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UNIVERSITY OF MEDICINE AND PHARMACY "CAROL DAVILA"-BUCHAREST

DOCTORAL SCHOOL

PHARMACY FIELD

PHYTOSYNTHESIS OF SOME SILVER NANOPARTICLES WITH POTENTIAL APPLICATION IN PERIODONTOPATHIES

Summary of PhD Thesis

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List of abbreviations

AgNP	silver nanoparticles
ADN	deoxyribonucleic acid
Ag+	silver ions
AgNO3	silver nitrate
CAL	*clinical attachment loss*
CaO	calcium oxide
CHX	encapsulated chlorhexidine
Cs	Camelliaa sinensis
CuO	copper oxide
DMEM	*Dulbecco's Modified Eagle's Medium*
Dp	average crystal size
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPH	test for the estimation of antioxidant activity
EDX	energy dispersion
FBS	fetal bovine serum
FDA	*U.S. Food and drug administration*
FTIR	Fourier transform infrared spectroscopy
GAE	gallic acid
GTR	*guided tissue regeneration*
HFIB-G	fibroblast cell line
Hi	Heterotheca imuloides
HM	microwave assisted extraction of Hyssopus officinalis
HPLC	high-performance liquid chromatography
HPLC-DAD	high-precision liquid chromatography equipped with diode arrays
HPMC	hydroxypropyl methylcellulose
HR-TEM	high resolution transmission electron microscopy
HT	classic Hyssopus officinalis extraction
ICDD	*The International Centre for Diffraction Data*
LDD	*local drug delivery*
LDH	lactate dehydrogenase
LDL	low density lipoproteins
MAE	microwave assisted extraction
MgO	magnesium oxide
MM	microwave-assisted extraction of Marrubium vulgare
MNP	magnetic nanoparticles
MT	classic Marrubium vulgare extraction
MTT	viability test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaBH4	sodium borohydride
NAD+	*nicotinamide adenine dinucleotie*
ND	not detected

NO	nitric oxide
NO2	nitrite
NP	nanoparticles
OFD	*open flap debridement*
OM	microwave assisted extraction of Origanum vulgare
OMS	World Health Organization
OT	classic extract of Origanum vulgare
PCL	Polycaprolactone
PGLA	polyglycolide-co-lactide
PHBA	hydroxybutyric acid
PLGA	polylactodecoglycolide
PRP	*platelet rich plasma*
RB	rice bran
RG	rice sprouts
RH	rice husks
ROS	reactive oxygen species
SAED	selected area electron diffraction
SD	the standard deviation of the mean
SDD	Subantimicrobials
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SH	sulfhydryl group
Si	Silicon
TBARS	substances reactive to thiobarbituric acid
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
THP-1	monocyte cell line
TiO2	titanium dioxide
TPA	12-O-Tetradecanoylphorbol-13-acetate
TPC	total content of phenolic compounds
UM	microwave-assisted extraction of Lamium album
UT	classic Lamium album extraction
UV	ultraviolet
XRD	X-ray diffraction
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ZnO	zinc oxide
ZnONP	zinc oxide nanoparticles

Introduction

Periodontal diseases are chronic inflammations that are associated with the accumulation of dental plaque (dental biofilm) and lead to progressive destruction of the periodontium. Periodontopathies are the result of complex dynamic interactions between bacterial pathogens, specific immunological factors and harmful environmental factors.

Given the increasing prevalence of these diseases in the population and the need to protect and restore periodontal health in order to maintain general well-being, the PhD studies focused on the development of therapeutic systems that play a functional role in the treatment of periodontal diseases.

Current drug treatments are based on the use of antimicrobial agents to eliminate pathogenic microorganisms on the one hand and on the use of anti-inflammatory agents to reduce the inflammatory response, i.e. a complex and complete correction of the oral environment that prevents the retention of bacterial plaque, on the other. However, their long-term administration leads to a number of side effects and is not recommended. Biocompatible alternative solutions that have a satisfactory therapeutic effect without negative consequences are currently being sought.

To ensure the success of the therapy, it is important to select the right antimicrobial agents and to use an effective delivery method or appropriate drug delivery systems.

Compared to conventional antibiotics, silver nanoparticles have antibacterial properties without creating bacterial resistance.

Due to their low toxicity, photothermal capabilities, immunotherapy, anti-inflammatory effects, antibacterial properties, simple manufacturing techniques and low cost, multifunctional metal nanoparticles with therapeutic activities are very promising in the field of periodontal disease treatment. Based on these properties, silver nanoparticles were selected as therapeutic systems in the present study.

Silver nanoparticles mediated by plant extracts offer a simple method for *in situ* synthesis and at the same time are very stable as they act as both reducing and stabilizing agents.

The herbal products were selected on the basis of the numerous data available in the literature on the antimicrobial, antioxidant and anti-inflammatory effects of the aerial parts of white horehound (*Marrubium vulgare* L.), hyssop (*Hyssopus officinalis* L.), oregano (*Origanum vulgare* L.) and nettle (*Lamium album* L.).

