THE UNIVERSITY OF MEDICINE AND PHARMACY
„VICTOR BABEȘ" FROM TIMIȘOARA
FACULTY OF PHARMACY
DEPARTMENT II- PHARMACOGNOSY

KIS I. BRIGITTA

ABSTRACT

Ph.D. supervisor

Prof. Univ. Dr. Corina Danciu

T i m i ș o a r a
2022

THE UNIVERSITY OF MEDICINE AND PHARMACY
„VICTOR BABEȘ" FROM TIMIȘOARA
FACULTY OF PHARMACY
DEPARTMENT II- PHARMACOGNOSY

KIS I. BRIGITTA

ABSTRACT

POPULI GEMMAE EXTRACT: PHYTOCHEMISTRY, THERAPEUTIC POTENTIAL AND GREEN SYNTHESIS OF SILVER NANOPARTICLES

Ph.D. supervisor

Prof. Univ. Dr. Corina Danciu

T i m i ș o a r a
2022

POPULI GEMMAE EXTRACT: PHYTOCHEMISTRY, THERAPEUTIC POTENTIAL AND GREEN SYNTHESIS OF SILVER NANOPARTICLES

I. INTRODUCTION

From past to the present, the Plant Kingdom offers numerous plant species that contain phytocompounds with undeniable therapeutic value. The evolution of science and technology has led to the discovery of the therapeutic potential of secondary metabolites extracted from various plant products. For this reason, interest in phytochemicals has grown significantly in recent years. Recent studies have described that more than 60% of drugs in use today are based on medicinal plants (1). A large number of natural compounds have demonstrated their efficacy in the prevention/treatment of various human health problems, including acute and/or chronic diseases (2, 3). Furthermore, the physico-chemical qualities and biological effects of precious and semi-precious metals such as Au, Ag, Cu, Zn, have been known since antiquity. Among these, silver is the most recognized due to its qualities as a catalyst, antimicrobial, anti-inflammatory or cicatrizing agent. The corroboration of modern science through interdisciplinary studies, with the field of nanotechnology, contributes to obtaining new nanoparticle-type compounds with rich pharmacological values. The use of nanotechnology in medicine has highlighted new ways to provide safer and more effective treatment options. Due to their small size (between 10 and 100 nm), nanoparticles can contribute to the targeted release of drugs in certain areas of the body, thus increasing their effectiveness and reducing their side effects (4).

Populus nigra L. (black poplar) is a species of the *Populus* genus, belonging to the *Salicaceae* family. Black poplar is a tall tree, its height can reach up to 30 m and its diameter up to 2 m. In Romania it grows in wet meadows and depressions, also appearing in the plains (5, 6). The buds, which represent the main vegetal product of black poplar, are large, 2 cm long and 5-8 mm thick, conical, elongated, the tip is sharp and slightly curved, yellow-brownish in color. On the surface, they have a viscous glue with a weak balsamic but aromatic smell. On the central axis of the buds there are 4-8 oval and sharp bracts, they are adherent due to the resins in the composition. Also due to the resins, which cover the buds, they have a shiny appearance (7, 8).

Regarding the chemical composition of the vegetal product of interest (*Populi gemmae*- further noted as Pg), different studies on the resin obtained from poplar bud proved the presence of flavonic derivatives (chrysol, tectocrizol, apigenol), flavonols (galangin, isalpinin, quercetol, kemferol) and other corresponding methylated derivatives (pinocembrine, pinostrombin). The presence of caffeic, dimethylcaffeic, isoferulic acids, as well as their esters was also proved. Esters of isoferulic acid with aliphatic and aromatic alcohols are present in large numbers and seem to predominate quantitatively in the extract. Fatty acids and aliphatic alcohols have also been identified. Among the terpene components identified, the most important is bisabolol, along with: γ-selinene, δ-cadinene, α-elemene and γ-cadinene (9, 10). In the literature, the absence of anthocyanin, saponins and quinones was mentioned (11).

Poplar species represent a subject of interest in the medical field due to their extensive phytochemical profile, but also due to their low toxicity. Different types of extracts obtained from the vegetal product have proven their effectiveness for the treatment of an increased number of conditions such as bronchitis, cough, trachea, laryngitis, sore throat, ulcers, hemorrhoids, anal fissures, rheumatism, etc. An increasing number of studies are describing new pharmaceutical applications for different types of extracts obtained from black poplar buds, among them are antioxidant, antidiabetic, anticancer, hepatoprotective and antimicrobial properties (12-19).
The objectives of this doctoral thesis:

- Evaluation of the Pg extract obtained from the western part of Romania regarding the phytochemical composition and antioxidant capacity.
- Evaluation of the therapeutic potential of Pg extract (*in vitro* studies on antimicrobial activity on selected strains (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Escherichia coli*, *Enterococcus faecalis*, *Candida albicans* and *Candida parapsilosis*); evaluation of the antiproliferative, cytotoxic, pro-apoptotic activity on MCF7 breast cancer cell lines, respectively lung adenocarcinoma A549; studies on the anti-angiogenic potential using the chick embryo chorioallantoic membrane technique; studies on the immunomodulatory activity of Pg extract.
- Evaluation of silver nanoparticles obtained through green synthesis of the extract of *Populus nigra* L. buds (physico-chemical characterization, *in vitro* studies on the antimicrobial activity on selected strains (*Staphylococcus aureus, Streptococcus pyogenes, Streptococcus mutans, Escherichia coli, Enterococcus faecalis, Candida albicans* and *Candida parapsilosis)* and antiproliferative on MCF7 breast cancer cell lines, respectively A549 lung adenocarcinoma.

