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New therapeutic opportunities in the treatment of chronic venous disease – nanoformulations with vegetal extracts

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INTRODUCTION

Chronic venous disease is one of the most frequent vascular conditions, bipedal position of the human body, as adaptive-evolutionary stage, being one of the main contributors to the affection. Incipient phases of clinical manifestations are often ignored or included into the context of other conditions, therefore the disease progresses to the most advanced phases (eg. varicose veins, ulcerations). All the signs and the symptoms are the consequence of cellular metabolism imbalances and are summed up by favoring the prooxidant, procoagulant and proinflammatory processes. Therapeutic options are varied, but invasive methods are often used, which lead to recurrences. Plant sources have always represented a starting point for vascular pathologies remedies. Among all, flavonoids are remarcable, as the main recommendation of therapeutic guidelines. Also, compressive therapy stands out and along with the administration of flavonoids, it is recommended for prophylactic purposes even after invasive therapies.

The association of several forms of therapy and several active ingredients, with the aim of annihilating the physiopathological mechanisms as comprehensively as possible, is a premise for research. Patient adherence to treatment is a primary factor in therapeutic success. This can be improved by designing formulations easy to administer by accessible and non-invasive routes such as transdermal, which could be used concurrently with compression materials (compression bandages/stockings).

The current research approach is based on the following main objectives: analysis of the present state of knowledge with the selection of plant sources with therapeutic potential; obtaining extracts and phytochemical characterization (qualitative, quantitative); the study of extracts' stability; highlighting the therapeutic implications (antioxidant activity, potential for interaction with targets at cellular level); cytotoxicity analysis (*in vivo*, *in vitro* and *in silico*); integration of extracts into nanoformulations with biopolymers; physico-chemical characterization of formulations; investigating the possibility of functionalizing a compressive material and dissemination of the results in the scientific literature.

In this context, the present work represents a preliminary stage of investigating the possibility of developing a complex pharmaceutical product as a first step towards new therapeutic opportunities for the treatment of chronic venous disease.

CURRENT STATE OF THE ART

1. GENERAL ASPECTS ABOUT CHRONIC VENOUS DISEASE

In this chapter was established the general context of chronic venous disease, supplemented by the description of the mechanisms by which the condition develops at the level of the vascular endothelium, of the vascular smooth muscles as well as at dermal level. The main therapeutic possibilities included in the management guidelines were described and also the implications of plant derivatives.

The definition of chronic venous disease is as follows: any morphological and functional abnormality of the venous system, of long duration, manifested by signs and symptoms that indicate the need for investigations and/or care [1]. According to therapeutic guidelines, the staging is made up through the CEAP classification (Clinic Etiologic Anatomic Pathophysiologic) [2–4]. In Romania, a study (carried out between June and July 2015 and published in 2018), highlighted a prevalence of 68.4% with an incidence of 36.9% [5].

The pathophysiology of chronic venous disease is complex, it is summarized by the triggering and self-feeding of oxidative, inflammatory and procoagulant processes. The mechanisms within this pathological cascade act, sometimes simultaneously, sometimes successively, in a damaging direction (figure 1.1.). Endothelial imbalance progresses to dermal destruction (tissue necrosis).

