusted to 0.5 McFarland (McF) standard using DensiCHEK Plus (BioMérieux Inc., Durham, NC, USA).

Agar Disc Diffusion Assay

The agar disc diffusion method was used to determine the antimicrobial activity of the commercial TP 1-4 on *S. sanguinis*, *S. mutans*, *L. acidophilus* and *C. albicans*, as described before [18]. In this method, agar plates were inoculated with a 100 μL of each of the standardized inoculum of the test microorganisms. Filter paper discs (6 mm in diameter) previously infused with 100 μL of each of the four commercial TPs (stock concentration of 500 μg/mL) and air-dried overnight were placed on the inoculated agar plates. Then, 0.2% chlorohexidine (CHX) and 5000 u Nystatin were used as positive controls for bacteria and fungi, respectively, and water was used as a negative control. The plates were incubated for 24 h at 37 °C and observed for zones of inhibition (ZOI) by measuring the diameters of ZOI formed around the filter paper disk using a Vernier caliper. The assay was carried out in triplicate for all tested organisms and repeated six times.

Microdilution Assay

The commercial TP with the least antimicrobial activity from the disc diffusion assay was used in this experiment. The lowest concentration of the commercial TP that visually inhibited growth of the four microorganisms (Minimum Inhibitory Concentrations, MICs) was determined according to the protocol set by the M07 of the Clinical Laboratory Standards Institute (CLSI) [45].

The 0.5 McF of the test microbes was added in 96-well plates (100 μL/well) and incubated at 37 °C for 24 h. The plates were rinsed with phosphate buffered saline (PBS; Sigma Aldrich) three times and 100 μL of MHB was pipetted into all wells. Then, 100 μL of TP (3.9–500 μg/mL) was transferred into the wells, except for the untreated controls, where 100 μL of MHB was added. The plates were incubated at 37 °C for 24 h. Then, the MICs were determined by sub-culturing 5 μL of samples from each well on a Tryptic Soy Agar plates (TSA; Sigma Aldrich) and incubating at 37 °C for 24 h following a previous method [46]. The growth or inhibition of *S. mutans* was qualitatively and quantitatively determined by visually examining the culture media and counting the total number of colonies formed (colony forming unit, CFU) on TSA to determine MICs. The experiment was carried out in triplicate and repeated three times.

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2.2. Synthesis of GA-AgNPs and Preparation of the GA-AgNPs_TP

2.2.1. Synthesis and Characterization of GA-AgNPs

The GA-AgNPs_0.4g used in this study were synthesized and characterized as previously described [18]. Briefly, 1.6 g of *Acacia senegal* gum arabic (GA) powder purchased from local vendors in North Kordofan (Sudan, Africa) was dissolved in 100 mL of boiling deionized water and filtered through 0.45 µm filters before use. Then, 4 g of silver nitrate ($AgNO_3$; Sigma Aldrich) dissolved in 100 mL of water was added to the GA solution. The volume of GA/$AgNO_3$ solution was adjusted to 400 mL with deionized water, quickly mixed by swirling the flask and autoclaved at 120 °C at 15 psi pressure for 20 min. The GA-AgNPs_0.4g were centrifuged at 9000 rpm for 45 min, resuspended in equal volume of deionized water then stored at room temperature covered in foil until further analysis.

Characterization of the GA-AgNPs_0.4g was previously reported. Briefly, the AgNPs were analyzed by ultraviolet-visible (UV-Vis) spectrophotometer on a POLARstar Omega plate reader (BMG LABTECH, Offenburg, Germany), dynamic light scattering (DLS) using Malvern NanoZS90 Zetasizer (Malvern Panalytical Ltd., Enigma Business Park, UK), Transmission Electron Microscopy (TEM) at the Electron Microscope Unit (University of Cape Town, South Africa) by a TecnaiF20 HRTEM (FEI Company, Hillsboro, OR, USA), and Fourier-Transform Infrared Spectroscopy (FTIR) at UWC School of Pharmacy using Perkin Elmer Spectrum Two FTIR spectrophotometer (Waltham, MA, USA) [18].

2.2.2. Preparation of the GA-AgNPs_TP-1

GA-AgNPs_TP-1 stock was prepared fresh by mixing 5 mL of TP-1 slurry (62.5 µg/mL) and 5 mL of 200 µg/mL GA-AgNPs_0.4g. The mixture was vortexed for 5 min. The concentration of TP-1 was kept constant at 62.5 µg/mL and used for further serial dilutions of GA-AgNPs_0.4g to yield 6.25–100 µg/mL (final concentration of TP-1 in GA-AgNPs_TP-1 treatments was 31.3 µg/mL).

2.3. Antimicrobial Activity of the GA-AgNPs-TP

2.3.1. Agar Disc Diffusion Method

The antimicrobial activity of the GA-AgNPs_0.4g and the GA-AgNPs_TP-1 was compared using the agar disc diffusion method, as described in Section 2.1.2, on the four microorganisms. Filter paper discs were infused with 100 µL of each of the two treatments at varying concentrations from 6.25 to 100 µg/mL and allowed to air-dry overnight. After spreading 100 µL (0.5 McF) of each inoculum on a sterile agar plate, the discs were placed on the agar surface. Then, 0.2% CHX and 5000u Nystatin were used as positive controls for bacteria and fungi, respectively, and the 62.5 µg/mL TP-1 was also used as a control. The plates were incubated for 24 h at 37 °C, then the plates were observed for ZOI and the diameters of ZOI were measured using a Vernier caliper. The assay was carried out in triplicate for all the tested microorganisms and repeated three times.

2.3.2. Microdilution Assay

The MICs for GA-AgNPs_0.4g and the GA-AgNPs_TP-1 were determined by broth microdilution assay as described above. Briefly, serially diluted concentrations of GA-AgNPs_0.4g and GA-AgNPs_TP-1 were prepared in MHB ranging from 6.25 to 100 µg/mL. MHB medium containing the standard inoculum was added to the serially diluted concentrations of the treatment. After 24 h of incubation at 37 °C, the wells were checked for any evidence of microbial growth by sub-culturing a sample from each well on TSA and incubated at 37 °C for 24 h. The growth or inhibition of the microbes was determined by visually examining the culture media followed by counting the total number of colonies formed (CFU) on TSA to determine MICs for the treatments.

