PN-III-P4-ID-PCE-2020-0620
Topical nanoformulations of selective vegetal extracts with high anti-inflammatory and analgesic properties - NANOGELEXPLORE

SCIENTIFIC REPORT SUMMARY

Objective 2. PHYSICAL AND CHEMICAL CHARACTERIZATION OF CRUDE EXTRACTS, NANOSTRUCTURED MIXTURES AND METALLIC NANOPARTICLES. EVALUATION OF BIOACTIVITY OF CRUDE EXTRACTS, ISOLATED PHYTOCOMPOUNDS AND NANOSTRUCTURED MIXTURES

Act. 2.1. Experimental development of cost-effective and time-efficient methods for the synthesis of Au and Ag nanoparticles using crude extracts as a reducing agent
Deliverables: Nanostructured mixtures, test report

Summary of the Research report on the biosynthesis methods of Au and Ag nanoparticles

Although they are very small in a big world, by their unique properties (high surface/volume ratio, high level of surface free energy, due to which they have high reactivity, both towards themselves, forming aggregates, and towards other molecules/biomolecules, nanoparticles (NPs) pose great problems (Loosli *et al*., 2015). The physicochemical interactions between NPs and biomolecules depend on several factors, such as the elemental composition of the NPs crown (Jackson *et al*., 2017), size, shape and surface, solid-liquid interface, contact surface with an organic molecule (Bhaumik *et al*., 2014), microenvironmental factors (Pulido-Reyes *et al*., 2017). Depending on this multitude of factors, bio-nano-interactions can induce a wide variety of cellular responses (Juárez-Maldonado *et al*., 2019). Several studies on the size of NPs have shown that the smaller they are, the more reactive they become and more toxic to cells (Liu *et al*., 2020). For the biosynthesis of Au (AuNPs) and Ag (AgNPs) nanoparticles, 33 experimental variants were organized defined by 8 variables, respectively the species from which the extract was obtained (*Aconitum toxicum* (Rchb.), *Helleborus odorus* Waldst. & Kit. ex Willd. and *Anemone nemorosa* L.), the plant organ used for the extraction of the active principles, the extraction method (ultrasound, microwaves), the type of solvent used to obtain the extracts (water: ethanol, respectively water: methanol, 40:60 v/v), extract concentration and HAuCl4/AgNO3 concentration, incubation time and temperature.

Bibliografie

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6. Liu W, Zeb A, Lian J et al (2020) Interactions of metal-based nanoparticles (MBNPs) and metal-oxide nanoparticles (MONPs) with crop plants: a critical review of research progress and prospects. Environ Rev 28(3):294 – 310

Act. 2.2. Quantitative and qualitative characterization of extracts, nanostructured mixtures and metallic nanoparticles
Deliverables: Research report

Summary of the research contribution regarding the quantitative and qualitative characterization of extracts by FTIR, UV-Vis, DPPH, Folin-Ciocalteu method

The size of NPs can influence the physicochemical properties of a substance, such as optical properties. In our research, the phytosynthesis of AgNPs was observed by the color variation of the variants supplemented with AgNO3, in the first hours after the induction of biosynthesis. From a light green color of the extracts after the biosynthesis of AgNPs they acquired colors from yellow to brown (Fig. 1).

Figure 1. Color variation of extracts after induction of AgNPs biosynthesis in extracts of *A. toxicum*, *A. nemorosa* and *H. odorus*

UV-vis analysis of extracts identified the maximum wavelengths specific to phenolic acids at 220–280 nm, flavonoids, quinones and furanocoumarins at 290–420 nm (Bungez et al., 2013).
The determination of the antioxidant activity by the DPPH test indicated a higher antioxidant activity of the extracts obtained by microwave-assisted extraction, regardless of the species and part of the plants used for extraction, compared to the extracts obtained with ultrasound.
The STEM-EDS analyzes were performed using the FESEM - HITACHI SU8230 microscope from the Regional Center for Research and Development for innovative materials, products and processes for the automotive industry (CRC&D - AUTO). For all analyzed samples, the presence of Ag particles with nanometric dimensions, below 50 nm, was confirmed. The morphology of the particles is approximately spherical, and the sizes and dispersion differ from one extract to another, depending on the biosynthesis conditions (Fig. 2).
X-ray diffraction analysis indicated the presence of the crystalline phase of metallic Ag, with no other detected impurities, the nanometric size of the Ag crystallites, as well as the absence of microdeformations of the crystalline network.