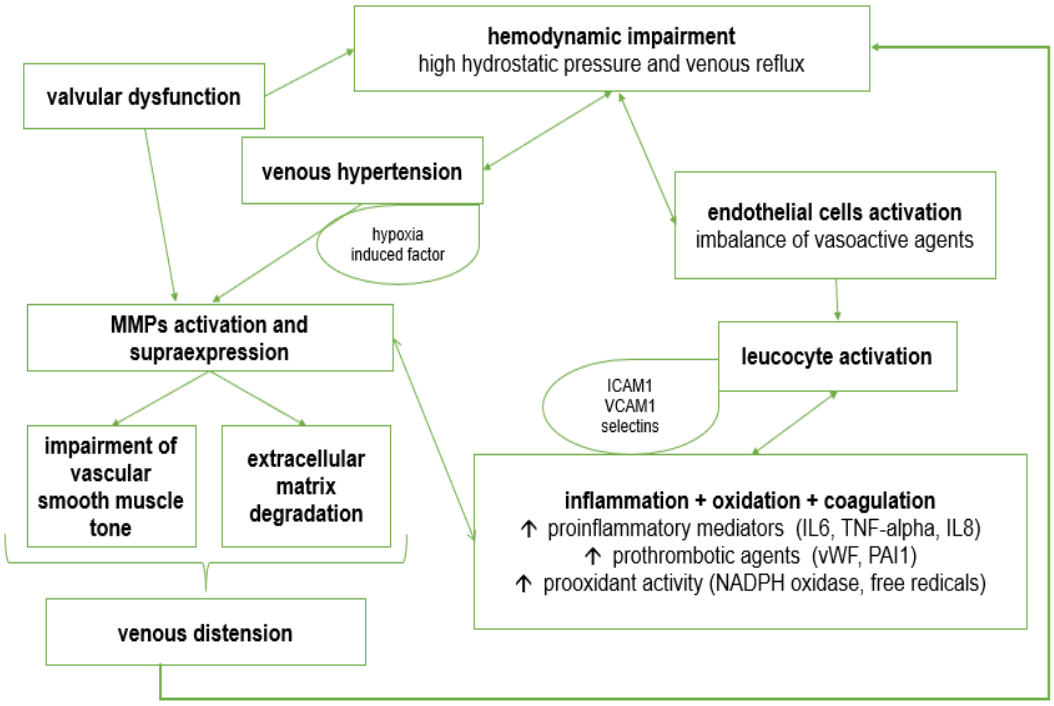


Figure 1.1. Vascular cascade of chronic venous disease, adapted from Mansilha et al. [6].

As for therapeutic measures, they are varied and are established according to the pathology stage. Compression therapy is the first-line recommendation (class I, level of evidence A/B) [2]. From the pharmacological point of view, purified and micronized flavonoid fractions (MPFF) are used as the basic medication (recommendation class IIa, level of evidence A) [2]. Among the invasive therapies, applied in the advanced phases, the following stands out: sclerotherapy (with liquid or foam) [7], laser therapy (EVLA) [6], endovenous ablation [8], surgical therapy as stripping or CHIVA ligation technique [9,10]. For the most advanced stages characterized by chronic ulceration, debridement and antibiotic treatments are practiced.

MPFFs remain a standard in the treatment of chronic venous disease [11], but there are also considered other therapies based on natural sources (eg. *Ruscus* extract [12], chestnut extract [13–15], red wine leaf extract [16], hydroxyethylrutoside complexes [17,18]).

The implications of plants in treatment can be summarized as follows:

- antioxidant activity: capture of free radicals (eg. singlet oxygen, superoxide, hydroxyl, peroxides), chelation of metal ions (eg. Fe^{2+}, Fe^{3+}), inhibition of catalytic enzymes (eg. xanthine oxidase), regeneration of cells' own antioxidant systems [19];
- anti-inflammatory activity: blocking signaling systems for inflammatory processes, inhibiting enzymes involved in stimulating monocytes and neutrophils to produce cytokines (eg. protein-tyrosine kinase is inhibited by genistein and beta-glucuronidases are strongly inhibited by luteolin, kaempferol, apigenin, quercetin) [20]; inhibition of cyclooxygenases (COX1, COX2), lipoxygenase (LOX) and iNOS with decreased production of pro-inflammatory cytokines (IL6, IL1-beta, IL8) and TNF-alpha; inhibition of nuclear factor NF-kB [21];
- antithrombotic activity: inhibition of adhesion molecules ICAM1 and VCAM1 expression, inhibition of platelet activation factor (PAF) (eg. ginkgolide B [22]), GPIIb/IIIa receptors interferences (eg. naringenin, naringin, coumarin, esculetin, fraxetin [23]);
- other actions: restoration of venous tone through vasoconstriction-vasodilatation balance (eg. inhibition of COMT metabolism), inhibition of high ET1 production, collagen stabilizing effects for vascular deformity prevention [22,24,25], favoring veno-lymphatic drainage [24], wound healing (eg. quercetin [26], *Calendulae flos* extracts [27,28] and *Centella asiatica* extracts [22,26]), reduction of matrix metalloproteinases activity (eg. rutoside, diosmin [29]).

Thus, plant sources have proven to be intensively studied for the treatment of vascular diseases and constitute a continuous research field.

2. NANOTECHNOLOGIES WITH PLANT DERIVATIVES AND TOPICAL-TRANSDERMAL APPLICATIONS

The chapter outlines the implications of plant sources in nanotechnology, general aspects regarding the topical-transdermal route and examples of applications reported in the specialized literature.