The main objective of the doctoral thesis was therefore the phytosynthesis of AgNPs with antimicrobial activity against oral pathogens, which form the basis for the development of

multifunctional products that can contribute to the prevention and treatment of dental infections and diseases and ensure less invasive therapy.

I. GENERAL PART

1. Current concepts regarding the treatment of periodontal diseases

1.1. General aspects regarding the therapy of periodontopathies

Periodontopathies are known to be multifactorial diseases involving both the presence of bacterial biofilms and a complex inflammatory response, including the production of cytokines, eicosanoids and metalloproteinases. Gramme-negative bacteria in the biofilm trigger an inflammatory response in the host that can lead to progressive, sometimes irreversible, destruction of soft and/or bone tissue [1-3].

Surgical therapy includes techniques such as Open Flap Debridement (OFD) or Fuided Tissue Regeneration (GTR; platelet-rich plasma PRP therapy) [2,3].

Non-surgical therapy consists of a series of procedures and interventions aimed at controlling the infection and preventing the progression of periodontal disease, including:

1. Scaling - may include the scaling procedure, which removes bacterial plaque and calculus from the surface of the teeth and below the gum line, and the root planing procedure, which smooths the roots of the teeth to remove bacterial toxins and create a clean and smooth surface that encourages the reattachment of healthy gums to the tooth.
2. Antibiotic and antimicrobial treatment – both systemic: oral administration or injection of antibiotics to fight infection; and topical: direct application of antibiotics or antimicrobial agents in the periodontal pockets to reduce certain bacteria, as described below.
3. Rigorous personal oral hygiene: proper brushing and flossing are essential to prevent plaque formation, as is the use of antiseptic agents such as antiseptic mouthwashes to reduce local bacterial load.
4. Treatment of risk factors: smoking cessation, as smoking is known to be a major risk factor for periodontal disease; control of blood glucose levels, as uncontrolled diabetes can exacerbate periodontal disease [4].

1.2. Systemic antibiotic therapy in the standard treatment of periodontopathies

In the non-surgical treatment of chronic parodontopathy and the prevention of severe complications, metronidazole is frequently used as a standard antibiotic either alone or in combination with other antibiotics such as amoxicillin, or amoxicillin with clavulanic acid or ciprofloxacin.

1.3. Local antibiotic therapy as the non-invasive option of first choice. Adjuvant topical therapies

The main cause of infections in periodontal diseases is the periodontal pockets, which is why the direct application of drugs in these areas has proven to be more effective than systemic treatments, or complementary to them, also reducing the complications associated with oral administration. To ensure the success of the therapy, it is essential to select the correct antimicrobial agents and use an effective method of administration, appropriate release systems of medicinal substances [2].

1.4. Modern systems of local delivery of active substances in periodontal disease therapy. Multiparticulate systems. Metallic nanoparticles.

Silver nanoparticles synthesized by phytochemical methods, known as **phytosynthetic silver nanoparticles (AgNPs)**, are the subject of current research for their potential applications in various medical fields, including dentistry. In periodontitis, AgNPs offer significant therapeutic benefits due to their potent antibacterial properties (they unbalance bacterial cell membranes, penetrate cells and disrupt metabolic functions and bacterial DNA replication). In addition to their specific antibacterial activity, AgNPs also have their own anti-inflammatory properties, which are beneficial in periodontal therapy [5]. The synthesis of these AgNPs by phytosynthesis is ecological and sustainable, using plant extracts that act as reducing agents for the formation of nanoparticles and as stabilizers, improving the bioavailability and tolerability of the nanoparticles [6]. Mainly plant extracts with active ingredients with intrinsic antioxidant, but also anti-inflammatory, antimicrobial and antiseptic activity are selected, which are presented in the next chapter of the general theoretical part of the thesis and which provided the ideas for the studies that are the subject of the personal contributions of the thesis.

AgNPs can be incorporated into various dental products such as mouthwashes and toothpastes to provide targeted treatment and prevention of periodontal disease. They are also useful in implants and coatings of some dental devices to prevent microbial colonization and biofilm formation.

2. Phytotherapeutic approaches in the management of periodontopathies

2.1. The importance of phytotherapy

There is a range of evidence to support the concerns regarding the valorization of plants in various fields of activity.

Due to the diversity of chemical compounds they contain, medicinal plants are expected to contribute to the development of new innovative therapeutic strategies as well as new molecular models with local antimicrobial, anti-inflammatory and antinociceptive effects.

For the treatment of periodontal diseases, the use of certain plant extracts could be beneficial, which could have significant therapeutic effects in a suitable formulation. For the analysis, we selected plants from the Lamiaceae family that are recommended in the literature for their

antimicrobial, antioxidant, anti-inflammatory and antifungal effects.