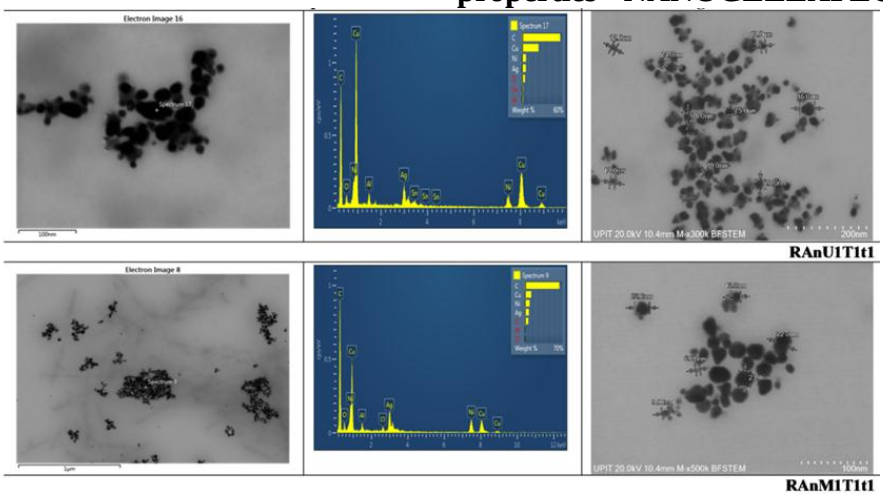

Figure 2. EDS analysis in point-scan. Micrographs of extracts supplemented with AgNPs

Act. 2.3. *In vitro* testing of plant extracts before and after phytosynthesis of metallic nanoparticles
Deliverables: Laboratory methodology, test report

Summary of the reaserch report on the *in vitro* evaluation of the cytotoxic and genotoxic effects of plant extracts before and after phytosynthesis of metal nanoparticles using plant-systems: the *Triticum* test, the *Allium* test, cell viability testing by the Evans blue/Trypan blue staining method, metabolic activity testing by the staining method with 2,3,5-triphenyl tetrazolium chloride

The *Triticum* test was also applied to evaluate the phytotoxicity of the extracts, with and without nanoparticles generated in different experimental conditions, applying the following methods: (1) after immersion in the test solution, the seeds were placed in Petri dishes on filter paper, periodically watered with distilled water and kept in the dark until the measurements are taken (5 days after the initiation of the experiment); (2) caryopses hydrated for one and a half hours were placed on filter paper moistened with distilled water in Petri dishes in the dark to germinate and form roots. After 3 days, the formed roots were exposed to the test solutions by spraying 1 ml of the solution uniformly over the entire surface of the filter paper (for 3 days). For both methods, only distilled water was used as the blank (Control). For each test variant, 10 seeds from each plant species were used, and the experiment had 3 repetitions. During the experiment, the air temperature was 19.6±5oC, the humidity 21±5%.
Results obtained by applying experimental method 1 showed that the influence of hydroethanolic extracts of *H. odorus* on root growth in *T. aestivum* was not significant compared to the control. In the case of the hydromethanolic extracts, in all variants, values higher than those determined in the control were obtained for root growth. Increasing the incubation temperature in the AgNPs biosynthesis process significantly stimulated the growth of the stem. The fresh biomass recorded in the variants with hydromethanolic extracts had, in general, higher values than that determined in the control.
By applying the Evans blue test to evaluate cell viability, in the experimental variants defined by *H. odorus* extracts supplemented with AgNPs, an increased permeability of cell membranes was highlighted compared to the control, as shown in Figure 3.
In the root meristematic cells exposed to the treatment with H. odorus extracts, with and without AgNPs, different chromosomal aberrations were identified, such as C-mitoses, anaphase and telophase bridges, micronuclei, etc. (Fig. 4).