2.4. Cytotoxicity Assay

Two experiments were performed in this assay. In the first experiment, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; Sigma Aldrich) assay was

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used to assess the cytotoxicity of TP-1, GA-AgNPs_0.4g and the GA-AgNPs_TP-1 on BMF cells, following a previous method [47]. The BMF cells is a Human Oral Fibroblast cell line established in the Oral and Dental Research Institute (UWC, South Africa). The cells were maintained and grown to near confluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone laboratories, South Logan, UT, USA) and 1% penicillin-streptomycin cocktail (Gibco, Germany). The cells were seeded in a 96 well plate (100 µL/well) at 2×10^4 cells per well and allowed to grow for 24 h in a water jacketed CO_2 incubator (Forma series 3-model 4111, Thermo scientific). The cells were treated with 100 µL of the treatments ranging from 6.25 to 100 µg/mL. The TP-1, GA-AgNPs_0.4g, and GA-AgNPs_TP-1 treatments were prepared in DMEM. After 24 h, the cell viability was assessed by adding 10 µL of 5mg/mL MTT solution to each well and incubated at 37 °C for 3 h. Then, 100 µL of DMSO (Sigma Aldrich) was added to all wells and the absorbance was measured at 570 nm using an RT-2100C microplate reader (Rayto Life and Analytical Sciences Co., Shanghai, China). The percentage of cell viability was calculated using the following equation:

$$\% \text{ Cell Viability} = (\text{sample absorbance}/\text{control absorbance}) \times 100\%$$

In the second experiment, the time point and concentration of the treatments where the cytotoxicity on the BMF cells started was investigated. The same MTT assay was repeated with GA-AgNPs_TP-1 and TP-conditioned medium (TCM). Different concentrations (25, 50 and 100 µg/mL) of the treatments were used and the absorbance was determined at 0 min, 5 min, 30 min, 60 min, and 24 h. For the preparation of TCM, GA-AgNPs_TP-1 were diluted in DMEM and were shaken vigorously. Then, the GA-AgNPs_TP-1 were centrifuged for 10 min, and the TCM was collected, filter sterilized, and used for the treatment of BMF cells as described before [48].

2.5. Time Dependent Growth Inhibition of the Microbes and the BMF Cells

The aim of this experiment was to determine the time and concentration at which there were less damage to the BMF cells with maximum damage to the microbes. Antimicrobial activity of the GA-AgNPs_TP-1 (25, 50 and 100 µg/mL) was studied by observing microbial growth and counting of the CFU of each sample at different time intervals (0 min, 5 min, 30 min, 60 min and 24 h). Different concentrations of the GA-AgNPs_TP-1 were used starting from 25 to 100 µg/mL. The rate and extent of growth inhibition was determined at each time interval for all the microbes [21]. These effects were compared to the GA-AgNP_TP-1 activity on BMF cells investigated by MTT assay as described in the second experiment of Section 2.4.

2.6. Statistical Analysis

All the experiments were carried out in triplicate and the results were analyzed for variance in their respective means using QI Macros 2022 Starter Kit software (KnowWare International, Inc., Denver, CO, USA). The data were presented as means ± SD according to the one-way ANOVA test followed by post hoc, and multiple comparisons (Turkey's) test. Data were considered statistically significant at p value < 0.05.

3. Results and Discussion

Oral health plays an important role in the overall health and social aspect of a human being [49]. A number of oral hygiene products are available for the maintenance of oral health, prevention of oral infections, and their progression to oral diseases. Due to the bystander effects of these products and ability of the microbes to develop resistance towards them, new technologies are evolving [41]. The use of AgNPs as alternative antimicrobial, remineralization, and anti-inflammatory agents in dental hygienic products have reached clinical trials [17,19,50]. To date in the NIH U.S. National Library of Medicine ClinicalTrials.gov, only one trial that used green synthesized AgNPs (i.e., thyme and carvacroll based AgNPs) was registered (NCT04431804), and the rest were formulated using

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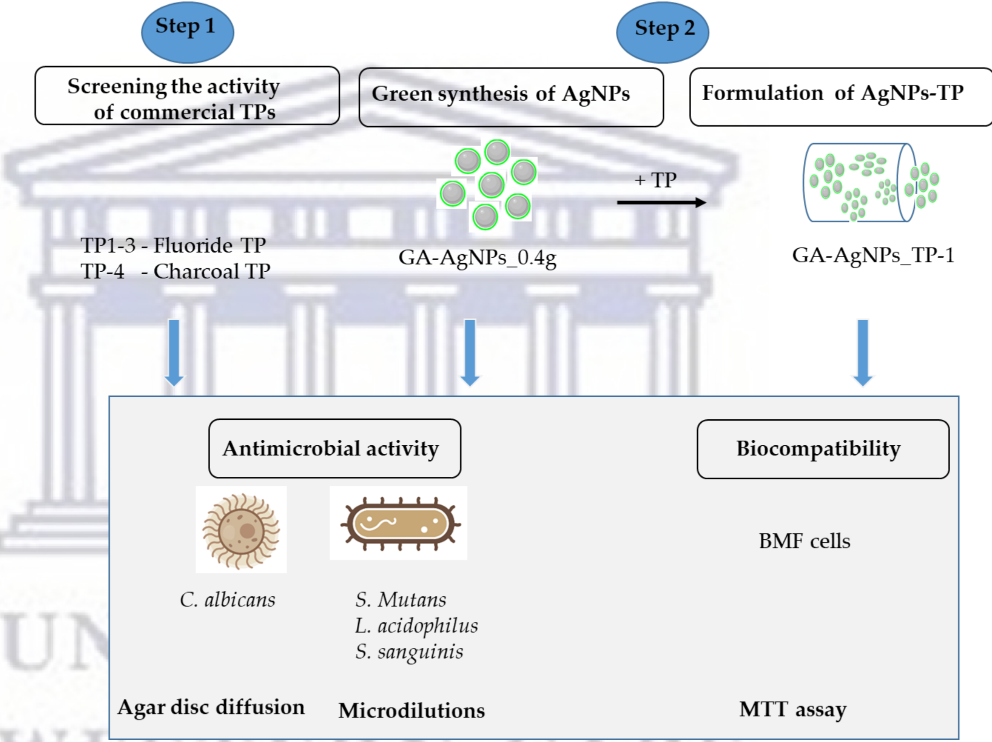
chemically-synthesized AgNPs. This suggests that green AgNPs can be used to replace the chemically-synthesized AgNPs and produce biocompatible products for clinical use.

The current study investigated the potential application of GA-AgNPs as antimicrobial agents in TP formulation; the steps are summarized in Scheme 1. The GA-AgNPs_0.4g were synthesized as previously described and demonstrated antimicrobial activities against Gram positive bacteria, Gram negative bacterial [18,51], and fungal strains. Moreover, these NPs were able to prevent the adhesion of *S. mutans* on human tooth enamel, which suggested that they can be used in dentifrices to prevent oral infections [18]. To confirm this claim, four commercial TPs were investigated for their antimicrobial activity on cariogenic and commensal oral microbes to identify the one with the lowest activity. Thereafter, GA-AgNPs_0.4g were incorporated into the selected TP to observe whether its antimicrobial activity was improved.



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Scheme 1. Steps towards GA-AgNPs-TP formulation, investigation of the antimicrobial and cytotoxicity activities. Step 1—screening of commercial TP antimicrobial activity. Step 2—formulation of GA-AgNPs based TP using a TP with least antimicrobial activity.