2.2. Lamiaceae Family

One of the plant families with a wide range of biological and medicinal applications is the Lamiaceae family, and among the best known plants with multiple uses are spices such as mint, basil, sage, rosemary, hyssop and oregano [7]. Due to their biological properties, the species of the Lamiaceae family are used in the pharmaceutical industry and in traditional medicine. Medicinal uses include stimulating blood circulation, diuretic applications and strengthening the central nervous system [8-10].

Due to the essential oils of Lamiaceae species, the biological applications are numerous and include antioxidant, anti-inflammatory, analgesic, antimicrobial, antifungal, antipuriginous, neuroprotective, antiseptic, antiallergic, antihepatotoxic activities, etc. [11-14].

II. PERSONAL CONTRIBUTION

3. The working hypothesis and general objectives

3.1. The working hypotesis

The development of phytosynthetic metal nanoparticles has become one of the most productive areas of research in nanotechnology. The number of annual publications on this topic has increased exponentially. Phytosynthetic nanoparticles have several advantages over nanoparticles obtained by classical methods (including improved antioxidant or antimicrobial properties and lower toxicity) and are now considered viable alternatives to conventional metal nanoparticles. The green synthesis of these nanoparticles uses extracts from a variety of plants that act as both reducing and filming agents.

3.2. The general objectives of the study

Based on these observations, the research in this PhD thesis was aimed at the ***phytosynthesis of silver nanoparticles using various extracts of indigenous plants***.

Despite the wide range of plants explored for this application, a review of the main scientific databases (SCOPUS, Web of Knowledge) revealed no studies on the use of extracts from *Marrubium vulgare* L. for the phytosynthesis of silver nanoparticles. In this context, ***the novelty*** of the conducted research refers to the fact that the ***extract of the species Marrubium vulgare L.,*** which is used in the production of silver nanoparticles, ***has not been mentioned as a reducing agent for the phytosynthesis process until now***. We used different plant extracts for the phytosynthesis of silver nanoparticles to comparatively analyse their performance.

The general objectives of this research were:

1. Obtaining plant extracts

2. Phytosynthesis of silver nanoparticles

3. Characterization of plant extracts and experimental nanoparticles

4. Evaluation of the biological effect, biocompatibility and cytotoxicity of the experimental nanoparticles

4. General research methodology

4.1. Materials used

The plant material used in this study, represented by the aerial parts of **White Horehound (*Marrubium Vulgare* L.), Hyssop (*Hyssopus officinalis* L.), Oregano (*Origanum vulgare* L.)** and **Nettle (*Lamium album* L.)** species, was collected from certified seeds and purchased from the local market.

4.2. The obtaining of the natural extracts

Two extraction methods were used to obtain the natural extracts, the choice of which is justified by the ability to ensure both the efficient extraction of the active ingredients and the possibility of scaling up for industrial applications, according to the literature [15].

- the classical method, which consists of mixing the plant material with the solvent and keeping this system at a certain temperature for at least 1 hour. In our case, the previously crushed plant product was subjected to a classical extraction at a temperature of 70ºC, using as solvent an alcoholic water mixture in a volumetric ratio of ethanol:water = 1:1; the Memmert UN 110 oven was used for the extraction, with an extraction time of 3 hours.
- the microwave-assisted method, in which the plant material and solvent were heated at a temperature of 70°C for 30 minutes using an Ethos Easy Advanced Microwave Digestion System.

In both methods, a hydroalcoholic mixture (ethanol:water 1:1 v/v) was used as solvent, and the ratio of plant material to solvent was kept at 1:10 (w/v).

After the extracts were collected and filtered, they were reduced using a Laborota 4000 Heildolph rotary evaporator to remove the volatile solvent by vacuum distillation. Once more than 90% of the solvent had been removed, the extract was finally dried by freeze-drying (using a Christ LSC Alpha 2-4-LSC freeze-dryer) in order to preserve the properties of the extract and extend its shelf life. The extracts dried in this way were stored in the freezer for further use.

For the production of silver nanoparticles, the extracts obtained were dissolved in water again. In phytosynthesis, equal amounts of natural extract and silver nitrate solution ($AgNO_3$, 10-3 M) are mixed, which is obtained by dissolving the corresponding amount of $AgNO_3$ (Chimreactiv,

Romania) in double distilled water. The double distilled water used for all experiments was prepared in the laboratory using a GFL 2102 distiller.

The extracts used were coded according to Table 1.

Table 1: Coding of the extracts obtained using as solvent the alcoholic mixture (according to the ratio shown above), with a plant material/solvent ratio (g/ml) of 1/10.

