ABSTRACT
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Topical nanoformulations of selective vegetal extracts with high antiinflammatory and analgesic properties - NANOGELEXPLORE

Stage 1/2021. Development of extraction and separation methods of phytochemical constituents from *A. toxicum Reichenb., H. odorus* Waldst. & Kit. Ex Willd and *A. nemorosa* L.

Used since ancient times and for the most diverse purposes, species of the Ranunculaceae family are recognized in traditional and conventional medicine as being rich in secondary metabolites with extremely attractive properties, from improving immunity to anti-cancer chemotherapy or reversing resistance to anti-tumor drugs.

Many nations and peoples use different species of Ranunculaceae for the most diverse purposes, from arrow poisoning (Teit, 1900, cited by Turner, 1984), to natural remedies, to the revitalization of unconscious people, to insecticides, etc., but the manipulation of these toxic plants it must be done very carefully, in order to minimize the harmful consequences. The toxicity and therapeutic potential of Ranunculaceae have been written about since the beginning of the last century, but for their exploitation and their safe use in the treatment of some ailments, additional and in-depth *in vitro* and *in vivo* studies and research are needed.

The Ranunculaceae family (Ranunculales order), includes 62 genera and 2525 species (Stevens, 2001; Cossard *et al*., 2016), known since ancient times due to their therapeutic properties due to secondary metabolites abundantly present in these species. The newest studies focus on the use of Ranunculaceae phytometabolites, such as alkaloids, terpenoids, saponins and polysaccharides, in anti-cancer chemotherapy, by blocking the cell cycle and inducing apoptosis of cancer cells (Khurshid *et al*., 2020), by improving immunity (Wu *et al*., 2012), by inhibiting cell proliferation, angiogenesis and metastasis, by reducing or reversing resistance to antitumor drugs (Schink *et al*., 2015). The separation of secondary metabolites from the plant body from their inactive components is carried out by extraction. There are two types of extraction methods: conventional or classic methods and modern methods, also called "green extraction techniques".

There are two types of extraction methods: conventional or classic methods and modern methods, also called "green extraction techniques". The most popular and common extraction technique is maceration. This involves placing the plant material in a container over which the specific solvent is poured until it is completely covered and kept at room temperature for at least three days (Abubakar and Haque, 2020). The maceration extraction technique is suitable for the extraction of thermolabile compounds.
In recent years, there has been an increase in the demand for natural extracts obtained through "green" extraction techniques, non-conventional processing of raw materials. These techniques were also developed to solve the problems that arise when using conventional extraction methods. The "green" technique can be defined as an "extraction method that is based on the discovery and design of extraction processes that will reduce energy consumption, allow the use of alternative solvents and renewable natural products and ensure a safe and high quality extract/product". Modern extraction techniques are microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid extraction.

The stage of development of extraction and separation methods of phytocompounds derived from *A. toxicum* Reichenb., *H. odorus* Waldst. & Kit Ex Wild and *A. nemorosa* L. consisted in the collection and authentication of the plant material, from which extracts were obtained using different solvents (H_2O:EtOH 40:60, H_2O:MeOH 40:60, water) and using four techniques extraction, maceration, ultrasound-assisted extraction (MAE), microwave-assisted extraction (UAE) and extraction with supercritical fluids (SFE) (methanol, cosolvent). Considering all the variables (plant material, solvent, extraction technique) a total of 35 extracts were obtained, which were characterized for the identification of phytocompounds with anti-inflammatory and analgesic properties. Depending on the species, various flavonoids and alkaloids were identified in the obtained extracts.

Magnoflorine and other alkaloids, cardiac glycosides, flavonoids with antitumor properties and cytotoxic activity against human tumor cells have been extracted from Ranunculaceae plants. *Aconitum* sp. have been used in traditional Chinese medicine to treat arthritis, paralysis and heart attack, but also duodenal ulcer and gastroenteritis. Antibacterial activity has been demonstrated on *Staphylococcus aureus* bacteria, the pharmacological effects being due to flavonoids, phenolic compounds, terpenoids, polysaccharides (Hao, 2019; Yin *et al*., 2019). Preparations obtained from anemone rhizomes are used in the treatment of dysentery, ulcers, malaria and other parasitic diseases, pharyngolaryngitis, hepatitis. The effect of the substances in the chemical composition of these plants is anti-inflammatory, antimicrobial, antitumor, sedative and analgesic, but also antihistaminic, anticonvulsant, antipyretic. Due to the high content of these substances, the rhizomes from the *Anemone raddeana* species are used to relieve neuralgia and rheumatic symptoms. *Anemona coronaria* has been widely used as antineuralgic and antirheumatic in traditional medicine in Lebanon (Raafat and El-Lakany, 2018). The powder obtained from the roots of *Pulsatilla chinensis* showed a pronounced antibacterial effect in the treatment of diarrheal syndrome induced in mice by inoculation of enterotoxigenic bacteria *E. coli* O101 (Yu *et al*., 2017). The use of these plants in various therapies is limited by the toxic effect they can have if the right concentrations are not observed (Wang *et al*., 2020).