Summary of the research report on the evaluation of the antimicrobial effects of plant extracts before and after the phytosynthesis of metallic nanoparticles using Gram positive and Gram negative bacteria

The method used to test the antimicrobial effect of the mentioned extracts was an adapted variant of the Kirby-Bauer diffusimetric method, in which sterile filter paper discs soaked with appropriate amounts of plant extracts were used instead of standard antibiotic-impregnated discs. The reference strains used: *Staphylococcus aureus ATCC 25923, Bacillus subtilis ISM 68/53* (echivalent *ATCC 6633*), *Escherichia coli ATCC 25922, Klebsiella penumoniae ATCC 700603, Candida albicans* ISM 76/32 (echivalent *ATCC 10231*). The negative control used was distilled water; the positive control was represented by antibiotic (Amikacin 30µg/disc, Gentamicin 120µg/disc, Cefoperazone 75µg/disc, Levofloxacin 5µg/disc) or antifungal (Nystatin 100 IU, Fluconazole 25µg/disc) in the form of standardized microtablets. Extracts obtained from rhizomes of *H. odorus* and *A. nemorosa*, with and without AgNPs, it was found that supplementation of the extracts with NPs enhanced the antimicrobial effect of the extracts regardless of the incubation variables for biosynthesis. *H. odorus* extracts had a more pronounced antimicrobial effect on *B. subtilis* and *C. albicans* strains, the weakest effect being shown against *E. coli*. Extracts of *A. nemorosa* obtained with ultrasound or microwaves, without silver nanoparticles, showed no antimicrobial effect. The most sensitive of the strains tested was that of *S. aureus*, followed by *K. pneumoniae* and *B. subtilis*, the strain of *E. coli* showing a low sensitivity to the action of these extracts.

Act. 2.4. *In vivo* testing of plant extracts before and after phytosynthesis of metal nanoparticles for their anti-inflammatory and analgesic activity
Deliverables: laboratory methodology, test report

Summary of research report on the *in vivo* evaluation of the anti-inflammatory potential of plant extracts before and after the phytosynthesis of metal nanoparticles in mice, after the induction of auricular/paw edema

The experiments were performed on CD1 mice, aged 20-21 weeks. The 1:3 diluted extracts were used, in doses of 20 ml/kg body weight (500 mg/kg body weight), administered by gavage. All tested *A. nemorosa* rhizome extracts with and without phytosynthesized AgNPs showed anti-

inflammatory activity in the experimental model of xylene-induced ear edema. The best results were obtained for the extracts made by the ultrasounds method. The anti-inflammatory action of the studied extracts was observed through a tendency to increase the number of erythrocytes and platelets, in parallel with the decrease in the number of leukocytes. Associated with the increase in the number of erythrocytes, there is an increase in the amount of hemoglobin and the hematocrit.