During time, several nanosystems with natural derivatives have been developed [30]: nanoparticles (nanospheres, nanocapsules, solid lipid nanoparticles - SLN, nanostructured lipid carriers - NLC), conventional liposomes or coated with chitosan and/or pectin (eg. secondary and tertiary liposomes [31]), ethosomes, transfersomes, invasomes, niosomes. Different types of extracts were integrated: aqueous (eg. from *Centella asiatica*, (nanoparticles), *Hibiscus sabdariffa* (liposomes), *Camelia sinensis* (nanoemulsion)), alcoholic (eg. from *Curcuma longa* (liposomes, etosomes, transfersomes), *Fraxinus angustifolia* (etosomes), *Vitis vinifera* (nanoemulsions)), hydroalcoholic (eg. from *Polygonum aviculare* (liposomes), *Glycine max* (nanoemulsions), *Salvia officinalis* (nanoparticles)) and oily (eg. from *Eucalyptus camadulensis* (liposomes), *Artemisia arborescens* (liposomes), *Melaleuca alternifolia* (nanoemulsion)) [32]; particular nanoformulations, called phytosomes (silybin (hepatoprotective), curcumin (anticancer), naringerin (antioxidant), quercetin (antioxidant) [33,34]).

Nanosystems' development intended for topical-transdermal administration gained advance based on two important arguments: a non-invasive way with a high degree of adherence, especially in the case of chronic conditions, and ensuring the release of the active substances at the site of action, especially for dermatological conditions [35]. There are three penetration possibilities: intercellular, transcellular, transfollicular. The transfollicular pathway has long been considered less important because hair follicles represent only 0.1% of skin surface [36–38], but it has been found that they can be permeation or storage routes [36]. The most advanced nanoformulations under research are the ultra-deformable type such as: transfersomes, invasomes, niosomes, ethosomes, aiming at their integration into complex pharmaceutical products (eg. gels, microneedles with transfersomes [39]).

Most studies have focused on the obtaining and analysis of nanoformulations with isolated active principles (ex. quercetin [40], rutoside [41]), less on extracts as a phytocomplex. Their results highlight significant research perspectives in this direction.

PERSONAL CONTRIBUTIONS

3. OBTAINING AND PRELIMINARY CHARACTERIZATION OF SOME VEGETAL EXTRACTS WITH POTENTIAL VASCULAR AND VENOPROTECTIVE ACTION

The experimental part begins with obtaining extracts belonging from the selected plant sources: *Sophorae flos*, *Ginkgo bilobae folium*, *Meliloti herba*, *Calendulae flos,* followed by the qualitative and quantitative analysis of the resulting extracts with the aim of highlighting the active principles of therapeutic interest.

3.1. The technological process for obtaining vegetal extracts – pilot line

Materials and method

A reflux extraction was performed with ethanol 80% (for *Sophorae flos,* rutoside has better solubility in concentrated alcohol [42]), respectively ethanol 70% (for *Ginkgo bilobae folium*, *Meliloti herba* și *Calendulae flos*) followed by rotary evaporator concentration and lyophilization.

Results and discussion

Dry extracts were obtained as powders with particular organoleptic properties depending on the vegetal source.

3.2. Determination of the obtained vegetal extracts' quality

The experimental activity carried out aims to identify and quantify the main phytoconstituents.

3.2.1. Qualitative screening by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS)

Materials and method

Used system: FTICR-MS solariX-XR QqqFTICR HR (Bruker Daltonics) with 15T superconducting magnet [43,44], electrospray ionization (ESI negative, ESI positive), liquid samples obtained by dissolving extracts in water and methanol. Ionization patterns were generated by Bruker Compass Data Analysis software. The compounds were identified in the spectrograms by comparison with the ionization patterns [43].

Results and discussion

The most compounds were identified for *Meliloti herba* extract - EM (16 compounds: rutoside, robinin, kaempferol-O-glucoside, kaempferol-O-rutinoside, isoquercitrin/hyperozide, coumaric acid, melilotic acid, medicarpin, apigetrin/afzelin, ferulic acid, isorhamnetin/rhamnetin, fisetin/kaempferol/luteolin, quercetin, quercitrin-O-rutinoside,

rosmarinic acid, daidzein/chrisin), followed by *Ginkgo bilobae folium* extract - EG (12 compounds: rutoside, bilobalide, quercetin, ginkgolide (A, B, C), kaempferol, isoquercitrin, isorhamnetin/rhamnetin, catechin, bilobetin, caffeic acid), then *Calendulae flos* extract - EC (10 compounds: rutoside, narcissin/calendoflavoside, thyphaneoside, chlorogenic acid, isoquercitrin, isorhamnetin/rhamnetin, quercetin, calendoflavoside (H, F/G, E)) and *Sophorae flos* extract - ES (8 compounds: rutoside, quercetin, isorhamnetin/rhamnetin, kaempferol/fisetin, isoquercitrin, sophoricoside, kaikasaponin I, kaikasaponin III). Correlated with data from the literature, these compounds have multiple implications in the treatment of chronic venous disease and shows therapeutic potential to the obtained extracts. Taking into account the belonging of the identified compounds to several phytochemical classes (phenolcarboxylic acids, flavonoids, polyphenols), the consecutive research steps are based on quantitative determinations for phytochemical classes.