In order to carry out the research, we used the underground and aboveground parts of the *Helleborus odorus* Waldst species. & Kit. ex Willd., *Anemone nemorosa* L. and *Aconitum toxicum* Reichenb.

The plant material from the species *Helleborus odorus* consisted of rhizomes and the flowering stem, coming from numerically well-represented populations, from several collection points spread over an area of about 30 ha from the site of community importance ROSCI0405, from the Strehaia-Bâtlanele hills, Mehedinti county. The GPS locations of the collection points are: N 44˚ 35' 19.2", E 023˚ 07' 38.1"; N 44˚ 35' 16.5", E 023˚ 08' 11.5"; N 44˚ 35' 13.0", E 023˚ 07' 56.9"; N 44˚ 35' 12.5", E 023˚ 07' 50.7".

The rhizomes and the flowering above-ground part of *Anemone nemorosa* L. were collected on April 25, 2021, as well as the rhizomes and the fruiting above-ground part, on May 6, 2021, from Trivale Forest in Pitesti municipality, with the following GPS location: N 44°51 '09.7" E 24°51'07.8"; N 44°51'17.6", E 24°51'25.5"; N 44°51'13.2", E 24°51'15.1".

Whole plants of *Aconitum toxicum* Reichenb. were collected during the flowering period from Valea Rudăriței (Leaota Mountains) – 31.07.2021, and from Valea Vâlsanului on 13.08.2021 and 20.08.2021.

Among the plants of *A. toxicum* (collected from Cheile Rudăriței), *A. nemorosa* and *H. odorus*, voucher specimens were chosen and pressed, which were registered in the herbarium collection of the Argeș County Museum, receiving the following numbers: *Aconitum toxicum* Reichenb. – specimen voucher no. 11376, *Anemone nemorosa* L. – specimen voucher no. 11377, *Helleborus odorus* Waldst et Kit. – specimen voucher no. 11378.

The rhizomes/ leaves/ flowers were used to obtain extracts with various phytocompounds, especially for the determination of plant parts rich in alkaloids and flavonoids. The solvents used for maceration were ethanol 96º and methanol 96º. Alcoholic extracts were prepared by grinding 10 g of plants in 100 ml of alcohol for 48 h at room temperature.

For MAE, UAE and SFE: solvent ratio used: H2O:EtOH 40:60, H2O:MeOH 40:60; plant:solvent ratio 1:10.

The steps of the primary processing of the plant material: (1) fresh plants were dried at 40 ℃ in a Memmert oven for 48 h; (2) with the help of the KERN MLB50-3N thermal balance, the relative humidity was determined for each part of the plant; (3) dried plants were pre-ground with the RETSCH GM200 laboratory mill for 3 minutes at 4000 rpm and ground for 10 seconds at 10,000 rpm; (4) the required amount of powder was weighed with METTLER analytical balance. MAE was performed for 5 min. in the NEOS MILESTONE GR extraction vessel under continuous stirring, 220W, 63-67 ℃. UAE was performed for 10 min in the cooling extraction vessel of the HIELSCHER UP200ST equipment, under the action of the sonotrode at an amplitude of 72 µm.

The obtained extracts were centrifuged at 6000 rpm for 5 min using the EBA200 - HETTIC device. After centrifugation, the supernatant was vacuum filtered through medium filter paper. Supercritical CO_2 extraction was performed on SFT-110 SFE SYSTEM equipment at the following parameters: CO_2: MeOH 20: 1; 3000 psi; flow rate: 6 mL/min CO_2; 0.3 mL/min MeOH; static extraction: 10 minutes (4 cycles); dynamic extraction: 10 minutes (4 cycles). All extracts obtained were stored at a temperature of 4 ℃.

