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

SCIENTIFIC AND TECHNICAL REPORT SUMMARY

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

Act. 1.1. Analysis of the scientific literature to strengthen the scientific framework for extraction and characterization Ranunculaceae derived natural products and the effects of extracts on *in vitro* and *in vivo* unicellular/ multicellular organisms.

Deliverables: Research report

Summary of the research report on methods of extraction and characterization of natural compounds derived from Ranunculaceae and their effects on unicellular and multicellular organisms *in vitro* and *in vivo*

There are two types of extraction methods: conventional or classical 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. Modern extraction methods include microwave-assisted extraction (MFA), ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE). Various extraction methods have been used in order to obtain the highest possible yield of phytochemicals, from certain representatives of the Ranunculaceae family. In brief, Baharetha *et al*. (2013), using SFE (60℃, 2500 psi) extracted thymokinone and thymohydroquinone compounds from *Nigella sativa* L. Abedi *et al.* (2017), using MAE, demonstrated that the essential oil obtained from *N. sativa* is clearly superior in terms of the yield of the compound thimoquinone and its antioxidant capacity, that obtained by the classical method of hydrodistillation. UAE has been shown to be highly effective in obtaining a significant amount of berries and palmatines of the species *Coptis chinensis* Franch. (Li *et al*., 2021).

The characterization of natural products derived from Ranunculaceae by spectrophotometric methods allows a good characterization of pigments, in quantitative determinations of solutions of transition metal ions and conjugated organic compounds involving photon spectroscopy in the UV-VIS region. For classes of biologically active compounds, determinations at specific wavelengths are often performed. Measurements shall be made either by measurement at a certain wavelength or by measurement in the wavelength range.. In the case of characterization of natural products by liquid chromatography, the phytochemical groups are extracted with the help of different solvents according to their polarity, and certain plant compounds can be recovered from the solid matrix with specific solvents. The range of polarities of plant secondary metabolites is very varied and there is no ideal solvent capable of fully extracting the target compounds. In addition, the additional characteristics of the solvents may bring restrictions or advantages in their use. Thus methanol, ethanol, acetone or mixtures thereof are a good approximation for the "ideal solvent", but methanol is a weak solvent for highly apolar compounds, and is a reactive solvent (forms methyl acetals), and acetone is a weak solvent for glycosylated compounds or for saponins and/ or anthocyanins (Toderaş *et al*., 2015; Jäntschi andNaşcu, 2009).

Magnoflorine and other alkaloids cardiac glycosides, flavonids with antitumor properties and cytotoxic activity against human tumor cells were extracted from Ranunculaceae plants (Kubo *et al*., 2015; Jung *et al.*, 2015). In traditional medicine *Trollius* species are used for their antibacterial effects in treating upper respiratory tract infections, pharyngitis, bronchitis, otitis media. Phenolic compounds are present in *T. chinensis* flowers and have an antibacterial effect (Hao, 2019). *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 bacteria of the species *Staphylococcus aureus*, the pharmacological effects being due to flavonoids, phenolic compounds, terpenoids, polysaccharides (Hao, 2019; Yin *et al*., 2019). Preparations obtained from rhizomes of *Anemone* 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 antihistamine, anticonvulsant, antipyretic. Due to the high content of these substances, the rhizomes of *Anemone raddeana* are used to relieve neuralgia and rheumatic symptoms. *Anemona coronaria* has been widely used as an antineuralgic and antirheumatic in traditional medicine in Lebanon (Raafat and El-Lakany, 2018). The powder obtained from the roots of *Pulsatilla chinensis* showed an antibacterial effect highlighted in the treatment of diarrhea syndrome induced in mice by inoculating 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). Şennazlı and Matur (2017) investigated histopathological changes in the liver, heart and skin of rats injected subcutaneously with plant material, aqueous extract and methanolic extract of *Heleborus orientalis*. Outbreaks of hepatocytes and myocardial degeneration in rats that received plant material and aqueous extract, liver necrosis, hyaline degeneration, myocardial edema, and vacuolar areas in some cells

were observed 10 days after administration. Ethanolic extract of root of *Thalictrum foliolosum* given as a single oral dose was not toxic to Wistar rats (Marslin, 2020).

Bibliografie

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Act. 1.2. Collection and preservation of plant material, characterization of biotope and pedoclimatic conditions, preparation and registration of voucher specimens.

Deliverables: Research report,Vouher specimens

Summary of the research report on the collection and preservation of plant material, characterization of biotope and pedoclimatic conditions, preparation and registration of voucher specimens

***Helleborus odorus* Waldst & Kit Ex Wild** rhizomes and the flowering stem were collected from several points spread over an area of about 30 ha: 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 area belongs to the Strehaia Hills, a subunit of the Getic Plateau, characterized by a temperate continental climate with Mediterranean influences, the average temperature is 1.95 °C. The highest rainfall falls in May, and the lowest in February-March. During our trip on March 5, 2021, the air temperature was 17 ° C, the soil temperature was 10 ° C and all the *H. odorus* plants were in bloom. The dominant soils are luvisols and molisols. The dominant ecosystem in the collection area is represented by oak forest (*Quercus petraea, Q. cerris, Q. frainetto*) mixed with *Tilia tomentosa, T. platyphyllos, T. cordata, Carpinus betulus, Acer campestre, A. platanoides, A. tataricum,* etc. with the well reprezented shrub layer (*Crataegus monogyna, Cornus mas, C. sanguinea*, etc.) and grass layer, which in the areas of where the material was collected had a coating with *H. odorus* of approx. 30%.