3.2.2. Quantitative spectrophotometric determinations

Materials and method

The Jasco V-530 spectrophotometer was used. The principles of the methods are as follows [45,46]:

- phenolcarboxylic acids (AFC): the formation of nitroso derivates with Arnow reagent and tautometization to red oximes (λ = 510 nm);
- flavones (FLV): the formation of yellow chelates with greenish fluorescence (λ=427 nm) in the presence of trivalent metal ions (Al^{3+});
- polyphenols (PF): oxidation of polyphenols under the action of Folin-Ciocâlteu reagent (Na_2WO_4/Na_2MoO_4), blue coloration (λ=725 nm);

The samples consists of successive dilution from extract solutions (extracts dissolved in extraction's solvent). The results were calculated based on standard curves, obtained under the same experimental conditions.

Results and discussion

The extracts contain important amounts of active principles of interest (AFC, FLV, PF). The results follow the sequences: 1. AFC (chlorogenic acid equivalents): ES (17.1399%) > EC (3.8073%) > EG (2.1722%) > EM (1.6453%); 2. FLV (rutoside equivalents): ES (37.4473%) >> EM (3.0600%) > EG (2.3765%) > EC (2.0129%); 3. PF (tannic acid equivalents): ES (28.4903%) > EG (7.2344%) > EC (4.9029%) [43]. For *Meliloti herba* extract, the polyphenol content could not be determined by the applied method due to the abundant precipitation. The recorded values for *Sophorae flos* extract are significantly higher than the rest of the extracts for all classes of active principles analyzed (especially flavones, values 10 times higher).

3.2.3. UHPLC-HRMS/MS chromatographic analysis

Materials and methods

The Thermo Scientific Dionex Ultimate 3000 UHPLC system with Chromeleon 7.2 (Thermo Fisher Scientific) software was used. Stationary phase: C18 column (l=150 mm, diameter 2.1 mm, particle size 2.6 μm). Mobile phase: A (ultrapure water + 500 μL/L formic acid) and B (methanol + 500 μL/L formic acid). Gradient elution was applied: 100% A 0-1 min; linear increase to 30% B 1-10 min; linear increase to 100% B 10-26 min, hold 4 min then linear decrease to 0% B 30-32.5 min and hold 2.5 min. Analysis parameters: 10 μL sample volume, mobile phase flow rate 0.3 mL/min, 35 min, 40°C.

Mass spectrometric system: Q-Exactive (Thermo Fisher Scientific) spectrometer with HESI-Heated ElectroSpray Ionization source. Ionization parameters: carrier gas flow rate (nitrogen) 8 units, auxiliary gas flow rate 6 units, capillary temperature and auxiliary gas heating temperature 300°C, voltage 2800 V, S chamber and RF level 50 [43,47]. Was used a fragmentation template generator software (ACDLabs MS Fragmenter 2019.2.1) [43,47].

Results and discussion

The qualitative analysis was carried out by general screening related to a series of approximately 150 compounds that could be present in plant matrices, belonging to different phytochemical classes.

In *Sophorae flos* extract (ES), 43 compounds belonging to the following phytochemical classes were identified: 35 flavonoids (6-methoxyluteolin, afrormosin, apigenin-7-O-glucosylglucoside, apigenin-7-rutinoside, apigenin, chryosoeriol-7-glucoside, cinnaroside (luteol 7-glucoside), diosmetin, phlorizin, formononetin, gallocatechin/epigallocatechin, genistin, ginkgetin, glycitein, hesperitin, hispidulin-7-rutinoside/isomers, hispidulin-O-glucoside/isomers, hyperoside (quercetin-3-galactoside), isoquercitrin/quercitrin (quercetin-3-rhamnoside), isorhamnetin-3-O-hexoside, isorhamnetin-3-O-rutinoside, kaempferol, kaempferol (or luteolin)-O-glucoside/isomers, myricetin, naringenin, naringin, pseudobaptigenin, quercetagetin (6-hydroxyquercetin), quercetin-3-O-rutinoside, quercetin, rhamnosyl-vitexin, robinin, rutoside (quercetin-3-rutinoside), sissotrin (biochanin-7-O-β-D-glucoside), sophoraflavanone G/isomers), 6 carboxylic acids (azelaic acid, caffeoylshikimic acid, hydroxyferulic acid, p-coumaric acid, quinic acid, syringic acid) and 2 terpenes (abscisic acid, calenduloside G/isomers) [43].