The extracts to be analyzed were filtered through 0.22 µm filters to remove possible deposits. Filtered extracts were transferred to disposable vials for HPLC analysis. An Agilent 1200 series LC system consisting of a quaternary pump, diode array detector, and autosampler. Analysis of aconitine was performed on a ZORBAX Eclipse plus C18 column (150 mm × 4.6 mm i.d.; particle size 5 µm; Agilent Technologies Inc., USA) at 30 °C. The solvents used for the HPLC separation of the target compound from the samples were acetonitrile (A) and a buffer solution (B) containing 40 mmol/mL ammonium acetate adjusted with ammonia solution at pH 10.0. The flow rate was 1.0 ml/min and the mobile phase elution gradient was 15 - 70% (A) in 0 - 45 min and 70% (A) in 45 - 50 min. Detection was performed at 240 nm. The calibration curve was constructed in the concentration range 0-50 mg/L. The standards used were aconitine, hypaconitine, mesaconitine and magnoflorine. Acetonitrile, water and ammonium acetate were of HPLC grade.

The natural compounds identified in the plant extracts were aconitine, mesaconitine, hypaconitine, magnoflorin, specific flavonoids and other phenolic compounds at 280-300 nm and 300-350 nm.

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STAGE 2/2022. PHYSICAL AND CHEMICAL CHARACTERIZATION OF CRUDE EXTRACTS, NANOSTRUCTURED MIXTURES AND METALLIC NANOPARTICLES. EVALUATION OF BIOACTIVITY OF CRUDE EXTRACTS, ISOLATED PHYTOCOMPOUNDS AND NANOSTRUCTURED MIXTURES

The properties of metal nanoparticles (NPs) and bio-nano-interactions are influenced by a multitude of factors, including elemental composition, size, shape and surface area, surface crystallinity, solid-liquid interface, contact surface with an organic molecule, factors of microenvironment. Depending on this multitude of factors, bio-nano-interactions can induce a wide variety of cellular responses.

Using extracts of *A. toxicum*, *H. odorus* and *A. nemorosa* as reducing agents, 33 experimental variants were developed and defined by 8 variables, the species, the plant organ, the extraction method, the type of solvent used to obtain the extracts, the concentration of the extract and HAuCl4/AgNO3 concentration, time of incubation and temperature of incubation, respectively.

The biosynthesis of metallic NPs was confirmed by the color variation of the extracts, by FTIR and UV-Vis spectral analyses, STEM-EDS analysis and X-ray diffraction analysis. Comparable to the extracts obtained with ultrasound, the DPPH test indicated a higher antioxidant activity of the extracts obtained with microwaves, regardless of the species and the plant organ.

Depending on the biosynthesis method, some extracts supplemented with metal nanoparticles showed phytotoxicity (inhibition of root and stem growth, inhibition of germination, reduction of biomass), cytogenotoxicity (inhibition of cell division, chromosomal aberrations in onion meristematic cells) and hepatotoxicity. The supplementation of the extracts with metal nanoparticles 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 obtained with methanol showed a weaker inhibitory effect than those obtained with ethanol.

Through the experimental model of ear edema induced by xylene, respectively of paw edema induced by carrageenan, it was observed that depending on the method of obtaining the extracts and biosynthesis of the metallic nanoparticles, the anti-inflammatory potential registered important variations, the best results being determined by the extracts obtained by UAE.

A number of 21 compounds were analyzed from a bioinformatic point of view (aconitine, hypaconitine, mesacontine, magnoflorine, gallic acid, catechin, caffeic acid, ferulic acid, chlorogenic acid, epicatechin, delphinidin, coumaric acid, daidzein, hyperoside, rutin, naringin, malvidin, quercitin, naringenin, genistein, syringic acid).

In order to evaluate the character of a possible drug, the compounds were processed in the Expasy database, and tested to comply with the rules of medical chemistry: the Lipinski, Egan, Muegge rules. For the mentioned compounds, the molecular mass, hydrophobicity, weight of hydrogen bond donor/ acceptor atoms, weight of the number of rotatable bonds, polar molecular surface, etc. were calculated. Through the bioinformatics calculation of the drug-like character, we found that some of the analyzed compounds respect the drug-like profile (gallic acid, catechin, ferulic acid, caffeic acid, chlorogenic acid, epicatechin, coumaric acid, quercitin, etc.).