***Anemone nemorosa* L.** rhizomes and the flowering above-ground part were collected on April 25, 2021, and rhizomes and the fruiting above-ground part, on May 6, 2021, from Trivale Forest, Piteşti: 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". The soil is of podzolic, clay-sandy, compact, permeable and fertile type. The average annual temperature of the researched region is 9.8 ° C. The average multiannual pressure is approx. 980.3 mm Hg. The collection area the present species are especially *Q. petraea* - 40%, *Q. robur* - 30%, to which is added *Fagus sylvatica* - 15% and in a small percentage (15%) other species.

***Aconitum toxicum* Reichenb.** whole plants were collected from several locations - from Rudăriței Valley (Leaota Mountains), on the date of collection - 31.07.2021, and from Vâlsanului Valley on 13.08.2021 and 20.08.2021.

The point of collection of *A.toxicum* plants from Rudăriţa is located at the edge of a predominantly deciduous forest where sporadically there are also conifers, which fix a calcareous debris, the phytocenoses belonging to the habitat 91V0 Dacian beech forests (*Symphyto-Fagion*): N 45°24´29,4"; E 25°15´50,2.The soil is slightly acidic, shady, with moderate humidity, the slope has a degree of inclination of about 45-50^o, the altitude being 1010 meters. The structure of the vegetal carpet is typical for the association *Pulmonario rubrae-Fagetum sylvaticae*. The collection point in the Vâlsan Valley are located in the Vâlsan Valley Protected Area - mixed nature reserve corresponding to UICN category IV and NATURA 2000 site: N 45°21'33.7"; E 24°43'32.1" with an elevation of about 890 meters. The substrate consists of a weakly stabilized calcareous marly rubble, interspersed with discontinuous portions of soil, covered from place to place

with lithoclasts. The harvested plant material was cleaned of impurities, divided into rhizomes, leaves and flowers, washed with tap water, rinsed with distilled water and stored in the freezer or dried at room temperature.

Among the collected the voucher specimens were chosen, pressed and registered in the herb collection of the Argeș County Museum (Fig. 4), receiving the following numbers: ***Aconitum toxicum*** **Reichenb. –**no. **11376; *Anemone nemorosa* L. –** no. **11377; *Helleborus odorus* Waldst et Kit. –** no. **11378.**

Act. 1.3. Research and experimental development of methods of extraction of phytochemicals from *A. toxicum* Reichenb., *H. odorus* Waldst. & Kit. Ex Willd and *A. nemorosa* L. to improve the therapeutic properties of crude extracts

Deliverables: Laboratory methodology, phytochemical constituents

Summary of laboratory methodology of extraction of phytochemicals from *A. toxicum* Reichenb., *H. odorus* Waldst. & Kit. Ex Willd and *A. nemorosa* L. – MAE; UAE; SFE

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

For MAE, UAE and SFE: the solvent ratio used: H_2O:EtOH 40:60, H_2O:MeOH 40:60; plant ratio: solvent 1:10.

Stages of primary processing of plant material: (1) fresh plants were dried at 40 ℃ in the Memmert oven for 48 hours; (2) with the help of the KERN MLB50-3N thermal balance, the relative humidity for each part of the plant was determined; (3) the 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 the METTLER analytical balance. Microwave-assisted extraction was performed for 5 minutes in the extraction vessel of NEOS MILESTONE GR under continuous stirring, 220W, 63-67℃. Ultrasound-assisted extraction was performed for 10 minutes 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 minutes using the EBA200 - HETTIC device. After centrifugation, the supernatant was filtered under vacuum through filter paper with medium filtration. Supercritical CO_2 extraction was performed on the SFT-110 SFE SYSTEM equipment at the following parameters: CO_2:MeOH 20: 1; 3000 psi; flow rate: 6 mL / min CO2; 0.3 mL / min MeOH; static extraction: 10 minutes (4 cycles); dynamic extraction: 10 minutes (4 cycles). All the extracts obtained were kept at a temperature of 4 ℃.

Act. 1.4. Isolation of phytochemical constituents from crude extracts of *A. toxicum Reichenb., H. odorus Waldst. & Kit. Ex Willd and A. nemorosa L.*

Deliverables: Laboratory methodology, phytochemical constituents

Summary of laboratory methodology on characterization of phytocompounds from crude extracts of *A. toxicum* Reichenb., *H. odorus* Waldst. & Kit Ex Wild and *A. nemorosa* L.

The extracts to be analyzed were filtered through 0.22 μm filters to remove any potential deposits. The extracts thus filtered were transferred to disposable vials for HPLC analysis. An Agilent 1200 series LC system was used to analyze the aconitine in the extracts, consisting of a quaternary pump, a diode array detector, and an automatic sampler. Aconitine analysis was performed on a ZORBAX Eclipse plus C18 column (150 mm × 4.6 mm i.d.; 5 μm particle size; 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 elution gradient of the mobile phase 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 range of 0-50 mg / L. The standards used were aconitine, hypaconitin, mesaconitin and magnoflorin. For the analysis of magnoflorin in the extracts, the detection was performed at 320 nm. Acetonitrile, water and ammonium acetate were of HPLC quality.
Natural compounds identified in plant extracts were aconitine, mesaconitin, hypaconitin, magnoflorin, specific flavonoids and other phenolic compounds at 280-300 nm and 300-350 nm, phenolic acids and their derivatives (flavones, flavonols, phenylpropene, quinones) - 250-450 nm and anthocyanins.

Dissemination of research results

For stage 1/2021, the web page of the project was created: http://nanogelexplore.upit.ro/#.
Although for the stage 1/2021 no other activities for disseminating the results were foreseen, additionally, the national and international communication and publication of the results was made, respectively by organizing the opening workshop of the project, participating in 4 scientific events, publishing 2 articles in ISI journals and publication of 2 chapters in books.
CONCLUSION: According to this report, the activities planned for 2021 have been fully realized, the 100% result indicators being met.

Project leader,

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