II. PERSONAL CONTRIBUTION. OBJECTIVES. MATERIALS AND METHODS. RESULTS.

STUDY 1: Phytochemical evaluation of Pg extract.

In recent years, experimental studies have shown that medicinal plants represent a rich source of phytocompounds with a wide variety of therapeutic effects (20, 21). The personal contribution to this study consists of obtaining the Pg extract collected from the western part of Romania, the analysis of the physicochemical profile and antioxidant screening.

1.1. Extraction and physicochemical characterization of Pg extract.

Black poplar buds were harvested from Timișoara and the extraction was carried out in the Department of Pharmacognosy (University of Medicine and Pharmacy "Victor Babeș"). To obtain the extract, 10 g of dried plant product and 10 mL of 70% ethanol were mixed, then covered with parafilm and left at room temperature. The extract was then taken to the ultrasonic bath for 10

minutes and later the solvent was removed using a rotary evaporator. With the help of LC-MS, and FT-IR techniques, a clear picture of the main compounds contained in the dry extract of Pg was obtained (22).

Regarding the obtained results, it was proven that the Pg extract contains a significant number of compounds from the classes of phenols, respectively phenolic glycosides. The following compounds were identified: dihydroxybenzoic acid, protocatechuic acid, 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeic and chicoric acids, apigenin-glucuronide, chrysoeriol-glucuronide, tremuloidin, salicin, pinostrobin and tremulacin.

1.2. Assessment of the antioxidant activity of Pg extract.

In recent years, a major emphasis has been placed on medicinal plants with antioxidant potential that may have numerous health benefits. The aim of this study was to evaluate the Pg ‘s extract antioxidant activity with the help of a frequently used method, the DPPH assay. The DPPH (2,2-diphenyl-1-picrylhydrazyl) method’s main advantage is that it is fast and easy to perform. This assay implies the oxido-reduction reaction between the DPPH radical and the natural antioxidants contained in the Pg extract. The purpose of this chemical reaction is to consider the change in color of the mixture from deep purple to pale yellow. The obtained antioxidant percentage of Pg extracts was compared to those obtained for standard reference of Vitamin C. The IC_{50} values alongside IC_{10}, IC_{20}, IC_{80} and IC_{90} were determined using the OriginPro 2020 software (23).

The obtained antioxidant percentage of Pg extract varies from 95%- 97.3%, whereas in the case of standard reference (vitamin C), it was between 97.5%- 98.9%. The kinetic reaction was examined for 1200 seconds for each concentration of Pg extract (50, 100, 250, 500, 1000 µg/mL). The results obtained in this study proved that the Pg extract showed a significant antioxidant activity, which is comparable to those obtained for the ethanolic solution of Vitamin C.

1.3. Inorganic element determination of Pg extracts by GF-AAS.

In order to establish the inorganic elements of Pg extracts, the GF-AAS method was used. The metal concentrations were established using a spectrophotometer novAA 400G and for each individual element, a calibration curve was previously registered with standard Merck solutions (24).

Related to the inorganic element determination of the Pg samples tested, the following elements were detected: cadmium 0.019 µg/g, chromium 0.79 µg/g, manganese 0.59 µg/g, nickel 3.28 µg/g, copper 6.66 µg/g, zinc 14.84 µg/g, iron 39 µg/g, aluminum 2109.87 µg/g. There were only 3 elements (lead, cobalt, arsenic) below the limit of detection. Therefore, it can be concluded that the extract does not contain toxic elements.

STUDY 2. Evaluation of the therapeutic potential of Pg extract.

The personal contribution to this study consists of the evaluation of Pg extract regarding the antimicrobial activity against selected microorganisms, the antiproliferative, cytotoxic, pro-

apoptotic, and anti-migratory activity on the selected cancer cell lines (MCF7 breast cancer, respectively A549 lung adenocarcinoma cell lines). Another contribution to this study was the evaluation of the antiangiogenic activity on the chorioallantoic membrane and the evaluation of the immunomodulatory activity of Pg extract (including cell viability studies as well as the the selected cytokine expression evaluation).

2.1. Evaluation of the antimicrobial and antifungal activity of Pg extract.

The antimicrobial activity was assessed using the disk diffusion and dilution method. The Pg extract (10, 25, 50, 75, 100, 150 µg/mL) was evaluated against 8 selected microorganisms: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida parapsilosis* (25).

Based on the antimicrobial results, it was shown that Pg extract can act as a bactericidal agent against *Staphylococcus aureus*, as a bacteriostatic agent against *Streptococcus pyogenes* and *Streptococcus mutants*, and as a fungicidal agent against all strains of *Candida* tested. The MIC values were the highest for *Staphylococcus aureus* (0.625 mg/mL) and *Enterococcus faecalis* (2.5 mg/mL). Lower values were recorded *for Streptococcus pyogenes* and *Streptococcus mutans* (0.312 mg/mL for each).

2.2. *In vitro* evaluation assays: screening of the antiproliferative/ cytotoxic/ pro-apoptotic potential of Pg extract on different cancer cell lines (MCF7 and A549 cells).