Chemical structures in Smiles format were loaded into MOE software and converted to files.mol2. These files were used for the calculation of physicochemical properties such as flexibility expressed in the number of rotatable bonds, refractivity (Å2), polar molecular surface area (Å2), hydrophobicity and water solubility. The results indicated that the flexibility is high, especially in the series of compounds from the alkaloid class. The compounds have a medium hydrophobicity and show a medium hydrophilic character.

Results on targets indicate high prediction on carbonic anhydrase isoforms, insignificant prediction on aconitines and derivatives.

The predictive results for the ADME-Tox profile showed that most compounds show good intestinal absorption; the compounds are not inhibitors for OCT2/OCT receptors, and in the case of the pharmacogenomic profile, a reduced activity as a substrate/inhibitor at the cytochrome CYP2D6 site was recorded.

A significant number of items representing toxicity were analyzed for the analyzed natural compounds: Ames (mutagenesis), carcinogenicity, toxicity to different species (crustaceae, bees, fish), nephrotoxicity, hepatotoxicity, cardiotoxicity, mitochondrial toxicity, toxicity to nuclear receptors, etc. The results showed that in the human body some compounds induce hepatotoxicity, they are not nephrotoxic and cardiotoxic.

STAGE 3/2023 Development of nanogels loaded with bioactive compounds and AuNPs/ AgNPs. Validation of technology for obtaining functionalized nanogels with natural extracts and metal nanoparticles

Nanogels or nanohydrogels are colloidal systems formed by a three-dimensional network of hydrophilic polymers, which can incorporate active substances into their matrix. Nanohydrogels show advantages as controlled drug release systems, such as physical stability, biocompatibility, solubilization capacity, and protection of active substances from degradation. Nanohydrogels can be applied to the skin, mucous membranes or can be injected into the body, depending on the therapeutic purpose (Chander *et al*., 2021; Wang *et al*., 2023).

To obtain nanohydrogels, it is necessary to choose an optimal formulation of excipients, which ensures the desired properties of the final product. Excipients are substances that do not have a pharmacological effect, but which contribute to the stabilization, preservation, improvement of bioavailability or facilitation of the administration of active substances.

The excipients used in the formulation of nanohydrogels can be classified into three categories: polymers, crosslinking agents and additives (Patel *et al*., 2020).

In the case of nanohydrogels with plant extracts, which have antioxidant, anti-inflammatory, antimicrobial or anticancer properties, the choice of the optimal formulation of excipients is influenced by the following factors: the compatibility between the plant extract and the polymer, the stability of the plant extract under the conditions of preparation and storage, the controlled release of of the plant extract depending on the pH and temperature of the environment, the bioavailability and bioactivity of the plant extract at the site of action (Kyriakoudi *et al*., 2021).

An example of optimal formulation of excipients for obtaining nanohydrogels with plant extracts is based on carbopol, Tween 80 and triethanolamine.

Carbopol is a synthetic polymer, derived from acrylic acid, which has gelling, viscosity, stabilization and mucoadhesive properties. Carbopol can form nanohydrogels by physical cross-linking, by pH variations, or by the addition of electrolytes. Carbopol is biocompatible, biodegradable and non-toxic, being used in various pharmaceutical, cosmetic and food applications (Bonacucina *et al*., 2004).

Tween 80 is a non-ionic emulsifier, derived from oleic acid, which has solubilizing, dispersing, wetting and stabilizing properties. Tween 80 can facilitate the formation of nanogels by reducing the surface tension and by improving the compatibility between the aqueous and oily phases. Tween 80 is biocompatible, biodegradable and non-toxic, being used in various pharmaceutical, cosmetic and food applications (Nielsen *et al*., 2016).

Triethanolamine is an alkaline substance, derived from ammonia, which has buffering, neutralizing, solubilizing and emulsifying properties. Triethanolamine can contribute to the formation of nanogels by adjusting the pH and by favoring the electrostatic interactions between the polymer and the plant extract. Triethanolamine is biocompatible, biodegradable and non-toxic, being used in various pharmaceutical, cosmetic and food applications (URL1).

The use of carbopol and triethanolamine in gel formulations represents a strategic choice for the development of pharmaceutical and cosmetic products with therapeutic properties. These components provide stability, controlled viscosity, optimal consistency as well as an effective release of active substances, thus contributing to the effectiveness and acceptance of the products by users.