For *Ginkgo bilobae folium* extract (EG) were found 42 compounds, classifed as follows: 32 flavonoids (2',6-dihydroxyflavone, 6-methoxyluteolin, amentoflavone, apigenin-7-O-glucosylglucoside, apigenin, baptigenin, cinnaroside (luteol 7-glucoside), chryosoeriol-7-

glucoside, daphnin, diosmetin, epicatechin, gallocatechin/epigallocatechin, ginkgetin, glycitein, hispidulin-O-glucoside/isomers, hyperoside (quercetin-3-galactoside), isoquercitrin/quercitrin (quercetin-3-rhamnoside), isorhamnetin-3-O-glucoside, isorhamnetin-3-O-hexoside, isorhamnetin-3-O-rutinoside, kaempferol, kaempferol (or luteolin)-O-glucoside/isomers, naringenin, naringin, narirutin (naringenin-7-O-rutinoside), quercetin-3-O-rutinoside, quercetin, robinin, rutoside (quercetin-3-rutinoside), sophoraflavanone G/isomers, tricin, vitexin (apigenin-8-C-glucoside)/isovitexin), nine carboxylic acids (azelaic acid, caffeoylshikimic acid, chlorogenic acid, gallic acid, hydroxyferulic acid, p-coumaric acid, quinic acid, syringic acid, cynarin (1,5-di-O-caffeoylquinic acid)) and a triterpenic compound (cinaropicrin) [43].

Meliloti herba extract (EM) comprises 32 compounds distributed by phytochemical classes as follows: 25 flavonoids (apigetrin (apigenin-7-glucoside), cirsimarin, chrysin, cumestrol, daidzein, diosmetin-7-O-rutinoside (diosmin), diosmetin, epicatechin, phlorizin, formononetin, hispidulin, hyperoside (quercetin-3-galactoside), irilone, kaempferol (or luteolin)-O-glucoside/isomers, kaempferol-3-O-rutinoside, liquiritigenin/isoliquiritigenin, medicarpin, naringenin, ononin, pratensein, procyanidin B1/B2, pseudobaptigenin, quercetin-3-O-rutinoside, robinin, rutoside (quercetin-3-rutinoside)), six carboxylic acids (azelaic acid, caffeoylshikimic acid, chlorogenic acid, ferulic acid, p-coumaric acid, cynarin (1,5-di-O-caffeoylquinic acid)) and a sesquiterpene (abscisic acid).

For *Calendulae flos* extract (EC), 35 compounds belonging to different classes were identified as follows: 17 flavonoids (apigenin-7-O-glucosylglucoside, apigenin, cinnaroside (luteolin-7-glucoside), chryosoeriol-7-glucoside, hispidulin-O-glucoside/isomers, hyperoside (quercetin-3-galactoside), isoquercitrin/quercitrin (quercetin-3-rhamnoside), isorhamnetin-3-O-hexoside, isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside, kaempferol-O-glucoside/isomers, naringenin, procianidin, quercetin-3-(6-malonyl)-glucoside, quercetin, robinin, rutoside (quercetin-3-rutinoside)), 11 carboxylic acids (azelaic acid, cafeic acid, caffeoylshikimic acid, chlorogenic acid, coumaroylquinic acid/isomers, ferulic acid, p-coumaric acid, quinic acid, sinapic acid, syringic acid, cynarin (1,5-di-O-caffeoylquinic acid), five terpenes (abscisic acid, calenduloside E/isomers, calenduloside G/isomers, carnasol, lactucopicrin) and two coumarins (cichorin, daphnin) [43].

From quantitative point of view, mainly flavonoid compounds and phenolcarboxylic acids were determined. In *Calendulae flos* extract, the highest amounts are represented by chlorogenic acid (20676.63 µg/g extract), isorhamnetin (11286.93 µg/g extract), rutoside (2165.42 µg/g extract). In *Ginkgo bilobae folium* extract were found high amounts of

isorhamnetin (5032.60 µg/g extract), quercetin (4504.66 µg/g extract) and rutoside (3907.47 µg/g extract). *Meliloti herba* extract was rich in rutoside (5233.24 µg/g extract), hyperoside (1318.87 µg/g extract) and p-coumaric acid (641.99 µg/g extract). *Sophorae flos* extract showed high content of rutoside (104186.77 µg/g extract), isorhamnetin (97049. 32 µg/g extract) and quercetin (46678.34 µg/g extract). Among all compounds quantified, five were found in all extracts: p-coumaric acid (ES>EG>EM>EC), gallic acid (ES>EG>EC>EM), hyperoside (ES>EM>EG>EC), rutoside (ES ≅2×EM≅3×EG≅5×EC) and abscisic acid (EM>ES>EG>EC).