Various TPs have different efficacy in controlling oral microbes [52,53]. This is influenced by their active ingredients. Differences in the antimicrobial activity of TPs have also been reported in other studies [54]. The selected TPs used in the present study also demonstrated microbial growth inhibition on the test oral microbes. Table 1 shows that all the TPs at 500 µg/mL (stock concentration) showed antimicrobial activity in all microbes, in the following order TP-4>TP-2>TP-3>TP-1. The antimicrobial activities of the TPs were not statistically significant in comparison to each other. However, TP-1 and TP-3 had lower ZOI ranging from 7.00 to 9.41 mm across all the tested microbes compared to the other TPs at 7.77–12.91 mm. Therefore, TP-1 and TP-3 were selected for further studies due to their lowest antimicrobial activity.

In order to select a TP that will be subsequently combined with GA-AgNPs_0.4g, TP-1 and TP-3 were serially diluted (3.9–500 µg/mL) and used in a spot-plating assay to determine the lowest concentration that had no effect on *S. mutans*. The rationale for using *S. mutans* among the four microbes that were studied was that it is the most implicated strain in cariogenesis [55]. TP-1 failed to completely inhibit the growth of *S. mutans* but

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showed a concentration-dependent reduction in CFUs from 13.3 μg/mL as shown Table 2. TP-3 was the most potent and completely inhibited bacterial growth from 62.5 to 500 μg/mL on the spot-plating assay. The resulting CFU counts from TP-1 and TP-3 treatments together with their MICs were further assessed. The observed MICs for TP-1 and TP-3 on *S. mutans* was 125 μg/mL and 31.3 μg/mL for TP-3 (Figure 1). The CFU counts of *S. mutans* exposed to TP-1 and TP-3 demonstrated significant differences in their activities, with TP-1 further being confirmed to have less activity than TP-3. TP-1 treatment kept the *S. mutans* in the lag phase longer than TP-3 treatment. This indicated that TP-3 was more effective in inhibiting *S. mutans* growth than the TP-1. Thus, TP-1 at 31.3 μg/mL was selected for further studies, and used in combination with the GA-AgNPs_0.4g for GA-AgNPs-based TP formulation.

Table 1. ZOI for commercial TP1-4 against oral microbes.

Microbes	TP-1 (mm)	TP-2 (mm)	TP-3 (mm)	TP-4 (mm)	0.2% CHX (mm)	Nystatin (mm)
S. sanguinis	9.32 ± 1.7	10.89 ± 0.76	9.41 ± 0.59	12.91 ± 0.40	17.36 ± 0.31	
S. mutans	8.70 ± 0.10	8.55 ± 1.62	8.36 ± 0.12	12.43 ± 0.34	16.22 ± 0.02	
L. acidophilus	9.00 ± 0.8	9.8 ± 1.4	9.25 ± 0.08	12.11 ± 0.55	16.99 ± 0.32	
C. albicans	7.00 ± 1.44	7.77 ± 3.62	8.19 ± 0.04	11.99 ± 0.35		15.75 ± 0.16

Table 2. Effect of TP-1 and TP-3 on *S. mutans* using spot-plating assay.

TPs	[TP] μg/mL							
	3.9	**7.8**	**15.6**	**31.3**	**62.5**	**125**	**250**	**500**
TP-1	+++	+++	+++	+++	++	+	+	-
TP-3	+++	+++	+++	++	-	-	-	-

Note: +, presence of bacterial on the TSA; -, absence of bacterial on the TSA; +++, growth comparable to untreated, ++ and + represent reduction of bacterial growth compared to untreated.

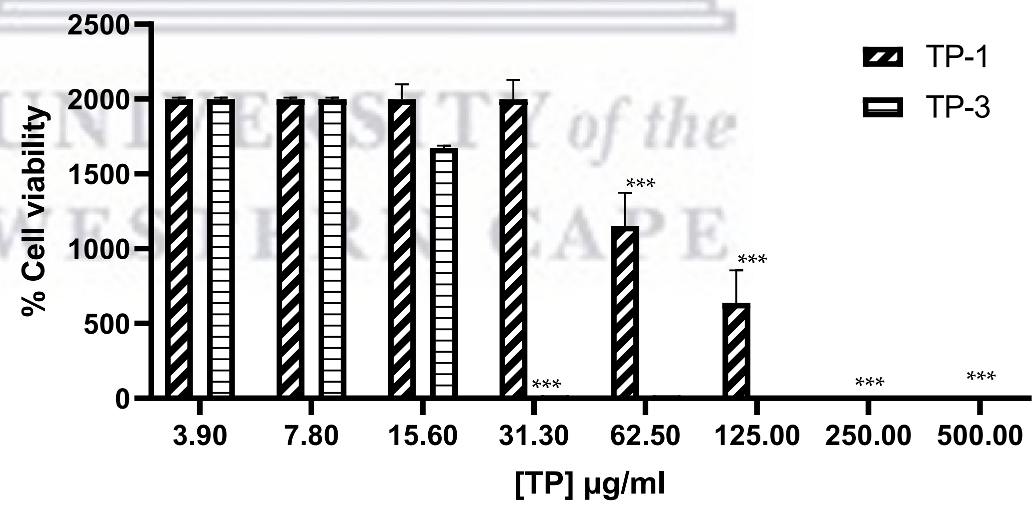

Figure 1. CFU count and MIC of TP-1 and TP-3 on *S. mutans*. $p \leq 0.05$ was considered as statistically significant, *** $p \leq 0.001$.

The synthesis and characterization of the GA-AgNPs_0.4g were previously reported; the NPs had a SPR of 425 nm, hydrodynamic size of 220 nm, with a core size range between 4 and 26 nm [18]. The formulation of the TP-1 with GA-AgNPs_0.4g yielded GA-AgNPs_TP-1, which was thereafter tested for antimicrobial activity to determine whether the addition of GA-AgNPs_0.4g to TP-1 will alter its activity. The effect of the GA-AgNPs_TP-1 on all microbes was significantly different (Table 3) compared to TP-1 alone, which had no activity at the test concentration (31.3 μg/mL). Furthermore, the effects of both GA-AgNPs_0.4g and GA-AgNPs_TP-1 on all selected microbes demonstrated

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a concentration-dependent response, with the highest antimicrobial activity at higher concentrations (50 and 100 µg/mL). The subsequent lower concentrations tested (6.25 and 12.5 µg/mL) demonstrated no activity on all microbes tested.

Table 3. Antimicrobial activity of GA-AgNPs_0.4g and GA-AgNPs_TP-1 measured as ZOI against selected microbes.