To obtain 50 mL of nanogel corresponding to each experimental variant of plant extract, the following were used: 5 mL of each type of extract, distilled water, Tween 80, carbopol and triethanolamine (TEA).

Thus, distilled water was mixed with the extract. Half of this volume was placed in a Berzelius beaker and stirred on the lab plate at 80 °C. When this temperature was reached, 6.25 mL of Tween 80 was pipetted in and allowed to stir for 15 min at the same temperature. The other half of the water-extact mixture was placed in a Berzelius beaker and stirred on the lab plate at 40 °C. When this temperature was reached, the amount of carbopol was added and stirring was continued until its medium solidification. Then, the first mixture was again subjected to agitation on the laboratory plate, also at 80 °C, after which the rest of the amount of Tween 80 was gradually added, continuing the agitation for another 5 minutes. Subsequently, the first mixture was added over the second, under stirring at 80 °C. After the two mixtures were incorporated, TEA was pipetted. The final mixture was homogenized using a laboratory homogenizer for 3 min.

The SEM-EDS analyzes of the gels obtained from the nanostructured phytochemical complexes of *A. nemorosa* L. were carried out using the HITACHI SU500 electron microscope from the Regional Research-Development Center for innovative materials, products and processes intended for the automotive industry (CRC&D - AUTO). The HITACHI SU500 electronic microscope is equipped with a cooling stage capable of bringing the sample down to -30 oC and maintaining it at the preset temperature during the analysis.

The working module in variable pressure (30Pa) and low vacuum allowed the morphological analysis of frozen gels on the cooling stage without the need for sputtering.

The working method was as follows: a gel sample was taken and a tiny amount was spread on the sample support (cooling stage); the sample support (cooling stage) was brought to the temperature of -20 oC until the gel sample froze; the microscope enclosure was closed and brought up to a pressure of 30Pa; SEM analysis of the gel morphology was performed; after the completion of the analysis, the enclosure is brought to atmospheric pressure, the sample holder is cleaned and the analysis is repeated for the next sample. In the following, micrographs at different magnifications obtained for the RAnU2 25% gel sample are presented (Fig. 1).

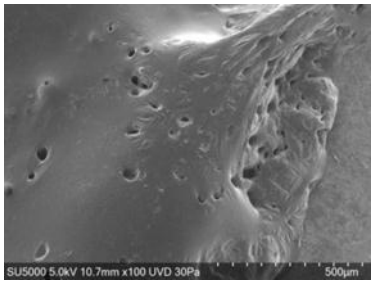

Figure 1. RAnU2-25% pore-gel sample. SEM micrograph x100

For hydroalcoholic extracts, the morphology of the particles is approximately spherical, and the sizes and dispersion differ from one extract to another. In the following, micrographs at different magnifications obtained for the RAnU2T1t1 sample are presented. The SEM-EDS analysis for the hydroalcoholic extracts confirms the presence of Ag particles with nanometric dimensions, below 50nm.

It was found that for the hydroalcoholic extracts the morphology of the particles is approximately spherical and the sizes and dispersion differ from one extract to another. Also, for the hydroalcoholic extracts, the presence of silver particles with nanometric dimensions, below 50 nm, was confirmed.

In vivo evaluation of the anti-inflammatory potential of nanogels in Wistar mice was performed bz two methods, as follow:

• Method of auricular edema. The animals were grouped into 4 experimental groups: the healthy group (treated with distilled water), the control group (treated only with TPA to induce edema), the experimental group (treated with TPA and the experimental gels), and the group treated with TPA and indomethacin.

Ear edema (right ear) was induced by multiple local application (for 6 days, once a day) of 2.5 µg/ear of TPA (12-O-tetradeca noylphorbol-13-acetate) dissolved in 20 µL ethanol (10 µL on both sides of the treated ear) (The gel treatment was done simultaneously with the application of TPA (20mg/day/ear for 6 days). The drug indomethacin (1mg/g/day/ear) was used as a reference of the anti-inflammatory effect. 4 hours after the last administration, the animals were sacrificed by cervical dislocation (after prior anesthesia with 0.01 ml diethyl ether - Sulaiman *et al*., 2008). The right and left ears of mice were weighed immediately (Gou et al., 2017). The difference in edema weight between the right and left ears of the same animal was calculated.