The results recorded in the chromatographic analysis confirm the chemical composition of the extracts which is appropriate for their implications in the treatment of vascular damage.

3.2.4. Extracts' stablility

Materials and method

Stability was determined by analyzing the variation of active principles amount over 12 months, with an intermediate determination at 8 months. The determinations were made according to the work techniques described for the spectrophotometric determinations. Statistical analysis: ANOVA and t-Student test, considering statistical significance for $p<0.05$.

Results and discussion

All analyzed extracts showed relatively good stability during a 12-month conditioning period even if the amounts of active principles followed statistically significant decreases either in the first 8 months or in the last 4 months, depending on the extract and the initial amount of active principles. For three of the analyzed extracts (ES, EG, EC) polyphenols had the most important variations. Although phenolcarboxylic acids can be considered more unstable, no significant variations were recorded, possibly due to the lower concentration of these active principles; only for the ES extract was a greater variation observed, but their quantity is several times higher (5-8 times) compared to the other extracts. For all extracts, the flavones have good stability, despite the variations, they remained within acceptable ranges.

4. *IN VITRO*, *IN VIVO* AND *IN SILICO* RESEARCH

This chapter is structured based on two directions: 1. evaluation of the therapeutic implications with emphasis on antioxidant activity and inhibition of matrix metalloproteinases and 2. evaluation of cytotoxicity considering the fact that a cytotoxic intake would accentuate the physiopathological mechanisms of chronic venous disease.

4.1. *In vitro* antioxidant action determination

The antioxidant action is one of the most important for the therapeutic impact of the analyzed extracts. It can develop in several ways, therefore the application of several determination methods leads to an overall perspective on the antioxidant activity [43].

Materials and method

The analysis was performed *in vitro* by three spectrophotometric methods (Jasco V-530):

1. ferric reduction power (FRAP): the reaction of the antioxidant in the sample with Fe^{3+} resulting in Fe^{2+} which has blue colour (λ=700 nm) [43];
2. inhibition of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH): the reduction of DPPH free radical (purple) to the corresponding yellow hydrazine (λ=515 nm) [43];
3. inhibition of the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS): reducing the intensity of the colour (turquoise) of the ABTS radical (λ= 734 nm) [43];

Results and discussion

The extract of *Sophorae flos* is noteworthy, with the lowest concentration necessary to ferric reduction, EC_{50}=129.38 μg/ml, followed by the extract of *Calendulae flos*, with the value EC_{50}=437.00 μg/ml, then the extract of *Ginkgo bilobae folium*, EC_{50}=480.55 μg/ml, and the highest value belongs to the *Meliloti herba* extract, EC_{50}= 871.20 μg/ml. Thus, the best antioxidant activity obtained by the FRAP method belongs to the extract of *Sophorae flos*, and the lowest is attributed to the extract of *Meliloti herba*. Regarding DPPH radical inhibition, the best inhibitory effect was also recorded for *Sophorae flos* extract (IC_{50}=94.78 μg/ml), then *Ginkgo bilobae folium* extract (IC_{50}=518.49 μg/ml) and *Calendulae flos* extract (IC_{50}=806.79 μg/ml), and the lowest effect for *Meliloti herba* extract (IC_{50}=972.30 μg/ml). Regarding ABTS radical inhibitory activity, *Sophorae flos* extract also shows the best activity (IC_{50}=71.51 μg/ml), followed by *Ginkgo bilobae folium* extract (IC_{50}=193.89 μg/ml) and *Meliloti herba* extract (IC_{50}=234.84 μg/ml); the lowest activity belongs to *Calendulae flos* extract (IC_{50}=369.34 μg/ml) [43].

Correlations were made between the results of active principles determinations (by UHPLC and spectrophotometry) and those recorded by the three *in vitro* antioxidant methods. Related to the flavonoid (FLV) and polyphenolic (PF) content, a very strong correlation was recorded ($|r| > 0.9$) for all methods of antioxidant activity assessment. About phenolcarboxylic acids (AFC) and the results from the UHPLC determination, a very strong correlation ($|r| > 0.9$) was obtained for FRAP and DPPH, respectively a strong correlation ($0.7 \leq |r| \leq 0.89$) for ABTS. Among the three applied methods, the FRAP method showed a statistically significant correlation related to the determination by UHPLC ($p = 0.004$, $p < 0.05$) as well as to the determination of phenolcarboxylic acids ($p = 0.022$, $p < 0.05$) and flavones ($p = 0.047$, $p < 0.05$). Even if not all correlations evaluated have statistical significance, very strong and strong correlations were observed between these methods which confirms the importance of determining antioxidant activity by several methods not only by one [43].