2.2.1. Characterization of the antiproliferative properties of Pg extract using MTT assay.

The personal contribution consists of the evaluation of the antiproliferative potential of Pg extract on MCF7 human breast cancer and A549 human lung adenocarcinoma cell lines using the MTT method. This method is used to determine cell viability and consists of the reduction of the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals (23).

The results obtained in this study shown that the Pg extract is selective on the tested cell lines, the IC_{50} values after 72 hours of stimulation with Pg extract were 72.49 µg/mL on the MCF7 cell line and 66.26 µg/mL on the A549 cell line.

2.2.2. Characterization of the distribution of the cells in the phases of the cell cycle following incubation with Pg extract.

The characterization of the distribution of MCF7 and A549 cells in the phases of the cell cycle following incubation with Pg extract (10, 25, 50, 75, 100, 150 µg/mL) was performed using flow cytometry (23).

Considering the results regarding the cell cycle arrest, similar outcomes were obtained in both A549 and MCF7 cells. Pg extract arrests cells in the G0/G1 phase, however, the percentage of cells in this phase is relatively small, thus the main antiproliferative mechanism of Pg extract is not related to the cell cycle phases.

2.2.3. **Evaluation of the anti-migratory effect of Pg extract using Scratch assay.**

The evaluation of the anti-migratory effect on MCF7 and A549 cells after incubation with Pg extract was conducted by Scratch assay. This method is based on the ability of a selected sample to inhibit cell migration, metastasis, growth and development of the tested cancer cell lines. By means of this method, the anti-migratory potential of Pg extract was determined *in vitro,* on MCF7 and A549 cell lines (26).

The obtained results on the screened cancer cell lines provide evidence that Pg extract has decreased tumor cell migration and produced changes in the cancer cell morphology. Pg extract caused a dose-dependent decrease in "Scratch" closure rate, which was 1.4% (100 µg/mL Pg extract) and 1.3% (150 µg/mL of Pg extract) in the case of the MCF7 cancer cell line. A similar conclusion was obtained in the case of the A549 cells line, Pg extract has decreased the "Scratch" closure rate in a dose-dependent manner, the highest closure rate was 14.1% (100 µg/mL Pg extract) and 12.5% (150 µg/mL Pg extract).

2.2.4. **Determination of the cytotoxic potential of Pg extract using lactate dehydrogenase (LDH) assay.**

In order to assess the cytotoxicity of Pg extract (10, 25, 50, 75, 100 and 150 µg/mL), the LDH assay was determined. The principle of this assay is based on the release of LDH enzyme into the medium, which through an enzymatic reaction produces formazan. The obtained amount of formazan is directly proportional to the LDH, which is an indicator of the cytotoxic effect (23, 25).

The results showed that Pg extract had a cytotoxic effect on both cancer cell lines. A significant cytotoxic effect was observed after 72 hours of stimulation at these concentrations: 25 µg/ml (13.5 ± 0.7%), 50 µg/ml (18.2 ± 0.9%), 75 µg/ml (22.9 ± 1.1%) and 100 µg/mL (29.9 ± 14%) in the case of MCF7 cells. On the other hand, the cytotoxic effect was reached at 25 µg/mL (11.3 ± 0.9%), 50 µg/mL (13.1 ± 1%), 75 µg/mL (18 ± 1.4%), but also 100 µg/mL (21.7 ± 1.6%) in the case of the A549 cell line. The cytotoxicity rate at 150 µg/ml was 37 ± 4.1% compared to the control cells (5 ± 1.1%) in the case of MCF7 cells, and it was 7.8 ± 1.3% compared to the control cells (2.9 ± 0.8%) in case of A549 cells. Therefore, slightly superior cytotoxic results were obtained for the MCF7 cell line compared to the A549 cell line.

2.2.5. Detection of the pro-apoptotic potential of Pg extract via 4 ,6-diamidino-2-phenylindole (DAPI) staining.

The assessment of the possible pro-apoptotic potential of Pg extract (10, 25, 50, 75, 100 and 150 µg/mL) on selected cancer cell lines (human breast adenocarcinoma MCF7 and lung cancer cell lines A549) was analyzed using the colorimetric DAPI method (4,6-diamidino-2-phenylindole) (23, 25).

Following the DAPI staining assay, the obtained results indicated that high doses of Pg extract presented signs of apoptosis and changed the tested cancer cell morphology. In addition, a

significant increase in chromatin condensation and also signs of nuclear membrane blebbing were observed.

2.2.6. Characterization of the pro-apoptotic potential of Pg extract (early/late) and the degree of necrosis by the Annexin V-PI assay.

In order to evaluate the process of cellular apoptosis, the Annexin V-PI staining was performed using fluorescence flow cytometry. Following incubation with Pg extract (10, 25, 50, 75, 100, 150 µg/mL) the MCF7 and A549 cell lines were evaluated related to early and late apoptosis, viable and necrotic cells (23, 25).