Microbes	[AgNPs] (µg/mL) Added in TP-1	GA-AgNPs_0.4g (mm)	GA-AgNPs_TP-1 (mm)	0.2% CHX (mm)	Nystatin (mm)
S. sanguinis	100	9.11 ± 0.14	9.17 ± 0.20	17.29 ± 0.36	
	50	8.95 ± 0.32	9.06 ± 0.08		
	25	8.25 ± 0.25	7.17 ± 0.18		
S. mutans	100	9.80 ± 0.19	9.56 ± 0.39	16.17 ± 0.16	
	50	9.87 ± 0.17	9.96 ± 0.20		
	25	7.19 ± 0.16	6.14 ± 0.14		
L. acidophilus	100	10.04 ± 0.18	10.23 ± 0.23	17.00 ± 0.39	
	50	9.09 ± 0.09	9.10 ± 0.09		
	25	7.22 ± 0.31	7.41 ± 0.34		
C. albicans	100	10.28 ± 0.18	10.33 ± 0.20		15.10 ± 0.15
	50	9.46 ± 0.22	9.71 ± 0.17		
	25	7.12 ± 0.13	7.31 ± 0.18		

It must also be noted that GA-AgNPs_0.4g and GA-AgNPs_TP-1 had comparable activities that were not statistically different from each other. This indicated that the activity of GA-AgNPs_0.4g was not affected by adding them to TP-1. AgNPs are highly reactive and might interact with the components of the TP and become inactive. The similarity in the antimicrobial activities of the two treatments suggested that GA-AgNPs can be incorporated in dentifrices and still retain its bioactivity. The GA-AgNPs_0.4g under study showed potential to serve as an antimicrobial agent against the four oral microbes, and prevented adhesion of *S. mutans* on human tooth enamels [18]. Several independent studies also highlighted the potential of AgNPs as alternative anti-caries agents when used in the formulation of dentifrices. Nanosilver (TruCareNanosilver) TP, composed of dicalcium phosphate dihydrate, sorbitol, hydrated silica, sodium lauryl sulphate, colloidal silver, menthol, carboxy methyl cellulose, sodium saccharin, flavor, and sodium benzoate compared to fluoride and chitosan TPs was more effective on *S. mutans*. The study indicated that nanosilver TP had the highest antibacterial activity, with ZOI of 20.14 ± 0.96 mm, followed by fluoride TP (16.01 ± 2.68 mm) and chitosan TP (10.84 ± 0.27 mm) [4]. In the literature, there was little evidence on applications of AgNPs as antimicrobial additives in TPs, although there were a significant amount of studies reporting on their enhanced antimicrobial activity using both chemical and plant extract-based AgNPs [9,56]. In a study by Adelere et al., they evaluated the antimicrobial activity of biogenic AgNPs synthesized using an aqueous extract of *Anacardium occidentale* stem bark and incorporated it into a commercial TP. They tested its antibacterial and antifungal activities against *E. coli* and *C. albicans*, respectively, and found that the AgNPs-based TP inhibited the growth of the microbes, while treatments without AgNPs failed to inhibit microbial growth. This demonstrated that the activity was due to the AgNPs added on the TP [22].

The MICs for GA-AgNPs_0.4g and GA-AgNPs_TP-1 were assessed on the four microbes in order to determine the inhibitory concentration that will be non-toxic to dental cells or tissues but still effective in inhibiting bacterial growth. Table 4 shows the response of *S. mutans* to the treatments. A similar response was also observed for the other three microbes (data summarized in Table 5). The spot-plating assay revealed a gradient-based reduction effect for the two treatments (GA-AgNPs_0.4g and GA-AgNPs_TP-1) on the selected oral microbes. Microbial growth following exposure to the two treatments was observed at ≤12.5 µg/mL in *S. sanguinis*, *S. mutans*, and *C. albicans*, and ≤25 µg/mL

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for *L. acidophilus*. TP-1 had no inhibitory effects on the growth of the microbes at the test concentration.

Table 4. Effect of GA-AgNPs_0.4g and GA-AgNPs_TP-1 using spot-plating assay.

TP Formulations	[Formulation] μg/mL				
	6.25	12.5	25	50	100
GA-AgNPs_0.4g	+++	++	-	-	-
GA-AgNPs_TP-1	+++	++	-	-	-

Note: +, presence of bacterial on the TSA; -, absence of bacterial on the TSA; +++, growth com-parable to untreated and TP-1, ++ represent reduction of bacterial growth compared to untreated.

Table 5. MIC_{50} for GA-AgNPs_0.4g and GA-AgNPs_TP-1.

Microbes	MIC_{50} (μg/mL)	
	GA-AgNPs_0.4g	GA-AgNPs_TP-1
S. sanguinis	10.09	15.66
S. mutans	12.26	11.89
L. acidophilus	7.12	20.89
C. albicans	10.47	12.67

The MIC_{50} on all microorganisms are presented in Table 5. There were no statistically significant differences between GA-AgNPs_0.4g and GA-AgNPs_TP-1 on individual microbes with reference to their MIC_{50}. The MIC_{50} values were slightly higher for GA-AgNPs_TP-1 compared to GA-AgNPs_0.4g. The activity of GA-AgNPs_TP-1 was attributed to the presence of GA-AgNPs_0.4g, as TP-1 was shown in Figure 1 to have no effect on the microbes at the concentration used. The above results suggested that the GA-AgNPs_TP-1 might have promising antimicrobial activity against oral microbes. However, concerns still remained as to whether the GA-AgNPs_TP-1 will have any adverse effects on the oral mucosa. This concern was addressed by testing the effect of GA-AgNPs_TP-1 on BMF cells.

There was a significant reduction in the number of viable cells after 24 h of treatment with GA-AgNPs_0.4g and GA-AgNPs_TP-1 at all tested concentrations (Figure 2). An interesting finding was that the effect of GA-AgNPs_TP-1 was lower compared to GA-AgNPs_0.4g, suggesting that formulating GA-AgNPs might aid in reducing cytotoxicity of the AgNPs while retaining its antimicrobial activity. This is based on the fact that a reduction in cell viability was also observed with TP-1 alone, which had 72% of viable cells at 31.3 μg/mL. However, it should be noted that the GA-AgNPs_TP-1 had a white deposit which could have influenced the opacity and the results of the assay. Therefore, the TCM was used in parallel with the GA-AgNPs_TP-1 to assess if there were discrepancies with the cytotoxicity assay results. There were no statistically significant differences between the bioactivity of cells treated with GA-AgNPs_TP-1 and TCM at all concentrations tested (Figure 3). Therefore, GA-AgNPs_TP-1 formulation was used for further testing as it had been used for all antimicrobial assays.