Negative Pearson coefficient values were noted between the amount of active compounds and antioxidant activity, suggesting an inverse correlation. Thus, the value of the inhibitory concentration on oxidative processes (assessed by IC_{50} or EC_{50}) is lower if the extract contains a high quantity of active compounds, showing a superior antioxidant action.

4.2. Activity on matrix metalloproteinases *in silico*

According to literature data, damage from chronic venous disease leads to increased expression and activity of matrix metalloproteinases. Even if MMP intervention is important in the reepithelialization process, their overexpression facilitates pathological processes [43]. In this context, it is proposed to evaluate the activity of some components from the extracts at the MMP level.

Materials and method

The crystal structures of the catalytic domains for the target proteins in complex with known inhibitors were prepared for docking (YASARA Structure [48]): MMP-1 (PDB ID: 1HFC, 1.50 Å resolution [49]), MMP-2 (PDB ID: 1HOV [50]), MMP-3 (PDB ID: 4G9L, 1.88 Å resolution [51]), and MMP-9 (PDB ID: 1GKC, 2.30 Å resolution [52]). The virtual library of the identified compounds was prepared, based on SMILES codes from PubChem. 3D structures were obtained using DataWarrior 5.2.1 [53]. AutoDock Vina v1.1.2 [54] docking algorithm was used. Predicted molecular interactions between ligands and target proteins were analyzed by BIOVIA Discovery Studio Visualizer (version 17.2.0, Dassault Systemes, 2016).

Results and discussion

The inhibition of matrix metalloproteinases is mainly based on the interaction with catalytic zinc. Although rhamnetin did not interact directly with the catalytic zinc, it had the

highest predicted affinity for MMP-1. Sophoricoside had the best affinity for the other isoforms (MMP-2, MMP-3, MMP-9), but formed metal bonds only with the catalytic zinc of the MMP-9 isoform. Seven phytocompounds interacted with catalytic zinc for MMP-1 (calendoflavoside, calenduloside G, isoquercitrin, kaikasaponin III, chlorogenic acid, narcissin, sophoricoside) and for MMP-2 (narcissin, calendoflavoside, calenduloside G and H, kaikasaponin III, ginkgolide B and rutoside). Bonds with the catalytic zinc for MMP-3 made ten compounds: calendulosides (E, F, G), narcissin, thyphaneoside, ginkgolide C, isoquercitrin, kaikasaponin III, catechin and rutoside. The most interactions with catalytic zinc were recorded for MMP-9 by 12 phytocompounds: calendoflavoside, thyphaneoside, narcissin, calendulosides (E, F, G), ginkgolides (A, B, C), kaikasaponin I, rutoside and sophoricoside. Among all analyzed phytocompounds, narcissin and calenduloside G achieved interactions with catalytic zinc at the level of all four MMP isoform. Thus, each phytocompound has a different affinity and establishes its own system of binding with various types of MMPs. MMP isoforms are involved in the damage from chronic venous disease, and vegetal extracts, assimilated as phytocomplexes of active compounds, can inhibit MMP isoforms through the polyvalence of the interactions developed by their constituents [43].

4.3. Cytotoxicity studies

An additional imbalance produced by the administration of cytotoxic substances must be avoided in order not to propagate endothelial destruction. Cytotoxicity studies are useful for isolated natural compounds, but especially for plant extracts that are phytocomplexes [55]. Some active compounds (eg. flavones) can be both antioxidants and prooxidants, depending on concentration, cell type, cell culture conditions or pre-existing pathological context [55,56]. Thus, the cytotoxicity of the extracts was studied, in the current research, in three ways: *in vitro*, *in vivo*, *in silico*.

Materials and method

1. *in vitro*: the study on endothelial cell lines type HPAEC (Primary Pulmonary Artery Endothelial Cells, ATCC, PCS-100-022) by MTT test with colorimetric purple formazan formation (Tecan Infinite 200 Pro reader, λ= 570 nm)[55];
2. *in vivo*: BSLA test – *Brine Shrimp Lethality Assay* using *Artemia salina* species (ARTOXKIT protocol), three types of effects were followed: lethal, behavioral and cytological [55];
3. *in silico*: prediction of cell membrane diffusion: performed with PerMM (Permeability of Molecules across Membranes) [57]; docking simulations with the following targets: caspase-3 (PBD ID: 1QDU [58]), caspaze-8 (PDB ID: 1F9E [59])

as homodimers in complex with peptide inhibitors and the c-Myc/Max heterodimer in complex with DNA (PDB ID: 1NKP [60]) extracted from the RCSB PDB database [61].