The inhibition percentage was compared with the control group, according to the following formula:

$$\%\ \text{Inhibition} = \frac{\text{edema weight (control)} - \text{edema weighti (test)}}{\text{edema weight (control)}} \times 100$$

The greatest anti-inflammatory effect of the tested gels was found in the gel variant with rhizome extract obtained by UAE and AgNPs incubated for 48 hours at 40 ºC (with a percentage of edema inhibition of 24.1%).

No significant anti-inflammatory differences were found between the application of gels without AgNPs depending on the extraction method, but the percentages of edema inhibition were lower compared to the administration of the extracts by gavage.

• Testing the anti-inflammatory effect through the experimental model of paw edema induced by carrageenan. The gels made from the tested *A. nemorosa* rhizome extracts, with and without phytosynthesized AgNPs showed anti-inflammatory action in the experimental carrageenan-induced paw edema model, except for the extract made with the help of microwaves (without phytosynthesized metal nanoparticles), for which the results were inconclusive. And in this case, the best anti-inflammatory results were obtained for the extracts made by the UAE method.

Overall, the percentage increase in paw volume was lower in the gel variants (compared to the anti-inflammatory results of the extracts reported in the earlier phase of the project).

Another aim of the experiments carried out in the present research was also to test the antimicrobial effect of nanogels in which were incorporated plant extracts of *A. nemorosa* rhizome obtained by MAE and UAE, with or without phytosynthesized nanoparticles, on six microbial strains (two fungal from the genus *Candida* and four bacterial: three Gram positive and one Gram negative) reference. The 6 experimental variants are presented in Table 1, and the reference microbial strains used are presented in Table 2.

The method used to test the antimicrobial effect of gels with plant extracts was an adapted variant of the Kirby-Bauer diffusimetric method (Balouiri, 2016), in which sterile filter paper discs were used instead of standard discs impregnated with antibiotics deposited the gels in a thin layer (in two repetitions), but also a diffusimetric method using wells made in the solid medium layer.

In addition to the gels with plant extracts, the following were used: the negative control (Tween 80) and the positive controls represented by the antibiotic (Gentamicin 10 µg/disc) and antifungal (Fluconazole 25 µg/disc) in the form of standardized microtablets.

Table 1. Experimental variants for testing the antimicrobial effect of nanogels

Experimental variants	**Extract type**	**$AgNO_3$ (mM)**	**Incubation temeprature (°C)**	**Incubation time (h)**	**Code**
RAnU2 25%	UAE	-	-	-	**1**
RAnU2T1t1		5mM (U2)	22°C (T1)	24h (t1)	**3**
RAnU2T2t1			40°C (T2)	24h (t1)	**4**
RAnM2 25%	MAE	-	-	-	**2**
RAnM2T1t1		5mM (M2)	22°C (T1)	24h (t1)	**5**
RAnM2T2t2			40°C (T2)	48h (t2)	**6**

Table 2. Reference microbial strains used

Reference microbial strains	The symbol used	The medium used for testing the antimicrobial effect
Staphylococcus aureus ATCC 25923	S.a.	Geloza Müeller Hinton
Bacillus subtilis ISM 68/53 (echivalent *ATCC 6633*)	B.s.	Geloza Müeller Hinton
Escherichia coli ATCC 25922	E.c.	Geloza Müeller Hinton
Streptococcus pyogenes	S.p.	Mueller Hinton Agar +5% Ram Blood
Candida albicans ATCC 10231	C.a.	Sabouraud
Candida parapsilosis ATCC 22019	C.p.	Sabouraud

Experiments performed with *A. nemorosa* rhizome extracts in the absence of AgNPs did not show antibacterial effect in previous experiments. The gels with such extracts used in the present experiment (coded 1 and 2) did not determine an antimicrobial action by the disc diffusimetric method, neither on the bacteria nor on the tested yeasts. Instead, the gel variants that had plant extracts with AgNPs in their composition exerted an antimicrobial effect highlighted by an area of inhibition.

The gel of variant 4 (RAnU2T2t1) had an effect on 3 of the 4 bacterial strains, and the gel of variant 6 (RAnM2T2t2) produced a slight inhibition of two bacterial strains (*S. aureus* and *B. subtilis*) and the strain of *C .parapsilosis*. Only one bacterial strain (Gram-negative) was slightly inhibited by Tween 80, but all inhibition zones determined were much lower than those produced by positive controls (antibiotic/antifungal). Although the extracts of *A. nemorosa* with AgNPs had a stronger antimicrobial effect when they were obtained with microwaves, in the form of gels those obtained with the help of ultrasound acted more intensively, especially through an incubation at 40 °C.