No.	Plant	Extraction method and codification			
1	Hyssop (*Hyssopus officinalis* L.)	Classical extraction	HT	Microwave-assisted extraction	HM
2	Oregano (*Origanum vulgare* L.)	Classical extraction	OT	Microwave-assisted extraction	OM
3	Nettle (*Lamium album* L.)	Classical extraction	UT	Microwave-assisted extraction	UM
4	White Horehound (*Marrubium vulgare* L.)	Classical extraction	MT	Microwave-assisted extraction	MM

4.3. Phytosynthesis of silver nanoparticles

For the phytosynthesis procedure, equal amounts of the prepared extract and silver nitrate solution (25 ml each) were mixed. This mixture was allowed to react for 24 hours at ambient conditions, with the final pH of the mixture being approximately 7, as determined using a Lab 870 pH meter (Analytic Laboratory). The reduction of Ag+ ions to silver nanoparticles (AgNPs) was achieved by adding the crude extracts to freshly prepared silver nitrate solution.

4.4. Characterization techniques

4.4.1. Determination of total phenol content (TPC)

Folin-Ciocâlteu reagent and sodium carbonate solution were purchased from Merck KGaA, Darmstadt, Germany. These reagents were used without further purification. Optical density was measured at 765 nm using a Rigol Ultra 3660 UV-Vis spectrophotometer from Rigol Technologies, Beijing, China. The results were compared with a standard curve prepared with gallic acid solutions, and the final values were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight [16].

4.4.2. High-precision liquid chromatography

The quantification of polyphenols and other compounds in the extracts was performed using an L-3000 HPLC system from Rigol Technologies Inc. in Beijing, China. This system was equipped with a diode array detector (HPLC-DAD) and a Kinetex EVO C18 column (150 × 4.6 mm, 5 µm particle size). The mobile phase consisted of a two-solvent system, where solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile. Elution was

performed in gradient mode with the following gradient: 2-100% solvent B at 30°C for 60 minutes at an elution flow rate of 1 mL/min. The analysis was performed at five different wavelengths (255, 280, 325 and 355 nm).

4.4.3. UV-Vis Spectrometry

A Rigol Ultra 3660 spectrophotometer from Rigol Technologies Inc. in Beijing, China, was used for these determinations. This device has an optical resolution of 0.5 nm and uses quartz cells with a path length of 1 cm. The analysis was performed in the wavelength range of 370-600 nm, according to the recommendations in the literature [17-19].

4.4.4. X-ray diffraction

X-ray diffraction analyzes were performed using a 9 kW Rigaku SmartLab diffractometer from Rigaku Corp., Tokyo, Japan. The instrument was operated at 45 kV and 200 mA, with CuKα radiation (λ = 1.54059 Å). The 2θ/θ scan mode was used, covering the angular range from 7 to 90° (2θ).

4.4.5. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) analysis was performed using a Titan Themis 200 image-corrected transmission electron microscope from FEI, Hillsboro, OR, USA. The microscope was equipped with a high brightness field emission gun (X-FEG) electron source and a super X detector for energy dispersive spectroscopy (EDX). The heterostructures were investigated at 200 kV using a high-resolution TEM (HR-TEM) in conjunction with areal electron diffraction (SAED) for structure identification.

4.4.6. Fourier Transformed Infrared Spectroscopy (FTIR)

FTIR measurements were performed using a JASCO FT-IR 6300 instrument from Jasco Int. co. Ltd., Tokyo, Japan. The instrument was fitted with a Specac ATR Golden Gate accessory from Specac Ltd., Orpington, UK, with KRS5 objective. Measurements were performed in the range from 400 to 4000 cm^{-1} with 32 accumulations at a resolution of 4 cm^{-1}.

4.4.7. Antioxidant activity

The antioxidant activity of the extracts and silver nanoparticles was determined using the DPPH assay (Sigma Aldrich, Burlington, MA, USA).

4.4.8. Biocompatibility evaluation of phytosynthesized silver nanoparticles

Human gingival fibroblasts (HFIB-G cell line, cat. no.: 1210412, Provitro, Berlin, Germany) were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin /streptomycin/amphotericin. Cells were maintained in a humidified atmosphere with 5% CO2 in air at 37°C. Cells (104 cells/well) were seeded in 96-well plates and allowed to adhere overnight. Then, fibroblasts were exposed to HT-AgNP, HM-AgNP,

MT-AgNP, MM-AgNP, UT-AgNP, UM-AgNP, OT-AgNP, or OM-AgNP for the next 24 and 48 h at 37°C, respectively with 5% CO2. The samples were previously diluted in Dulbecco's modified Eagle complete medium, and the obtained concentrations of phytosynthesized NPs were 0.1% (equivalent to a concentration of about 0.54 µg Ag/mL), 2.5% (equivalent with a concentration of 13.5 µg Ag/mL) and 5% (equivalent to a concentration of about 27 µg Ag/mL). The final volume was 300 µL for each well. The control samples were represented by cells not exposed to the phytosynthesized NPs. At the end of each incubation time, cells were examined using an Optika IM-3 inverted microscope.