Pg extract was shown to induce a modest apoptosis phenomenon. The MCF7 cell line was more sensitive than the A549 cell line. Results on MCF7 cells indicated that statistically significant values of late apoptotic cells were observed starting at 50 µg/mL (the percentage was 0.44% ± 0.32 vs. control cells 0.04% ± 0.03) and gradually increased at 75 µg/mL (0.27% ± 0.25), 100 µg/mL (1.74% ± 0.25) and 150 µg/mL (3.11% ± 3.08). Early apoptotic cell values were 2.63% ± 3.72 (10 µg/mL), 3.73% ± 4.49 (25 µg/mL), 8.85% ± 9.55 (50 µg/mL ml), 113.33% ± 11.12 (75 µg/mL), 28.83% ± 4.68 (100 µg/mL) and 45.53% ± 6.50 (150 µg/mL). Signs of necrosis were recorded starting from the lowest doses, 6.64% ± 1.09 (10 µg/mL), 9.53% ± 2.11 (25 µg/mL), 9.98% ± 4 .30 (50 µg/mL), 14.97% (±75.14%) µg/mL), 9.90% ± 3.14 (100 µg/mL), and 5.01% ± 4.07 (150 µg/mL).

Regarding the A549 lung cancer cell line, the Pg extract demonstrated a weak phenomenon of apoptosis. The percentage of early apoptotic cells increased from 1.34% ± 0.33 (control) to 1.70% ± 0.47 (10 µg/mL Pg), 1.27% ± 0.12 (25 µg/ mL Pg), 1.34% ± 0.3 µg (50 µg/mL Pg), mL Pg), 1.97% ± 0.77 (75 µg/mL Pg), 1.47% ± 0.42 (100 µg/mL Pg), and 2.68% ± 0.62 (150 µg/mL Pg). Related to late apoptosis, a slight increase in cells was outlined, starting from 1.43% ± 0.14 (control) to 5.15% ± 1.02 (150 µg/mL Pg), however, the rest of the tested concentrations did not produce remarkable growth. The percentage of necrotic cells increased from 0.12% ± 0.12 (control) to 0.60% ± 0.11 (50 µg/mL Pg) and 0.62% ± 0.02 (75 µg /mL Pg). On the other hand, the percentage of viable A549 cells decreased from 97.12% ± 0.04 (control) to 92.01% ± 0.02 (150 µg/mL).

2.2.7. Evaluation of the antiangiogenic profile of Pg extract using the chorioallantoic membrane technique (CAM).

Angiogenesis is considered an important factor in cancer progression; moreover, it is responsible for the cell invasion process and metastasis. The aim of this study was to evaluate the *in ovo* antiangiogenic effect of Pg extract on the chorioallantoic membrane. This method requires the use of fertilized chicken eggs (*Gallus gallus domesticus*). The effect on CAM of 150 µg/mL Pg extract was followed in normal development, the control being represented by 0.1% DMSO. The stereomicroscopic evaluation was performed daily and the captured images were further processed by the Zeiss ZEN software (27).

Pg extract was very well tolerated on CAM, no signs of toxicity were observed at the level of the vascular plexus. After 24 hours of treatment with Pg extract, a low degree of capillary

interconnection was observed inside the plastic ring (where the extract was applied). A small amount of newly formed vessels was detected inside the ring. However, the untreated parts (outside the ring) presented a higher degree of vascularization development. It was observed that using 150 µg/mL of Pg extract on the CAM is well tolerated. In conclusion, Pg extract showed an antiangiogenic potential on the chorioallantoic membrane.

2.2.8. Immunomodulatory effects of Pg extract. Evaluation of dendritic cell viability and IL-10 and IL-23 cytokine expression.

2.2.8.1. Dendritic cell viability evaluation

The aim of this study was to evaluate the immunomodulatory potential of Pg extract on peripheral blood mononuclear cells (PBMC) differentiated into dendritic cells (CD). The assessment of cell viability was performed by the FACS colorimetric method with DAPI (24 hours of stimulation), while the evaluation of the apoptotic activity was performed by the Annexin V-7AAD test. DMSO was used as a control in the presence or absence of inflammatory stimulation with LPS. The obtained results were examined and interpreted using the Flow Jo 7.6.5 software (23).

The obtained preliminary results indicated that higher concentrations of Pg extract could affect the immune response, therefore, lower concentrations of Pg (1-10 µg/mL) were also included. DAPI colorimetric method demonstrated that there were no cytotoxic effects of Pg extract (low and moderate concentrations) on naïve and inflammatory CD. Only at the concentrations of 75 and 100 µg/mL Pg extract, cellular toxicity phenomena were identified (but they did not reach statistically significant differences). It was also demonstrated that cells treated with the lowest concentrations of Pg extract (1–10 µg/mL) did not enter into the apoptotic pathway. High concentrations of Pg extract led to some changes in cell morphology. Therefore, the use of low concentrations of Pg extract is substantially better, avoiding the occurrence of apoptosis phenomena.

2.2.8.2. Evaluation of IL-10 and IL-23 cytokine expression.

The aim of this study was to evaluate the immunomodulatory potential of Pg extract on DCs, by analysing their activity on the selected cytokines. In order to demonstrate whether Pg extract had valuable consequences on DCs, the ELISA assay was conducted. In this experiment, two significant interleukins, namely IL-10 and IL-23 were selected (23).

The results on cytokine expression and release of Pg extract indicated that low concentrations of the extract presented an increase of IL-10 and IL-23 secretion on the inflammatory DCs. Moreover, higher concentrations of Pg (starting from 75 and 100 µg/mL) decreased the cytokine production of inflammatory DCs. Therefore, Pg extract may inhibit the proinflammatory responses of immune cells.