Due to the cytotoxicity portrayed by the GA-AgNPs_TP-1 on BMF cells, a further cytotoxicity assessment was performed to determine a reference (equivalence) point or the lowest concentration where the treatments show least toxicity to BMF cells but still retain antimicrobial activity. Thus, the doses of GA-AgNPs_0.4g and GA-AgNPs_TP-1 were benchmarked at the lowest concentration that showed antimicrobial activity on the microbes, but had little (~ 80% of viable cells) or no observable adverse effects on the cells. It is desirable that the reference concentration, while active against microorganisms, must be biocompatible and not induce any cellular damage or health risks to humans [23]. In clinical applications, humans are likely to come into contact with the AgNPs through various routes, including oral and skin contact; hence, their biocompatibility must be

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validated [57]. The analysis of variance for all microbes and BMF cells revealed significant differences, as shown in Figure 4. Treatment with GA-AgNPs_TP-1 demonstrated dose and time dependent effects on the microorganisms (*S. mutans* was used as a representative) and the BMF cells. Both microbial and cell growth declined drastically over this time span. The desired concentration was the one that yielded at least ≥80% BMF cell viability; however, this point occurred much earlier, before 5 min, while all microbes were still at their respective stationary phases. The point of equivalence between the cells and microbes-exposed GA-AgNPs_TP-1 is indicated by an arrow on the graphs (Figure 4), which occurred within the first 30 min for all concentrations of the GA-AgNPs_TP-1; at this point, the cell viability was at 50%. The non-selective effects of GA-AgNPs_0.4g were reported previously [51]: although these effects can be somehow masked in TP formulation (Figure 2), their toxicity still persists. This is quite unexpected since the use of plant-based AgNPs was expected to prevent cytotoxicity associated with chemically-synthesized AgNPs [3,13].

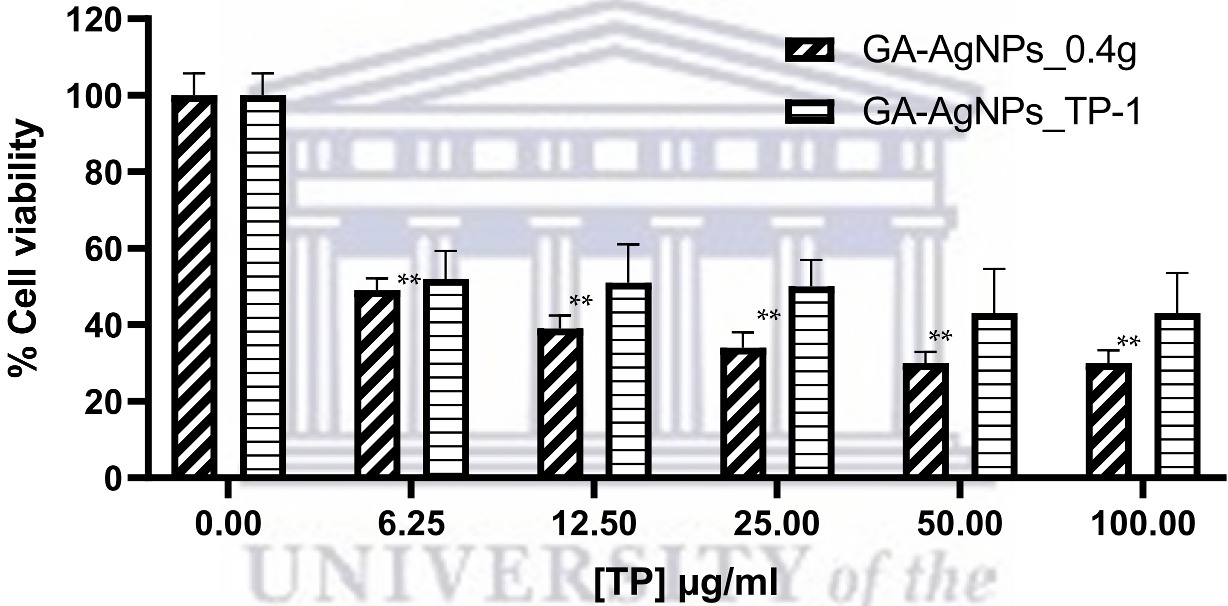

Figure 2. Cytotoxicity effects of GA-AgNPs_0.4g and GA-AgNPs_TP-1 on BMF cells. Cell viability was assessed by MTT assay after treatment for 24 h. $p \leq 0.05$ was considered as statistically significant, ** $p \leq 0.01$.

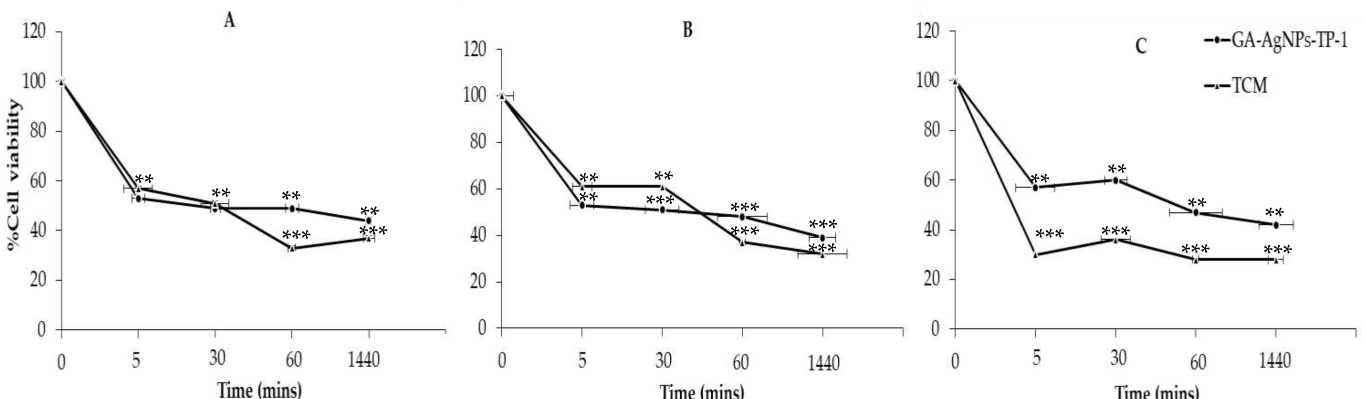

Figure 3. Cytotoxicity effects of GA-AgNPs_TP-1 compared to TCM on BMF cells. Cell viability was assessed by MTT assay after treatment with 25 (**A**), 50 (**B**) and 100 (**C**) µg/mL for various time points up to 24 h. $p \leq 0.05$ was considered as statistically significant, ** $p \leq 0.01$ and *** $p \leq 0.001$.