4.4.9. Assessment of cell viability

Human fibroblast cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Thermo Fisher Scientific, Eugene, OR, USA) assay, which is based on the reduction of the tetrazolium compound MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) of yellow color, to purple formazan crystals, by enzymes (mitochondrial succinate dehydrogenases – NAD(P)H dependent) metabolically active cells. (Hansen; Nielsen; Berg, 1989). The growth medium was removed after 24 and 48 h of incubation, respectively, and then, 100 µL of DMEM with 10 µL of MTT was added to each well for 4 h at 37°C. After incubation, 100 µL/well of SDS-HCl solution (10%) was used to dissolve the purple formazan crystals, and after another 4 h, the absorbance was measured at 570 nm in the FLUOstar® Omega multimode microplate reader from BMG LABTECH (Ortenberg, Germany).

4.4.10. Determination of nitric oxide (NO) production by the Griess method

The levels of NO accumulated in the cell culture medium were measured after 24 and 48 h of incubation, respectively, using the nitric oxide assay kit (NO, Thermo Fisher Scientific, Vienna, Austria) based on the Griess reagent (naphthyl dihydrochloride ethylenediamine 0.1% and sulfanilamide 1% in 5% H3PO4). Absorbance at 540 nm was measured using the FLUOstar® Omega BMG LABTECH (Ortenberg, Germany) multi-mode microplate reader and the results were compared to the control.

4.4.11. Toxicity assessment by LDH (Lactate dehydrogenase) test

This assay was used to measure the lactate dehydrogenase released by HFIB-G cells after 24 and 48 h respectively in the presence of phytosynthesized NPs. Thus, 50 µL of culture medium from each tested well was mixed with 50 µL of reaction mixture (substrate and cofactor) and incubated at room temperature for 30 minutes in the dark. Absorbance was measured at 490 nm (reaction product specific) and 680 nm (for background correction) using the FLUOstar® Omega multimode microplate reader from BMG LABTECH (Ortenberg, Germany). To calculate the percentage of

cytotoxicity, the absorbance at 680 nm was subtracted from the absorbance at 490 nm and the results were compared to the control.

4.5. Statistical analysis

The experimental results from parallel multiple determinations (as indicated for each method) and the primary data obtained were analyzed for statistical significance using analysis of variance (one-way ANOVA) and Tukey's test to determine significant differences between means. Significant differences were set at $p \leq 0.05$. Results are means ± standard error of the mean (SE) of independent determinations. MTT, NO and LDH test results were expressed as means with standard deviations. Quantitative independent variables were tested between groups using Student's t test. Values of $p < 0.05$ were considered statistically significant. Graphical representations were created using OriginPro 2018 data analysis and graphing software (OriginLab Corporation, Northampton, MA, USA).

5. The characterization of the extracts and phytosynthesized silver nanoparticles

5.1. The characterization of natural extracts

The results of the phytochemical analysis and the HPLC analysis show the influence of the extraction method on the composition of the extract. Thus, total phenolic compounds (expressed as mg GAE/g dry weight) are significantly higher in microwave-assisted extraction; oregano extracts consistently show higher TPC values compared to hyssop, fenugreek and nettle extracts. Data from the literature show some comparable results. For example, maceration at room temperature in a hydroalcoholic mixture resulted in a TPC of approximately 2.45 mg GAE/g, while various extraction methods applied to oregano plant material resulted in TPC below 20 mg GAE/g [20].

Among the constituents identified by HPLC, the HT sample shows significantly higher concentrations for the group of phenolic acids (gallic acid, rosmarinic acid and protocatechuic acid) compared to the other extracts. Rosmarinic acid was also identified in a higher content than in other studies presented previously [21]. The group of flavanols is consistently found in higher concentration in the microwave extracts as well as in the oregano extracts, the same trend was found for the flavonoid standards (with the notable exception of naringenin), which were identified in higher amounts in the extracts of hyssop, although again microwave-assisted extraction resulted in a higher yield. Significant exceptions were found for isoquercitrin (not detected in the OT sample) and rutin (not detected in the OM sample). Resveratrol represents an interesting case. It was identified in higher

amounts in hyssop extracts and shows a higher yield in classical temperature extraction; this could be explained by its thermal lability, which can be enhanced in microwave-assisted techniques [22].

A particular observation related to the HPLC results is that the only two compounds found in higher amounts in the MT sample (compared to MM) are catechin-flavan-3-ol and, to a lesser extent, hydroxycinnamic acid o-coumaric acid (the other quantified isomers, epicatechin and p-coumaric acid, are found in higher amounts in MM). These observations suggest a certain selectivity of extraction methods that could influence nanoparticle formation and biomedical applications.

5.2. The characterization of silver nanoparticles

UV-Vis absorption spectra were used to monitor the phytosynthesis of silver nanoparticles, which showed characteristic peaks of AgNPs at about 423 nm for the MT-AgNP sample and at 430 nm for the MM-AgNP sample. In the UV-Vis spectra recorded for the MT-AgNP and MM-AgNP samples, the occurrence of absorption maxima in the 400-500 nm range indicates phytosynthesis of the nanoparticles. Although the UV-Vis absorption spectra were obtained at low concentrations (using diluted samples), they not only confirm the phytosynthesis process but also indicate a slightly larger size of the nanoparticles in the case of the MM-AgNP sample. Comparing the absorption spectra of the diluted extracts and the silver nitrate stock solution, neither the extracts nor the silver nitrate show absorption maxima in the region of interest.