STUDY 3. Green synthesis of silver nanoparticles (AgNPs).

The personal contribution to this study consists of obtaining silver nanoparticles using Pg extract, evaluating the antimicrobial effect on selected strains (*Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Pseudomonas aeruginosa, Candida albicans* and *Candida parapsilosis*) using the disc-diffusion and the disc dilution method, evaluating the antiproliferative potential on MCF7 and A549 cancer cell lines using the MTT method.

3.1. Green synthesis of AgNPs from Pg extract.

The purpose of this study was to use an environmentally friendly "green" method in order to synthesize nanoparticles (AgNPs) using Pg extract. This method was developed to obtain nanoparticles using the ethanolic extract of Pg (Pg-AgNPs). The physicochemical examination of the obtained AgNPs was performed using the following techniques: TG-DSC analysis, FT-IR and electron microscopy investigations (TEM and SEM-EDX). These methods reveal the stability, the functional groups on the surface of the nanoparticles, as well as the size and shape of the freshly synthesized Pg-AgNPs (28).

The results showed that due to different temperatures during the synthesis process and to various concentrations of an aqueous solution of $AgNO_3$ (Pg-AgNPs_S1 at 25 °C and 1M and Pg-AgNPs_S2 at 60 °C and 5M), different shapes of silver nanoparticles were obtained. Pg-AgNPs_S1 are spherical or quasi-spherical; in comparison, Pg-AgNPs_S2 have an irregular shape with different forms such as rhombohedral, triangular and spherical. The physicochemical investigation showed that the obtained Pg-AgNPs are stable, and their size is small, which is relevant for biomedical applications. It was also demonstrated that the shape and size of nanoparticles can be influenced by controlling the metal salt concentration and the reaction temperature.

3.2. Antimicrobial activity of Pg-AgNPs using disk diffusion and dilution method.

The *in vitro* antimicrobial activity of Pg-AgNPs (Pg-AgNP_S1 and Pg-AgNP_S2) was evaluated by the disc diffusion and the disc dilution method. The selected strains were: *Staphylococcus aureus, Escherichia coli, Streptococcus pyogenes, Pseudomonas aeruginosa, Candida albicans* and *Candida parapsilosis* (29, 30).

Pg-AgNPs_S1, which have a spherical or quasi-spherical shape and size between 3 and 60 nm, had a weak activity on *Pseudomonas aeruginosa* and *Escherichia coli* (Gram-negative), but had significant results on *Streptococcus pyogenes, Staphylococcus aureus* (Gram-positive) and *Candida* species. Similarly, Pg-AgNPs_S2, which have an irregular shape and size between 5 and 150 nm had a weak activity on *Pseudomonas aeruginosa*, *Escherichia coli* (Gram-negative) and *Candida* species (*Candida albicans* and *Candida parapsilosis*), but a significant activity on *Streptococcus pyogenes* and *Staphylococcus aureus* (Gram-positive). Both Pg-AgNPs presented significant antimicrobial activity on Gram-positive bacteria and fungi. Comparing the antimicrobial activity of the two nanoparticles (Pg-AgNPs-S1 and Pg-AgNPs-S2), the difference may be representative due to their particle size.

3.3. Antiproliferative activity of Pg-AgNPs using MTT assay.

The aim of the study was to evaluate the antiproliferative activity of Pg-AgNPs_S1 and Pg-AgNPs_S2 on MCF7 breast cancer and A549 lung adenocarcinoma cell lines, using the MTT method. The same protocol as in the previous study mentioned above was followed. Different concentrations of Pg-AgNPs (10, 25, 50, 75, 100, and 150 μg/mL) were tested on selected cancer cell lines with incubation periods of 24 and 72 h (31).

It was demonstrated that Pg-AgNPs showed an antiproliferative potential on both cancer cell lines (MCF7 and A549), but among the two tested nanoparticles, Pg-AgNPs_S2 showed superior activity on the MCF7 cell line (compared to A549 cell line). In the case of Pg-AgNPs_S1, a dose-dependent decrease in cell viability was observed for both cell lines, especially for the A549 line.

The antiproliferative activity of the tested poplar nanoparticles was manifested in a dose- and time-dependent manner, however, Pg-AgNPs_S2 demonstrated a much stronger antiproliferative potential on both studied cancer cell lines.

III. CONCLUSIONS

Considering the obtained results from the studies within this doctoral thesis, we can accomplish the following aspects regarding the Romanian Pg extract:

1. The LC-MS analysis highlighted that *Populus nigra* L. buds are rich in various compounds that are mainly belonging to the phenol and phenolic glycosides classes, such as protocatechuic acid, 3-caffeoylquinic acid, caffeic acid, chicoric acid, salicin, pinostrobin, and tremuloidin.
2. Pg ethanolic extract is a competitive inhibitor of Vitamin C and it consumes all of DPPH in the first 20 seconds. The selected concentrations (50, 100, 250, 500, 1000 μg/mL) had a strong antioxidant activity, comparable to Vitamin C. The percentage of inhibition varied from 95%-97.3% whereas, in the case of Vitamin C this percentage was between 97.5%-98.9%.
3. Related to the safety profile, it can be concluded that Pg extract presented some of the essential trace elements (such as Fe, Cu, Zn, Mn). The elements with toxic potential were under the detection limit (such as As, Cd, Co, Pb). Therefore, it can be concluded that the extract does not contain toxic elements being safe for the administration in the correct dosage.
4. Tested concentrations of Pg extract (10, 25, 50, 75, 100, 150 μg/mL) presented a significant antimicrobial activity against *Streptococcus pyogenes, Streptococcus mutans* (MIC= 0.312 mg/mL for both strains), *Staphylococcus aureus* (MIC= 0.625 mg/mL), and candida species such as *Candida parapsilosis, Candida albicans* (MIC= 1.25 mg/mL).