In the case of the well method, there was a slight inhibition of *E. coli* and *B. subtilis* bacteria exerted by the gels with extracts not supplemented with AgNPs (6.5 mm diameter), but the antimicrobial effect was more obvious in the case of those with AgNPs. Again gel variant 4 inhibited bacterial growth more than the other variants tested. The tested yeasts were not sensitive to the action of the gels used.

In vitro testing of plant extracts before and after phytosynthesis of metal nanoparticles using normal and tumor human cell cultures, quantitative analysis for the alkaline comet assay, respectivelly, showed an insignificant increase in the comet tail size after treatment with *A. nemorosa* extracts, with and without AgNPs indicating the absence of DNA damage. Quantification of the tail moment confirmed its decrease in the experimental variants characterized by the presence of AgNPs, the lowest value being characteristic of the RAnU2T2t2 sample. At the same time, the amount of DNA in the head of the comets was higher in the case of cells treated with A. nemorosa extracts supplemented with AgNPs, compared to the raw extracts, suggesting their genoprotective effect.

Predicting the benefits of topical nanoformulations of selective plant extracts with anti-inflammatory and analgesic properties

Inflammation is associated with various diseases, and persistence of inflammation systemically indicates dysfunction and damage to various organs.

Except for the anti-inflammatory and analgesic effects, the expected benefits of topical nanoformulations of selective plant extracts include:

- increasing the skin penetration capacity of hydrophilic/lipophilic active substances and controlling the release of active phyrocompounds;
- metal nanoparticles can reduce skin inflammation induced by allergens;
- due to the composition rich in phenolic compounds, it exerts an antioxidant effect, protecting the skin from the attack of free radicals that are formed under the effect of UV radiation and that can cause cell damage;
- antibacterial effects and prevention of wound infection;
- improving the bioavailability of active principles;
- protection of active principles against enzymatic and hydrolytic degradation and improvement of stability.

Documentation regarding the benefits of topical nanoformulations of selective plant extracts with anti-inflammatory and analgesic properties, proposals and projections of future studies

Among the tested extracts, those of *A. nemorosa* stood out for the highest content of rutin, catechin and malvidin. Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid for which a series of pharmacological activities have been demonstrated, such as antioxidant, cytoprotective, vasoprotective, anticancer, cardioprotective activities, including the prevention of neuroinflammation (Khan *et al*., 2009; Ganeshpurkar and Saluja, 2017). Also, catechins derived from plants ensure the stabilization of the anti-inflammatory response based on an excellent antioxidant activity and are effective in suppressing short- and long-term inflammatory stress (Kim and Heo, 2022).

Moreover, the most pronounced antioxidant activity (DPPH) was determined for the RAnU2 variant, defined by the hydromethanolic extracts obtained from the rhizome of *A. nemorosa* by UAE.

For all the analyzed samples, after the phytosynthesis of metallic nanoparticles, the presence of Ag particles with nanometric dimensions, below 50 nm, was confirmed.

At the same time, the extracts of *A. nemorosa* had a more pronounced inhibitory effect against the Gram negative and positive bacteria tested compared to the other extracts obtained from *H. odorus* and *A. toxicum*.

A. nemorosa extracts supplemented with AgNPs expressed an obvious inhibitory effect in all experimental variants compared to extracts without AgNPs, in the absence of AgNPs the antimicrobial effect was null. *The E. coli* strain was the least sensitive to the action of *A. nemorosa* extracts, and the most pronounced antibacterial effect was exerted on the *K. pneumoniae* strain. By comparison with the antibiotics used as positive controls, namely Amikacin 30 µg for *S. aureus*, *B. subtilis*, *K. pneumoniae* and Gentamicin 120 µg for *E. coli*, the antimicrobial action of the extracts was weaker.

Regarding microwave extracts of *A. nemorosa*, in the absence of AgNPs (RAnM), the antimicrobial effect was absent. For all other extracts obtained the antimicrobial effect was present.