The phytosynthesis process was confirmed by X-ray diffraction analysis. The Ag0 phase was identified by comparison with ICDD PDF card No. 01-077-6577. Although the presence of other XRD peaks is easily recognised due to the contribution of the amorphous halo to the overall appearance of the diffractograms, detailed analysis using PDXL software confirmed their presence.

Evaluation of the samples by TEM, HR-TEM, SAED and particle size distribution analysis showed that the nanoparticles are generally spherical with an average diameter of 14.4 nm for the MT-AgNP sample and 18.8 nm for the MM-AgNP sample. The use of MM extract led not only to the formation of nanoparticles with larger diameters but also to a great morphological diversity. While quasi-spherical nanoparticles were predominant, other distinct morphologies were also identified, including triangular, hexagonal and ellipsoidal shapes in the MM-AgNP sample. These results emphasise the advantage of using MT extract for the synthesis of nanoparticles with smaller size and more uniform morphology, which is crucial for biomedical applications [23].

TEM images revealed a quasi-spherical morphology for all analyzed samples, and the diameter distribution was consistent with XRD determinations: HT-approximately 18.5 nm, HM-10.5 nm, OT-16.5 nm and OM- 10 n.m.

FTIR analysis was performed on both nanoparticles and parent extracts to evaluate the phytoconstituents involved in the process. Both extracts and nanoparticles revealed the presence of functional groups corresponding to alcohols and phenols (O–H), carboxylic acids (C–O stretching), methyl and aldehyde groups (C–H bond stretching), alkenes (C=C stretching), and aromatics (C–C stretch). The FTIR peaks observed in both extracts can be assigned as follows: 3383/65 cm^{-1} for free hydroxyl groups, 2979 and 2902/3 cm^{-1} for C-H stretching vibrations and 1652 cm^{-1} for carbonyl group vibrations. In addition, the bands at 880 and 1453 cm^{-1} (present only in MM) could be attributed to stretching vibrations of CO_3^{2-}. Peaks from 1382/4 to 1044/5 cm^{-1} represent asymmetric and symmetric stretching vibrations of PO-2 and phospholipids, while the peak at 1404 cm^{-1} corresponds to C-O-C stretching of nucleic acids and phospholipids. Furthermore, the peak at 632 cm-1 indicates out-of-plane bending of CH vibrations, and the bands in the 700-900 cm^{-1} region correspond to out-of-plane bending vibrations, while those in the 500-600 cm^{-1} region are attributed to ring stretching vibrations strongly mixed with in-plane bending CH vibrations.

When comparing the results obtained for nanoparticles (NPs) with extracts, an increase in the peaks corresponding to organic compounds (1400–1650 cm^{-1}) is observed. Specifically, three peaks appear in the FTIR spectra of NPs or experience a significant increase in intensity: 1639/1651 cm^{-1}, 1608 cm^{-1} (more visible in MM-AgNPs) and 1516/5 and 1450/3, 1272/ 48 , respectively 1172/3. Literature data attribute the appearance of the FTIR peaks at 1172/3 and 1272/48 cm^{-1} to the presence of AgNPs. The increase observed for the peak at 3363/31 cm^{-1} is most likely due to the presence of silver nanoparticles. The remaining significant changes in the FTIR spectra could be assigned as follows: 1639/1651 cm^{-1} (C-N and C-C stretching indicating the presence of proteins), 1608 cm^{-1} (more visible in MM-AgNPs, corresponding to the amide I band of the protein), 1516/5 (in-plane CH bending vibration in phenyl rings) and 1450/3 (N–H stretching vibration present in protein amide bonds). These results suggest a synergistic action of several classes of phytoconstituents in the phytosynthesis process, including phenolic compounds and proteins [24].

6. The assessment of biomedical potential and cytotoxicity

6.1. The evaluation of the antioxidant activity of silver nanoparticles

Fig. 1 shows the results of the antioxidant potential assessment test for extracts and dispersions containing metal nanoparticles.