5. Pg extract has been shown to possess antiproliferative properties on the MCF7 human breast adenocarcinoma cell line as well as on the A549 human lung carcinoma cell line in a dose- and time-dependent manner. In set of experimental conditions within this study, the IC_{50} values recorded were 72.49 µg/mL (MCF7 cells) and 66.26 µg/mL (A549 cells).
6. Pg extract stopped the proliferation of MCF7 and A549 cells in the G0/G1 phase, but the percentage of these cells was relatively small, therefore we can conclude that the antiproliferative mechanism of Pg extract is not related to the phases of the cell cycle.
7. According to the Scratch assay, Pg extract showed an anti-migratory effect on both tested cell lines. This phenomenon was recorded at 50 µg/mL, 75 µg/mL and 100 µg/mL concentrations of Pg extract.
8. The evaluation of the cytotoxic activity of Pg extract on the tested cell lines revealed that after 72 hours of stimulation a significant cytotoxic effect was observed at 75 µg/mL (22.9 ± 1.1%), and 100 µg/mL (29.9 ± 1.4%) in case of MCF7 cells, respectively at 75 µg/mL (18 ± 1.4%), and 100 µg/mL (21.7 ± 1.6%) in the case of A549 cell line. The highest cytotoxicity rate achieved was 37% ± 4.1% vs. Control (5 ± 1.1%) in the case of MCF7 cells but overall, the conclusion is that the cytotoxic activity is relatively weak.
9. It was demonstrated by DAPI assay that chromatin condensation was increased in a dose-dependent manner. After incubation with high doses of Pg extract (100 and 150 µg/mL), signs of apoptosis were observed for the tested cancer cell lines. DNA fragmentation was not detected in MCF7 or A549 cells.
10. Following the Annexin-PI method can be concluded that Pg extract induces a modest phenomenon of apoptosis. MCF7 cell line was more sensitive than the A549 cell line.
11. Related to the antiangiogenetic profile of Pg extract it can be concluded that the extract led to a low degree of capillary interconnection; only a small number of newly formed vessels was observed. These are characteristics of an extract with antiangiogenic potential. At the highest tested concentration (150 µg/mL) Pg extract was well tolerated and did not presented signs of toxicity.
12. The evaluation of DCs activity following incubation with Pg extract led to the following conclusions: Pg extract induced cell toxicity only at the highest tested doses, namely from 75 to 100 µg/mL (but did not reach statistical differences). To go more in detail, cells were evaluated further for late apoptosis. It was revealed that cells treated with the lowest concentrations of Pg extract (1-10 µg/mL) did not enter the apoptotic pathway. Therefore, low doses of Pg extract may be adequate for further tests regarding the immunomodulatory activity.
13. By means of ELISA immunomodulatory assay, the following aspects can be highlighted: the lowest concentration of Pg extract, namely 10 µg/mL, revealed significant immunomodulatory activity by upregulating the expression of the IL-12 and IL-23 subunits p19, p35 levels. However, it is not clear which immune response will be promoted by the extract, therefore, this aspect will need to be further evaluated.

14. Through the studies within this thesis, green synthesis of AgNP using an ethanolic extract of Pg was conducted. Results have shown that the Pg-AgNPs were stable, and the particle sizes varied between 3 and 150 nm. Pg-AgNPs_S1 had a spherical or cvasi-spherical shape, and Pg-AgNPs_S2 had an irregular shape. The data obtained within the study demonstrated that the designed Pg-AgNPs were suitable for further biomedical applications.
15. Regarding the *in vitro* antimicrobial activity can be concluded that Pg-AgNPs_S2 presented a significant antimicrobial activity against Gram-positive bacteria (*Streptococcus pyogenes, Staphylococcus aureus*) and *Candida albicans, Candida parapsilosis*, but a weaker activity on Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*). Pg-AgNPs_S1 presented a weaker activity, with notable effect on Gram-positive bacteria (*Streptococcus pyogenes* and *Staphylococcus aureus)*, and much weaker antibacterial activity on the Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The difference between the two tested Pg-AgNPs, may be due to their various physicochemical properties, such as shape or size. Pg-AgNPs-S1 had a small particle size and Pg-AgNPs-S2 had a bigger particle size, so the contact surface was enlarged.
16. The results regarding the antiproliferative activity revealed that Pg-AgNPs_S2 presented a stronger antiproliferative potential on MCF7 and A549 human cancer cell lines than the Pg-AgNPs_S1. This activity was manifested in a time and dose-dependent manner.