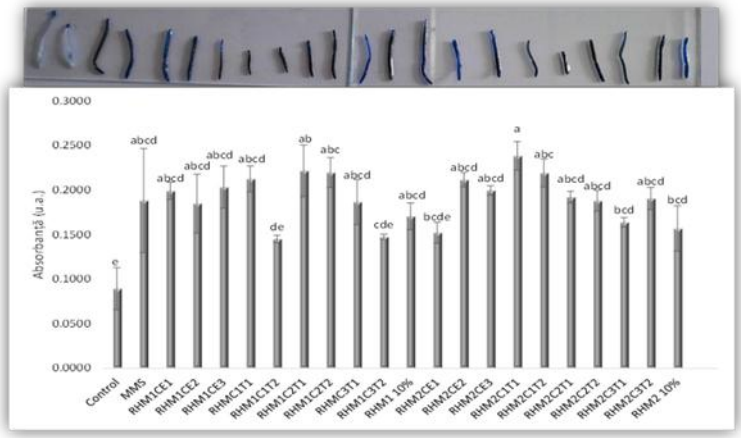

Figure 3. Viability of root meristematic cells of *A. cepa* incubated in hydroalcoholic extracts of *H. odorus* and nanostructured mixtures (a, b, c, d, e – interpretation of the significance of differences by the Duncan test, $p < 0.05$)

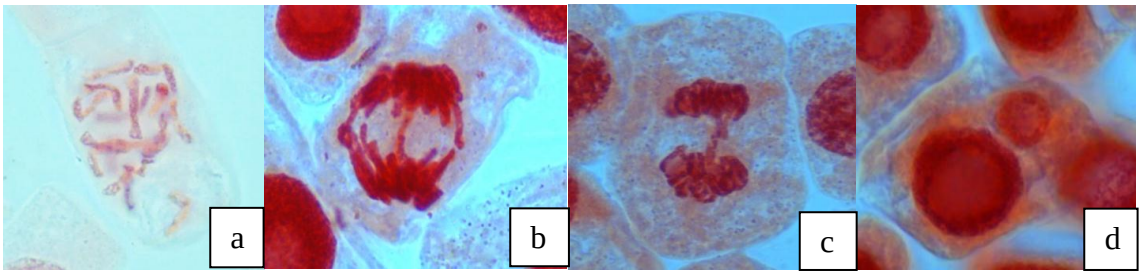

Figure 4. Chromosomal aberrations observed in the meristematic cells of A. cepa treated with H. odorus extracts with and without AgNPs; (a) C-mitosis; (b) anaphase bridges; (c) telophase bridges; (d) micronucleus

Act. 2.6. Identification of the character of a potential drug and of a compound with high biological activity
Deliverables: Research report

Summary of the research report on the identification of compliance with medicinal chemistry rules

In order to evaluate the character of a possible drug, the compounds were processed in the Expasy database [https://www.expasy.org/], and tested to comply with the rules of medical chemistry: the Lipinski rule [https://www.expasy.org /], Veber [https://www.expasy.org/], Egan [https://www.expasy.org/], Muegge [https://www.expasy.org/]. Chemical compounds for which excess is identified are considered not to meet the drug-like condition. Through the bioinformatics calculation of the drug-like character, we found that the following compounds respect the drug-like profile:, gallic acid, catechin, ferulic acid, caffeic acid, chlorogenic acid, epicatechin, delphinidin, coumaric acid, daidzein, malvidin, quercitin, naringenin etc.

Act. 2.10. Computational pharmacokinetic profiles
Deliverables: Research report

Summary of the research report regarding the identification of the absorption, distribution, toxicity of the studied compounds

At the level of the human body, these compounds: (1) develop hepatotoxicity (exception - hypaconitin, catechin, ferulic acid, caffeic acid, chlorogenic acid, epicatechin, coumaric acid, syringic acid); (2) they are toxic at the mitochondrial level - with the exception of gallic, ferulic, caffeic, coumaric, syringic, rutin, naringin acid. The compounds are not nephrotoxic (exception – delphinidin, coumaric acid, daidzein).

Dissemination of research results

For stage 2/2022, the web page of the project was updated.
Although for the stage 2/2022 no other activities for disseminating the results were foreseen, additionally, the international communication and publication of the results was made, respectively by participating in 2 scientific events, publishing 4 articles in ISI journals and the elaboration of 4 undergraduate theses and 1 doctoral scientific research report.
CONCLUSION: According to this summary report, the activities planned for 2022 have been fully realized, the 100% result indicators being met.

Project leader,
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