By incubating cultures of S. aureus and *B. subtilis* (Gram positive bacteria) with sonicated *A. nemorosa* rhizome extracts, inhibition zones of up to 14 mm were formed. The temperature applied for the synthesis of AgNPs in *A. nemorosa* extracts influenced the antibacterial effect exerted by them on the Gram positive bacteria used.

Regarding the Gram negative bacteria tested, it was found that the extracts of *A. nemorosa* had a more pronounced inhibitory effect on the *K. pneumoniae* strain than on the *E. coli* strain.

Important to emphasize is the fact that all tested *A. nemorosa* rhizome extracts, with and without phytosynthesized AgNPs, showed anti-inflammatory action in the experimental model of xylene-induced ear edema and carrageenan-induced paw edema. The best results were obtained for the extracts made by the ultrasonic method. Also, 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 globular percentage (hematocrit). A slight increase in the number of figured elements was recorded in the case of the administration of plant extracts containing phytosynthesized nanoparticles.

Physico-chemical characterization and quantification of the anti-inflammatory and analgesic properties of alkaloids and flavonoids from the extracts of *A. toxicum*, *H. odorus* and *A. nemorosa*, standardization of the phytosynthesis of metal nanoparticles, definition of the most effective excipients according to the active biocompounds and the type of metal nanoparticles they made a significant contribution to the enrichment of knowledge in the field. The project represents an important source of information for the standardization of methods for the extraction and isolation of alkaloids and flavonoids from members of the Ranunculaceae family.

The results of this project also **open other research directions**: *in vitro* micropropagation of *A. toxicum*, *H. odorus* and *A. nemorosa* species in order to control the content of active constituents, holistic *in vivo* approaches, changes in the transcriptome and proteome, and clinical trials, industrial-scale production of useful drugs and new products in the field of smart specialization. The results of the project direct the valorization of local natural resources and revitalize traditional medicine in accordance with modern medicine.

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THERE WERE NO FAILURES REGARDING THE ESTIMATED RESULTS

• The estimated impact of the results obtained, emphasizing the most significant result obtained.

The integration of data resulting from experiments on the extraction and separation of phytochemical principles (alkaloids, flavonoids), phytosynthesis of metal nanoparticles, *in vitro*, *in vivo* and *in silico* evaluation of the bioactivity of extracts and nanoformulations and from the study of the pharmacodynamics and pharmacokinetics of active biocompounds made possible the

selection and improvement of the anti-inflammatory and analgesic properties of the topical nanoformulations of *A. nemorosa*. The most significant result obtained in the conducted research was the highlighting of a significant anti-inflammatory and analgesic effect of the extracts obtained from the rhizomes of *A. nemorosa* by UAE, with and without biosynthesized AgNPs. It is important to emphasize that the most important anti-inflammatory and analgesic activity was determined by the RAnU2T2t2 nanoformulations, obtained by incubating *A. nemorosa* extracts with 5 mM $AgNO_3$ solution for the biosynthesis of AgNPs, at a temperature of 40 °C, for 48h. At the same time, this nanoformulation, as well as the corresponding nanogel, also induced the most important antimicrobial effect, as well as the lowest cytotoxicity on normal human fibroblasts. The screening of the specialized literature highlights the fact that this is the first study that demonstrates through *in vitro*, *in vivo* and *in silico* studies the anti-inflammatory and analgesic potential of the extracts obtained from the rhizomes of *A. nemorosa* and their nanoformulations.

The project allowed the development of a human cell culture laboratory and the creation of a competitive research team, able to approach, develop and quantify the study of selective extracts with anti-inflammatory and analgesic properties and increase research excellence. Thus, further studies on the manipulation of metal nanoparticles to control the release of active phytocompounds and improve the bioavailability of the active principles, the protection of the active principles against enzymatic and hydrolytic degradation and the improvement of stability, as well as the development of antiallergic/ antitumor/ antioxidant/ antimicrobial nanoformulations can be approached by means of the new equipment laboratory equipment and research team members.

Promotion and dissemination of results at the project level

Within the project, numerous activities took place to promote and disseminate the results obtained at the project level, respectively: 2 workshops that marked the opening and closing of the project, participation in 19 national and international scientific events, publication of 13 scientific papers in ISI journals, 4 book chapters and the submission to OSIM of two patent applications. Also, the project was promoted through the web page http://nanogelexplore.upit.ro/diseminare_en.html.

Project manager,
(Şuțan Nicoleta Anca, Signature)