The originality of the present study consists in:

- Investigating for the first time the phytochemical profile of *Populus nigra* L. buds, collected from the western part of Romania (Timisoara).
- Highlighting the inorganic elements present in Pg extract.
- Conducting a study regarding the antioxidant activity of the species.
- Testing *in vitro* the antimicrobial activity of the Pg extract on different bacterial and fungal strains.
- *In vitro* evaluation of the antiproliferative/pro-apoptotic/cytotoxic activity of the Pg extract on MCF7 human breast adenocarcinoma and A549 human lung cancer cell line.
- Highlighting the antiangiogenic potential of Pg extract using an *in ovo* technique.
- Investigating the immunomodulatory activity and cytokine expression of Pg extract through complex techniques on human dendritic cells.
- Synthesis and detailed characterization of Pg-AgNPs.
- Evaluation of the antimicrobial activity of Pg-AgNPs.
- Analysis of the antiproliferative effect of Pg-AgNPs.

Research perspectives

The results of the research undertaken within the doctoral thesis justify the continuation of studies in the following directions:

- Supplementary evaluation of Pg extract as well as the green synthesized nanoparticles from black poplar bud on other cancer cell lines and microbial strains.
- Further *in vivo* evaluation of Pg extract as well as the green synthesized nanoparticles from black poplar bud on experimental animal models.
- Looking forward, in terms of positive results, new lines of research can be opened toward clinical trials.

BIBLIOGRAPHY

1. Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery. *Metabolites*. 2019; 9, 258. doi: 10.3390/metabo9110258.
2. Jantan I, Bukhari SNA, Seyed Mohamed MA, Wai LK, Mesaik MA. The Evolving Role of Natural Products from the Tropical Rainforests as a Replenishable Source of New Drug Leads. Drug Discovery and Development- From Molecules to Medicine. London: *IntechOpen*. 2015. Available from: https://www.intechopen.com/chapters/47844 doi: 10.5772/59603.
3. Mintah SO, Asafo-Agyei T, Archer M, Junior PA, Boamah D, Kumadoh D, Appiah A, Ocloo A, Boakye YD, Agyare C. Medicinal Plants for Treatment of Prevalent Diseases. *Pharmacognosy - Medicinal Plants*. London: IntechOpen; 2019. Available from: https://www.intechopen.com/chapters/64420 doi: 10.5772/intechopen.82049
4. Khan I, Saeed K, Khan I. Nanoparticles: Properties, applications and toxicities. *Arabian Journal of Chemistry*, 2017. doi:10.1016/j.arabjc.2017.05.011
5. de Souza Silva JE, Santos Souza CA, da Silva TB, Gomes IA, Brito Gde C, de Souza Araújo AA, de Lyra-Júnior DP, da Silva WB, da Silva FA. Use of herbal medicines by elderly patients: A systematic review. *Arch Gerontol Geriatr*. 2014; 59, p. 227-33. doi: 10.1016/j.archger.2014.06.002.
6. Rédei K, Keserű Z, Szulcsán G. Early Evaluation of Promising White Poplar (Populus alba L.) Clones in Hungary. Acta Silvatica et Lignaria Hungarica. 2010; 6.
7. Tiziana D, Michele I, Elena C, Simona P, Antonietta F, Severina P. White poplar (Populus alba L.) leaf waste recovery and intercropping outcome on its polyphenols. Industrial Crops and Products. 2021; 171, p. 113866. https://doi.org/10.1016/j.indcrop.2021.113866.
8. Cottrell, Joan. Conservation of black poplar (*Populus nigra* L.). Forestry Commission Information Note, 2004.
9. Vanden BA, Cox K, Van Braeckel A, Neyrinck S, De Regge N, Van Looy K. Reintroduced Native Populus nigra in Restored Floodplain Reduces Spread of Exotic Poplar Species. *Frontiers in Plant Science*, 2021; 11. Doi:10.3389/fpls.2020.580653
10. Stanciauskaite M, Marksa M, Liaudanskas M, Ivanauskas L, Ivaskiene M, Ramanauskiene K. Extracts of Poplar Buds (Populus balsamifera L., Populus nigra L.) and Lithuanian Propolis: Comparison of Their Composition and Biological Activities. *Plants* (Basel). 2021; 10, p. 828. doi: 10.3390/plants10050828.
11. **Kis B**, Avram S, Pavel IZ, Lombrea A, Buda V, Dehelean CA, et al. Recent Advances Regarding the Phytochemical and Therapeutic Uses of *Populus nigra* L. Buds. *Plants*, 2020; 9, 1464. doi:10.3390/plants9111464
12. Jerkovic I, Mastelić I. Volatile compounds from leaf-buds of Populus nigra L. (Salicaceae). *Phytochemistry*. 2003; 63, p. 109-13. 10.1016/S0031-9422(02)00706-9.
13. Wang K, Zhang J, Ping S, Ma Q, Chen X, Xuan H, Shi J, Zhang C, Hu F. Anti-inflammatory effects of ethanol extracts of Chinese propolis and buds from poplar (Populus×canadensis). *J. Ethnopharmacol*. 2014; 155, p. 300–311.