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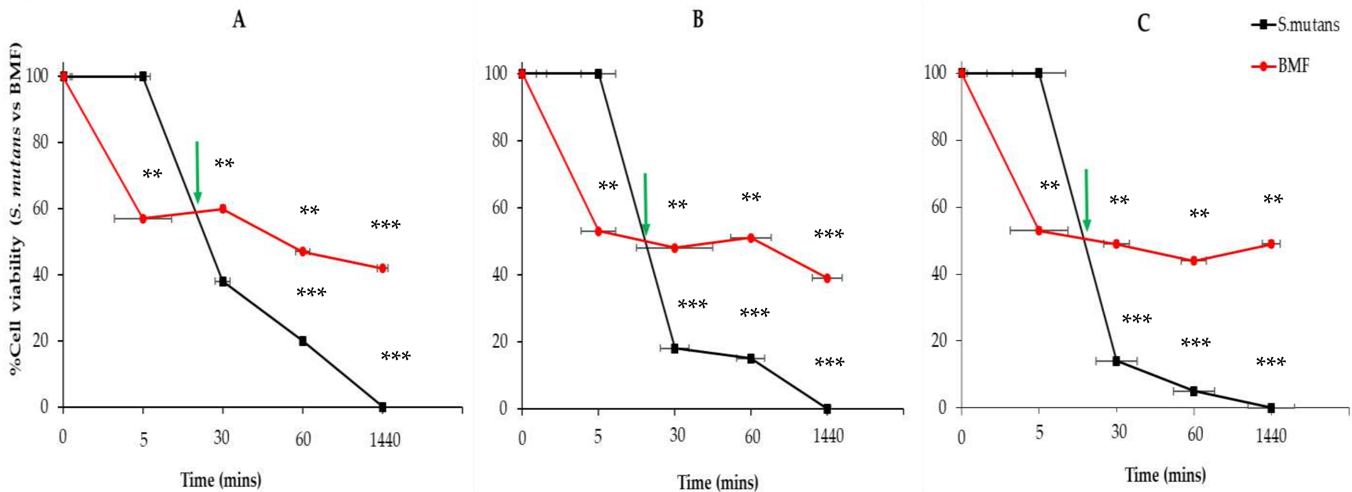

Figure 4. Comparison of the survival of *S. mutans* versus BMF cells after exposure to GA-AgNPs_TP-1. *S. mutans* and BMF cells were treated with 25 (**A**), 50 (**B**) and 100 (**C**) µg/mL for various time points up to 24 h. Viability was evaluated by MTT assay. The green arrow points at an equivalence point of the GA-AgNPs_TP-1. $p \leq 0.05$ was considered as statistically significant, ** $p \leq 0.01$ and *** $p \leq 0.001$.

AgNPs toxicity is widely reported in the literature [41,58–60], from ingested to cosmetic products, including TPs [16,42]. Their cytotoxicity appears to be time and concentration dependent [43], which was also demonstrated by the current study. Of interest was that the addition of GA-AgNPs_0.4g to TP-1 slightly reduced the cytotoxicity of the GA-AgNPs_0.4g. This effect might be further improved by using other formulations without any additives; of note, TP-1 used in this formulation is a fluoride-based TP and AgNPs are known to be highly reactive and capable of interacting with media components. In this case, it is possible that fluoride can interact with the AgNPs [61]. Nonetheless, the known or common practice of maintaining good oral hygiene is the use of dentifrices for two minutes and rinsing it off [62]. It could therefore be speculated that the GA-AgNPs_TP-1 can be used within the first two minutes of brushing and rinsed without any adverse effects.

4. Conclusions

Green synthesis of AgNPs for use in dentifrices is an economical, eco-friendly and promising approach in combating antimicrobial resistance in oral microbes, which will ultimately cause oral diseases. In the current study, GA-AgNPs_0.4g were incorporated into commercial TP at an inactive concentration and demonstrated antimicrobial activity against oral microbes. The antimicrobial activity of GA-AgNPs_0.4g was not altered after being combined with TP-1; the GA-AgNPs_0.4g-TP-1 inhibited the growth of the microbes between 5 min and 24 h of exposure. Although GA-AgNPs_0.4g -TP-1 and GA-AgNPs_0.4g were also shown to be cytotoxic to the oral mucosa-representative (BMF) cells at the same concentrations and the same time intervals that were lethal to the microbes, they can still be used in dentifrices. This is because dentifrices are only used for a short time, are not swallowed, and are rinsed off after a minute of use. Similarly, the GA-AgNPs-based dentifrices can be applied with such cautions. GA-AgNPs_TP-1 has promising prospects for use in dental care to control pathogenic oral microbes, and concerns about its cytotoxicity can be masked or reduced with the addition of biopolymers such as chitosan and poly(ethylene glycol) before its clinical application [63]. Of more interest in this study will be the use of chitosan, which also has antimicrobial activities [4,5]. Therefore, more rigorous studies need to be carried out on oral biofilms and capping off cytotoxicity of GA-AgNPs-based dentifrices. In addition, the phytochemicals involved in the synthesis and capping of the GA-AgNPs_0.4g need to be identified to elucidate the potency, stability, and toxicity of the AgNPs.

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2.4.c. Peer review

Reviewer 1:

Reviewer Comments	Responses
1. Section Results and Discussion: "This was further confirmed in Figure 1C, the MIC50 for TP-1 and TP-3 on S. mutans were at 250 µg/ml and 31.3 µg/ml, respectively. Thus, TP-1 at 31.3 µg/ml was selected for further studies and used in combination with the GA-AgNPs_0.4g for GA-AgNPs-based TP formulation." It is difficult to accept this statement because Figure 1C is only cited in the text but not presented n figure 1.	Changed
1. Some language errors were detected, e.g. :" The similarity in the ZOIs for the two treatments suggested that GA-AgNPs can be used in dentifrices without losing its bioactivity." (lines 295-297) or "Although the GA-AgNPs-TP-1 and GA-AgNPs_0.4g were also shown to be cytotoxicity to the oral mucosa representative (BMF) cells" (lines 404-405)	Changed

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Reviewer 2:

Reviewer Comments	Responses
In abbreviation ´GA-AgNPs_0.4g´, remove 0.4g through the text	This is a continues study with other manuscript therefore, we wish to keep the abbreviation as it talks to our previous studies done with these nanoparticles.
In a table, provide composition of all the ingredients of toothpaste used in this study and composition of Gum Arabic (powder natural form) if authors know from the published literature, and discuss context of their composition in results outcome.	Because there will be conflict of interest, we decided not to reveal the ingredients of the toothpaste. However, the identity of the toothpaste maybe available to any researcher who wants to replicate this study. Composition of Gum Arabic

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Line 52-55 with references 9 which is a review article, cite a research paper with https://doi.org/10.1371/journal.pone.0175428 which demonstrated how nanomaterial are able to interact with cellular membrane causing anti-microbial activities.	Changed
In **Materials and method**, please add catalogue number supplier, country/region of all chemicals/reagents (e.g. Cat # missing *Acacia senegal* (North Kordofan, Sudan), ATCC or acquisition number of all bacterial species procured in this study, kit/assay catalogue no, supplier, instrument with name details, model number, version etc. This is mandatory as COEP guidelines to add full details of material to replicate the experiment independently in any lab who wish to cross verify or reproduce the results	All the information was added.
In section *2.3. Cytotoxicity Assay,* it is unclear whether authors performed assay on bacterial cells or human cell lines/primary cells? If human origin cells were used, please provide details of cells and media reagents.	Changed and added.
Can authors present schematically the steps in protocol step by step to comprehend better, for example, showing schematically microdilution steps and GA-AgNPs_TP-1 synthesis steps will add better understanding.	The protocol will be made available for anyone who wants to replicate the study upon request.
Provide which version of QI Macros 2022 was used for the Statistical Analysis?	Version provided
What is the unit for those numbers shown in Table 1, for ZOI for commercial TP1-4 against oral microbes (mm, cm, mm^2 cm^2)?	Changed and corrected.
Line 202-205, it will be better cite https://doi.org/10.3390/ijms22136866 as reference for the viability assay quantification to refer the readers.	Changed and added.
Figure 1-2, in pteridisches, those Arabic numbers showing treatments are jumbled in	Changed and explained in more details.