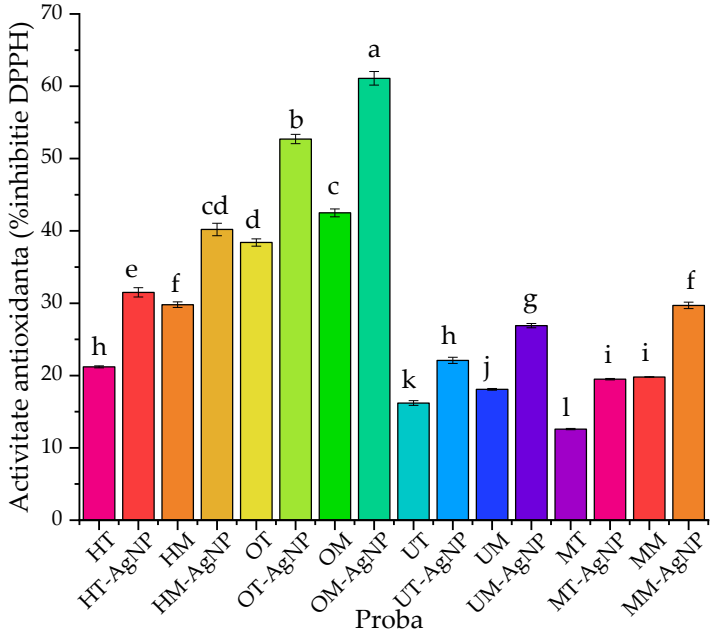

Fig. 1. Results of the antioxidant potential assessment test (DPPH inhibition). Values are mean ± SEM, n = 5 per treatment group. Means not sharing a common superscript letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the TUKEY test.

6.2. The evaluation of morphology, biocompatibility, viability and cytotoxicity

6.2.1. The analysis of the dynamics and morphology of human gingival fibroblasts (HFIB-G) exposed to phytosynthesized silver nanoparticles

Gingival fibroblasts incubated with phytosynthesized silver nanoparticles at low concentrations of 0.1% and short exposure time (24 hours) show little morphological change. The cells generally retain their elongated shape, but a slight reduction in cytoplasmic extensions is observed. The nucleus remains intact and the cytoplasm shows no obvious signs of contraction. The fibroblasts still adhere well to the substrate, but small cytoplasmic vacuoles or the beginnings of aggregation of nanoparticles can be observed around the cell.

At high concentrations of 5% and prolonged exposure to phytosynthetic silver nanoparticles, fibroblasts undergo dramatic morphological changes. The cells are completely rounded, especially the white horehound ones, a sign of loss of adhesion, and the cytoplasm is significantly reduced. The nuclei appear fragmented or even absent in some cells, indicating cell death.

Deposits of silver nanoparticles are visible both inside and outside the cells, suggesting internalization of the nanoparticles and severe damage to the cell structure.

6.2.2. The analysis of the viability of human gingival fibroblasts (HFIB-G) after incubation with phytosynthesized silver nanoparticles by the MTT technique

The results of the MTT assay show that there are no significant changes in the viability of

HFIB-G cells exposed to low concentrations of 0.1% HM-AgNPs and HT-AgNPs compared to the control group. In contrast, incubation of human gingival fibroblasts with higher concentrations of HM-AgNPs and HT-AgNPs for 48 hours resulted in a significant decrease in cell viability, with a p-value < 0.001 for 2.5% HM-AgNPs and HT-AgNPs, $p < 0.01$ for 5% HM-AgNPs and $p < 0.05$ for 5% HT-AgNPs.

The concentrations of 2.5% and 5% OM-AgNPs and OM-AgNPs caused a progressive decrease in the viability of the tested HFIB-G cells, both after incubation at 24 hours ($p < 0.05$ for 2.5% OM-AgNP and OT-AgNP; $p < 0.01$ for 5% OM-AgNP; $p < 0.001$ for OT-AgNP) and after 48 hours of incubation ($p < 0.001$ for 2.5% OM-AgNP; $p < 0.01$ for 2.5% OT-AgNP; $p < 0.001$ for 5% OM-AgNP and OT-AgNP).

The results obtained after a 48-hour exposure to 2.5% and 5% MM-AgNP and MT-AgNP, respectively, are consistent with those obtained for OM-AgNP and OT-AgNP. Also, UM-AgNP and UT-AgNP at high concentrations (2.5% and 5%) cause a significant decrease in the viability of human fibroblast cells in a dose- and time-dependent manner.

The MTT assay result showed that both crude extracts and their corresponding nanoparticles exhibited a concentration-dependent decrease in cell viability, with microwave-assisted extracts (MM) and microwave-synthesised nanoparticles (MM-AgNP) showing more pronounced effects than nanoparticles obtained by classical extraction. In particular, treatment with MM-AgNP reduced the viability of HFIB-G cells by 37.72% after 24 hours and by 56.85% after 48 hours at a concentration of 5%, while with MT-AgNP the reduction was 18.08% after 24 hours and 38.86% after 48 hours.

The comparative analysis of the cytotoxic effects between the crude extracts and their respective nanoparticles revealed a consistent pattern, with the crude extracts showing greater efficacy. In particular, the MM extract resulted in a significant 69% reduction in cell viability after 48 hours, exceeding the reduction observed with its nanoparticle counterpart (56.86%). This trend highlights the enhanced cytotoxic capabilities of the crude extracts compared to their nanoparticle formulations, suggesting a potential concentration of bioactive compounds in the extracts that may be attenuated when processed into nanoparticles.

After MTT testing of the hyssop and oregano samples, it was found that the tested phytosynthesized nanoparticles had no effect on cell viability at the lowest concentration after 24 and 48 hours of incubation. However, at higher concentrations, especially after 48 hours of incubation, a decrease in cell viability was observed compared to the control (cells without exposure to phytosynthesized nanoparticles).