14. Stanciu G, Chirila E, Dobrinas S, Negreanu-Pirjol T. Studies Regarding the Determination of Antioxidant Properties of New Plant Extracts for Cosmetic Purposes. *Rev. Chim*. 2010; 61, p. 45.
15. Benedec D, Oniga I, Muresan B, Mot AC, Damian G, Nistor A, Silaghi-Dumitrescu R, Hanganu D, Duma M, Vlase L. Contrast between Water- and Ethanol-Based Antioxidant Assays: Aspen (*Populus tremula*) and Black Poplar (*Populus nigra*) Extracts as a Case Study. *J. Food Qual*. 2014; 37, p. 259–267.
16. Vardar-Ünlü G, Sibel S, Unlu M. Composition and in vitro antimicrobial activity of Populus buds and poplar-type propolis. *World J. Microbiol. Biotechnol*. 2007; 24, p. 1011–1017.
17. De Marco S, Piccioni M, Pagiotti R, Pietrella D. Antibiofilm and Antioxidant Activity of Propolis and Bud Poplar Resins versus Pseudomonas aeruginosa. *Evid. Based Complement. Altern. Med*. 2017; 2017, p. 1–11.
18. Sofowora A, Ogunbodede E, Onayade A. The role and place of medicinal plants in the strategies for disease prevention. *African journal of traditional, complementary, and alternative medicines*, 2013; 10, p. 210–229. https://doi.org/10.4314/ajtcam.v10i5.2.
19. Boumghar N, Behidj N, Ksouri R. Antimicrobial and antibiofilm activities of phenolic compounds extracted from *Populus nigra* and *Populus alba* buds (Algeria). *Braz. J. Pharm. Sci*. 2019; 55, p. e18114.
20. Salmerón-Manzano E, Garrido-Cardenas JA, Manzano-Agugliaro F. Worldwide Research Trends on Medicinal Plants. *International journal of environmental research and public health*, 2020; 17, 3376. https://doi.org/10.3390/ijerph17103376
21. Atanasov AG, Zotchev SB, Dirsch VM. et al. Natural products in drug discovery: advances and opportunities. *Nat. Rev. Drug Discov*. 2021; 20, p. 200–216. https://doi.org/10.1038/s41573-020-00114-z
22. Venugopal K, Ahmad H, Manikandan E, Thanigai AK, Kavitha K, Moodley MK, Bhaskar M. The impact of anticancer activity upon Beta vulgaris extract mediated biosynthesized silver nanoparticles (ag-NPs) against human breast (MCF-7), lung (A549) and pharynx (Hep-2) cancer cell lines. *J. Photochem. Photobiol. B Biol*. 2017, 173, p. 99–107.
23. **Kis B**, Pavel I, Avram S, Tăculescu EA, Herrero M, Schwiebs A, Radeke H, Muntean D, Diaconeasa Z, Minda D, Oprean C, Bojin F, Dehelean, C, Soica C, Danciu C. Antimicrobial activity, in vitro anticancer effect (MCF-7 breast cancer cell line), antiangiogenic and immunomodulatory potentials of *Populus nigra* L. buds extract. *BMC Complementary Medicine and Therapies*. 2022; 22. Doi: 10.1186/s12906-022-03526-z.
24. Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature*. 1985; 181, p. 119901299.
25. **Kis B**, Pavel I, Haidu D, Stefanut M, Diaconeasa Z, Tăculescu EA, Dehelean C, Șipos S, Ivan A, Danciu C. Inorganic Element Determination of Romanian Populus nigra L. Buds Extract and In Vitro Antiproliferative and Pro-Apoptotic Evaluation on A549 Human Lung Cancer Cell Line. *Pharmaceutics*. 2021; 13. Doi: 986. 10.3390/pharmaceutics13070986.
26. Buda V, Brezoiu AM, Berger D, Pavel IZ, Muntean D, Minda D, et al. Biological evaluation of black chokeberry extract free and embedded in two Mesoporous silica-type matrices. *Pharmaceutics*. 2020; 12, p. 838. https://doi.org/10.3390/pharmaceutics12090838
27. Moacă EA, Farcaş C, Ghiţu A, Coricovac D, Popovici R, Cărăba-Meiţă NL, Ardelean F, Antal DS, Dehelean C, Avram Ş. A Comparative Study of Melissa officinalis Leaves and

Stems Ethanolic Extracts in terms of Antioxidant, Cytotoxic, and Antiproliferative Potential. *Evid. Based Complement. Alternat. Med*. 2018; 2018, p. 7860456. doi: 10.1155/2018/7860456.
28. Avram S, Ghiulai R, Pavel IZ, Mioc M, Babuta R, Voicu M, Coricovac D, Danciu C, Dehelean C, Soica C. Phytocompounds Targeting Cancer Angiogenesis Using the Chorioallantoic Membrane Assay. *IntechOpen*. 2017. doi: 10.5772/intechopen.68506
29. Ruíz-Baltazar AJ, Reyes-López SY, Larrañaga D, Estévez M, Pérez R. Green synthesis of silver nanoparticles using a *Melissa officinalis* leaf extract with antibacterial properties. Results Phys. 2017; 7, p. 2639–2643.
30. Kakakhel MA, Sajjad W, Wu F, Bibi N, Shah K, Yali Z, Wang W. Green synthesis of silver nanoparticles and their shortcomings, animal blood a potential source for silver nanoparticles: A review. *J. Hazardous Materials Advances*. 2021; 1, p. 100005. doi:10.1016/j.hazadv.2021.100005.
31. Wahab S, Khan T, Adil M, Khan A. Mechanistic aspects of plant-based silver nanoparticles against multi-drug resistant bacteria. *Heliyon*, 2021; 7, p. e07448. doi:10.1016/j.heliyon.2021.e07448.