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some colony shown. For examples in fig.2, concentration 6.25 µg/ml are jumbled out and make it difficult to interpret. I-IV: are concentration of the treatments compared to V-VI which are the controls.	
Provide a list of all abbreviations used in this manuscript as some of them are used without padded full forms, which makes it hard to follow.	All abbreviations are added in front of each.
In addition, I find discussion of results have a weak connection in the text. For wexmaples, authros emphasized the value of green synthesized silver but they also consider the toxciicty of unknown plant based products which are used in the synthesis? Further how gum Arabic would make sense to develop such formulation for dental toothpaste, how the different alkaloids (composition) principally will affect suitability of such paste in view of gingiva health is unclear and needs to be discussed.	Added in the conclusion.

Reviewer 3:

Reviewer Comments	Responses
Abstract: lines 17-18, The Authors write that AgNPs are biocompatible. Really, Silver nanoparticles show citotoxicity	Some literature reported that AgNPs are biocompatible (Plant extract synthesized silver nanoparticles: An ongoing source of novel biocompatible materials, The antibacterial mechanism of silver nanoparticles and its application in dentistry), however, in this study are found that AgNP showed cytotoxicity.
Lines 18-19: "In the present study, gum arabic AgNPs (GA-AgNPs_0.4g) were added into a commercial toothpaste (TP) with sub-lethal antimicrobial activity to produce GA-AgNPs_TP" what's the meaning of sublethal? What does the Authors want to say? It should be explained.	Changed and explained.
The aim of the paper is not comparing the antimicrobial activity of NPs with the formulation. This are to distinct phases. Te NPs are active, the MIC is determined and then a formulation is made and	Changed and revised.

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antimicrobial activity is measured. This should be revised	
Lines 46-47: "sodium lauryl sulfate, an anionic surfactant which is used as foaming agent for texture" sodium lauryl sulfate does not act as a texture agent. It is a foaming surfactant with detergent action. Please find other references which should be added.	Changed and other references were added.
Lines 51-54: "There is a growing interest for the use of natural products to combat microbial infections [8]. Studies have shown that the activity of these compounds was enhanced when used as reducing agents in the synthesis of nanoparticles (NPs) due to their small size that permits them to interact and penetrate cellular barriers [9]". These two sentences is not clear and there is a lot of confusion. Having a reducing activity and being able to combat microbial infections are two different properties. Some reducing natural agents are used to induce silver reduction and metallic nanoparticles formation. Maybe that this is what the Author want to say?. It is not clear, and this part should be improved and better explained.	Improved and explained better.
Lines 60-61: some references on antimicrobial activity of nanoparticles should be reported. Which is peculiar of nanoparticles? References 14 and 15 are not enough. Other references should be reported describing antimicrobial activity of silver nanoparticles	References were added.
Lines 77-78: there is still much scientific debate about what size and shape are best to have antimicrobial activity. So the authors should be less categorical and in the introduction this sentence is not relevant and should be deleted	Changed.
Lines 83-84: is "True Care 83 Nanosilver" the correct name? Please check and revise	Corrected.
Lines 91-92: this sentence is meaningless: the cytotoxic profile and antimicrobial activity are two different profiles of silver nanoparticles which have to be further studied.	Changed.
Lines 94-96: silver nanoparticles are not used for oral administration. Silver is highly toxic and is a poison. Its use is only for local administration. In fact the reference 34 is	Changed.

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about silver toxicity due to ingestion of silver nanoparticles due to exposure for consumers to silver present in industrial product such as food. It is not the administration of silver which, in pharmaceutical field, is related to its systemic use. It should be revised. The cytotoxicity towards gengival mucosa is a local action and not an oral administration which the administration of silver using the oral route as a route of administration.	
Paragraphs 2.2.1. and 2.2.2. cannot be a part of 2.2. Antimicrobial activity of the GA-AgNPs_TP.	Changed.
Line 155: how GA powder was obtained?	Commercial GA powder was obtained from North Kordofan Sudan
Silver nanoparticles characterization: by ultraviolet-160 visible (UV-Vis) spectrophotometer, dynamic light scattering (DLS), Transmission Elec-161 tron Microscopy (TEM), and Fourier-Transform Infrared Spectroscopy (FTIR). Which is the instrumentation? Which are the results. All these are missing.	The instrumentation and the results are cited in another paper by Ahmed et al 2022.
Line 164: how were they mixed? In which way?	Explained.
Lines 126-127 and 172: The agar diffusion tests were performed in a different way for commercial toothpastes and for AgNP added toothpaste. In fact in the case of AgNPs toothpaste, the infused paper filters were overnight dried. Why this different treatment?	Changed and corrected.
Which is the origin of BMF cells?	Added.
Line 234: -carvacroll is not correctly written - what does it mean thymol and carvacrol between brackets? Are they used as antimicrobials or other function? The sentence should be written again.	Corrected.
There are no proofs that silver nanoparticles maintain their stability in the toothpaste. Chemical-physical characterization is needed to confirm their presence in the final toothpaste. Did the toothpaste changes color during storage?	This part will be investigated in subsequent studies.
Moreover a toothpaste containing a different form of silver such as $AgNO_3$ or other form should be used for comparison as concerns antimicrobial and cytotoxic activies. Maybe that activity of this refering toothpaste could	This paper is still in the development stage, and further study will include comparison with AgNPs toothpaste. Advantages of toothpaste has been addressed in the paper.