In the case of deadnettle extracts, the results of the MTT test show that cell viability decreases in a dose-dependent manner after incubation with both types of nanoparticle solutions after both 24 and 48 hours compared to the control. A lower viability is observed in samples obtained with the temperature extraction method than with the microwave-assisted extraction method.

6.2.3. The evaluation of nitric oxide (NO) production by the Griess method

In the tests with phytosynthesized nanoparticle solutions using *Marrubium vulgare* L extracts, it was found that, in contrast to crude extracts, the NO concentration increased with both extraction methods and remained elevated at the highest concentration (5% of the original extract) after 24 and 48 hours, indicating a sustained inflammatory response compared to the control. Remarkably, the most significant increase in NO, 27%, was observed after 48 hours of incubation with the MM extract, which is consistent with the results of the MTT cytotoxicity assay.

In the tests performed with solutions of phytosynthesized nanoparticles using deadnettle extracts, nitric oxide levels increased after 24 hours of incubation of the cells as the dose of phytosynthesized nanoparticles increased. In contrast, the nitric oxide level decreased compared to the NO level in the control after 48 hours, indicating an anti-inflammatory effect of the tested samples on human gingival fibroblasts.

The highest anti-inflammatory effect was observed at lower concentrations of HT-AgNPs after 48 hours of incubation, where nitric oxide levels decreased by about 12% compared to the control. In contrast, a slight decrease in NO levels was observed with AgNPs derived from oregano extracts after 48 hours of exposure, especially at a concentration of 0.1% compared to the control. In addition, it was found that the lowest nitrite content was recorded in the samples obtained by the temperature extraction method.

6.2.4. The evaluation of cytotoxicity by the LDH method

After exposure to HT-AgNPs, membrane integrity was not significantly impaired. However, incubation with HM-AgNPs significantly impaired membrane integrity, especially after 48 hours, and showed an increase in LDH levels of about 15 compared to the control. The highest increase was observed after 48 hours of incubation with OM-AgNPs (45.97 % increase compared to the control), while the highest value measured after incubation with OT-AgNPs was 30.1 % compared to the control after 24 hours.

In the solutions of phytosynthesized nanoparticles with white horehound extracts, there are no significant changes compared to the control in terms of LDH content released into the environment.

At higher concentrations (2.5 %, 5 %), a decrease in LDH levels is observed for both samples compared to the control, suggesting a protective effect of the tested samples on the membrane integrity of the HFIB-G cell line.

In the samples of phytosynthesized nanoparticles with dew nettle extracts, in terms of the amount of LDH released into the medium after loss of membrane integrity, a lower amount of LDH was observed compared to the control at the higher concentrations of the tested samples, especially after incubation for 24 hours, suggesting a protective effect of the tested samples on the membrane of the tested human gingival fibroblasts. The lowest LDH level was measured after a 24-hour incubation of the fibroblasts at a concentration of 5% UM-AgNP and UT-AgNP, with 21.47% and 23.47%, respectively, compared to the control. There are no significant differences in LDH concentration when the cells are incubated for 48 hours.

7. Conclusions and personal contributions

In this doctoral thesis, we pursued the realization and physicochemical and biological characterization of some silver nanoparticles obtained by phytosynthesis to develop modern antimicrobial systems with potential efficacy in the prevention and local treatment of periodontal diseases.

The results of the study show that it is possible to use natural extracts for the phytosynthesis of silver nanoparticles. The application of two different extraction methods, namely classical temperature extraction and microwave-assisted extraction, resulted in the extraction of natural extracts with remarkable differences in composition. These differences in composition, together with the differences in the composition of the different plants, had a significant impact on the morphology and size of the synthesized nanoparticles.

The novelty of the work lies mainly in the phytosynthesis of silver nanoparticles using extracts of *Marrubium vulgare* L (white horehound) obtained by two different extraction methods: classical temperature extraction (MT) and microwave-assisted extraction (MM). The use of natural extracts for the development of metallic nanoparticles is a constantly evolving field. The approach presented in this dissertation is in line with the principle of "green chemistry".

The present study confirms the potential of *Marrubium vulgare* L extracts in the field of nanotechnology. Considering the known biomedical potential of this plant, the developed nanomaterials could have applications in various biomedical fields, but further studies are needed.

The research conducted in this dissertation represents a validation of the application potential of plant extracts, in particular *Marrubium vulgare* L., in the context of nanotechnology, emphasising a focus on the basic principle of "green chemistry". This approach aims to reduce hazardous residues

and limit the use of reagents with negative effects on the environment and human health. However, further research is essential to fully assess the toxicity of nanoparticles produced by phytosynthetic processes.

Personal research has provided a range of evidence on the complex interactions between nanomaterials and biological systems, highlighting the need for further comprehensive assessments of their biocompatibility and therapeutic potential.

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