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be the same of those containing silver nanoparticles. Which are the advantages of using silver nanoparticles. The advantage of using silver nanoparticles should be explained or confirmed.	
Line 252: The differences in the activities of TP compared to each other and the positive control were not statistically significant. This sentence is not clear. Does it mean that there were no statistical differences in activities in comparison to the positive control? The ZOI of positive controls are very higher. As concerns ZOI against Candida: 7.00 ± 10.44 for TP-1 and 7.77 ± 13.09 for TP-2. Is it ZOI ± SD? Is it correct? The SD is higher than the ZOI?	Changed and corrected.
Lines 316-318: The MICs for GA-AgNPs_0.4g and GA-AgNPs_TP-1 were assessed on the four microbes in order to determine the inhibitory concentration that will be non-toxic to dental cells or tissues but yet effective in inhibiting bacterial growth. This sentence is meaningfull: the MICs were evaluated to know the antimicrobial activity, then, in the following experients, cytotoxicity was evaluated. Before performing the cytotoxicity tests , it is not possible to know which is the non toxic concentration	Changed and corrected.
Cell viability with TP-1 alone should be reported in the Figure 3	Corrected and reported
Line 403, "GA-AgNPs_0.4g augmented the bioactivity of TP-1 at inactive concentration": what is the meaning?	Changed.
Lines 410-411 "the concerns about their cytotoxicity can be addressed by addition of biopolymers before its clinical application": what does it mean? Why the biopolymer addition? When has been demonstrated that the addition of a biopolymer has effects on cytotoxicity?	It has been addressed in other papers (Broad Spectrum Anti-Bacterial Activity and Non-Selective Toxicity of Gum Arabic Silver Nanoparticles)

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Chapter 3: Conclusions

3.1 Executive summary

This chapter provides concluding remarks, proposes suggestions for further investigations (recommendations), and limitations of the study. The GA-AgNPs_0.4g can be incorporated into a toothpaste making it effective. However, further study needs to be done on cytotoxicity and capping toxicity thereof.

3.2 Conclusions

The main aim of this project was to work towards the development of toothpaste containing green synthesized AgNPs-based toothpaste. The purpose of this toothpaste was to enhance control of oral flora without completely eradicating essential microorganisms. Therefore, from the inception of the nanoparticles through to their addition to the toothpaste, antimicrobial assays were conducted in parallel with cytotoxicity tests predominantly on buccal mucosa cells.

The following is a summary of this project's main findings:

- The literature reviews revealed few studies related to topical application of AgNPs, where 3 studies used mouthwashes and only one study used toothpaste. All these studies demonstrated that AgNPs has antimicrobial effects against oral microbes tested, which are comparable to the standard antimicrobial agents used. It was also found that there exists a high level of heterogeneity among study methods and that there is a need for standardization of protocols with respect to organisms used and the concentration of the nanoparticles along with the comparators due to the diversity in the outcomes.

- In the literature, the use of chemically produced AgNPs is fraught with difficulties and issues, which spurs the creation of more affordable and environmentally friendly processes for AgNP synthesis. Utilizing natural and renewable resources, plant-mediated AgNP synthesis is an economical and environmentally responsible method for creating novel, bioactive, and biocompatible AgNP-based products for human use. Therefore, the biogenic synthesis of AgNPs may be used to manage oral flora.

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- Given the results from the literature about the plant-mediated synthesis of AgNPs, the aim was to synthesis AgNPs from a plant extract (gum Arabic) which yielded GA-AgNPs. In contrast to chemically manufactured AgNPs with NaBH4 (C-AgNPs) and the combination of NaBH4 and GA AgNPs (GAC-AgNPs), the GA-AgNPs showed better anti-bacterial activity on both Gram positive and negative bacteria, but with increased toxicity across all cell lines examined. Two fractions of GA-AgNPs (0.4g/0.1g) were further described and tested against a variety of oral bacteria because of the enhanced broad spectrum antibiotic activity of GA-AgNPs. When tested against oral microorganisms, GA-AgNPs_0.4g and GA-AgNPs_0.1g both demonstrated antimicrobial activity, but GA-AgNPs_0.4g had a higher level of antibacterial activity. After that, GA-AgNPs_0.4g was chosen to test for long-lasting adhesion to the tooth enamel and showed antibiofilm formation capability on it.

- Afterwards, the cytotoxicity of both GA-AgNPs_0.4g/ GA-AgNPs_0.1g were tested against the buccal mucosa fibroblast cell line (BMF) and the KMST-6 normal skin fibroblast cells, as excellent at simulating the oral environment. The cytotoxicity was concentration dependent with GA-AgNPs_0.1g the least toxic. It was non-toxic at concentrations below 12.5 µg/ml to both cell lines with ±80% cell survival, while the GA-AgNPs_0.4g were toxic to the KMST-6 cells at all concentrations tested. GA-AgNPs_0.4g demonstrated reduced toxicity on BMF cells at concentrations ≤ 6.25 µg/ml with above 60% survival.

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- Subsequent to determining that GA-AgNPs_0.4g had the higher antimicrobial activity, it was tested whether it can augment the antimicrobial activity of a toothpaste. The GA-AgNPs_0.4g were added to a selected commercially available toothpaste with lowest antimicrobial activity. This combination was assayed for both antimicrobial and cytotoxicity potentials. Both antimicrobial and cytotoxicity activities were significantly increased, compared to those of the toothpaste in its commercial form.

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3.3 Recommendations and future research

- In recent research, the cytotoxicity of GA-AgNPs_0.4g should be masked by the addition of certain additives and further tested on different oral cell lines including epithelial cells to ensure the safety of GA-AgNPs_0.4g for oral application.
- Synthesis of standardized toothpaste with masked GA-AgNPs_0.4g (GA-AgNPs_0.4-TP).
- Testing the antimicrobial activity of GA-AgNPs_0.4g-TP against composite biofilm of the oral cavity simulating the oral environment.
- Evaluating the cariogenic inhibiting potential of the GA-AgNPs_0.4g-TP by assessing features such as acidity, texture, viscosity, stability, foamability and spreadability, longevity (shelf life) before making it available to the market.
- Determining the remineralization and staining potential of GA-AgNPs_0.4g-TP.
- Use of animal studies to evaluate dose dependent effect of GA-AgNPs_0.4g-TP.
- A limited pilot study on humans evaluating the recolonization on tooth enamel, antimicrobial activity, and cytotoxicity after the use of GA-AgNPs_0.4g-TP.
- A larger clinical trial may be required to confirm the findings of this investigation.
- Commercial launch of the GA-AgNP_0.4g-TP if all the above parameters are met.

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3.4 Limitations of the study

- The search strategy for the first literature review was broad, hence yielded diverse results. The search strategy can be narrowed to allow a review to be done with more potential of producing stronger evidence within a more focused approach.
- Cytotoxicity of GA-AgNPs was not resolved because conventional toothpaste was used, and it could not be altered (This limitation will be addressed in subsequent research).
- The pH effect of the GA-AgNPs on the toothpaste was not evaluated.
- The studies were limited to single species biofilm and were not extended to composite oral biofilm.
- The studies were limited to the use of fibroblast cells due to their availability in the laboratory where the study was performed.
- Time and financial constraints limited rigorous testing and scalability of the project.

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