Results and discussion

On HPAEC cell lines, *Ginkgo bilobae folium* extract was the most cytotoxic (IC_{50}=11.17 µg/ml), followed by *Sophorae flos* extract (IC_{50}=44.37 µg/ml), then *Calendulae flos* extract (IC_{50}=66.43 µg/ml), and the *Meliloti herba* extract proved to be the least cytotoxic (IC_{50}=136.87 µg/ml).

Even if through the BSLA test, from a cytological point of view, body deformations and cell inclusions were observed, the analyzed extracts did not affect the viability of *Artemia salina* larvae at concentrations below 1200 µg/mL, so they can be considered non-toxic [55].

The results of *in silico* studies showed low permeability for: calendoflavoside, calendulosides (G, H), chlorogenic acid, rutoside, sophoricoside, and better permeability for: calenduloside (E, F), isorhamnetin, ginkgolide (A,B,C), bilobalide, bilobetin, quercetin. After diffusion through the membrane, the analyzed compounds are localized at the mitochondrial level. Interactions with components of the apoptotic pathway were also highlighted: caspase-3, caspase-8 and the c-Myc-Max heterocomplex [55].

The *in silico* analysis focused more on the cytotoxicity of the compounds from *Ginkgo bilobae folium* extract, because according to the *in vitro* (HPAEC) and *in vivo* (BSLA) results, it proved to be the most cytotoxic, thus bilobalide and bilobetin proved potential caspase-3 activators, and ginkgolides potential inhibitors. The compounds from the *Calendulae flos* extract are also remarked: the calendulosides had cytotoxic potential (caspase activators), but their presence in the phytocomplex does not seem to show significant cytotoxicity as the *Calendulae flos* extract was the least cytotoxic according to *in vivo* analyzes (BSLA) and weakly cytotoxic *in vitro* (HPAEC), compared to the other extracts. Quercetin, isorhamnetin and rutoside, present in all the extracts obtained, can imprint cytotoxicity, but according to the *in silico* results they have limited implications. Therefore, the analyzed extracts can be considered suitable for integration into complex pharmaceutical forms to highlight their therapeutic potential.

5. PRELIMINARY STUDY OF EXTRACTS' INTEGRATION IN NANOFORMULATIONS AND DEVELOPMENT OF A PROTOTYPE OF TOPICAL-TRANSDERMAL PHARMACEUTICAL PRODUCT

This chapter outlines the inclusion of extracts in nanoformulations based on biopolymers (PHB, PLGA), the physico-chemical characterization of the formulations and the analysis of the possibility of impregnation on the surface of a compression stocking matrix.

5.1. The process of obtaining nanoformulations and pH determination

Materials and method

Formulations with extracts were made by oil-water emulsification and solvent evaporation. Oil phase: nanoparticle-forming polymer (PHB, PLGA, PHB:PLGA 1:1 m/m mixture, Merck) dissolved in chloroform. Aqueous phase: ES, EM, EC, EG and mixture of extracts 1:1:1:1 dissolved in ethanol. 100 ml of 3% polyvinyl alcohol was added to the mixture of the two phases. Evaporation of the solvent was carried out by maintaining for 2 hours at 1000 rpm (magnetic stirrer) [62]; pH was determined using indicator paper (Merck).

Results and discussion

15 suspension type samples with particles dispersed in polyvinyl alcohol are obtained. Drying in a thin layer on a flat surface at room temperature (12h) resulted in a polymer film loaded with extract-biopolymer particles. All samples have a pH of 5.5, adequate for topic-transdermal administration [63].

5.2. Extensibility assay

Materials and method

Ojeda-Arbussa method was used: device consisting of two overlapping plates, with millimeter scale on the outer side of the lower plate [63,64]. The results were expressed as the surface on which the sample is spread under the action of known weights.

Results and discussion

Regarding biopolymers, the samples with PHB had better stretchability than those with PLGA. The largest spreading surface was noted for the EC-PHB sample (141.72 ± 7.24 cm^2). The association of PHB with PLGA in the polymer mixture reduces the extensibility of PHB, the values corresponding to formulations with PHB-PLGA 1:1 being intermediate (PHB>PHB-PLGA>PLGA) [63]. Regarding the extracts, samples with EC and EG had better stretchability than those with EM and ES. The hydroxyl (-OH) and carboxylic (-COOH) groups present in the extracts' phytoconstituents molecules can have a significant contribution to the extensibility by forming bonds with the polymer chains. Also, the molecules of natural compounds are large

in size and through steric interaction can change the conformation and flexibility of polymer chains [65,66].

Even though variations were noted depending on the biopolymer and the extract, all samples show adequate extensibility for spreading on the skin and on the surface of the compression stocking material.

5.3. Attenuated total reflection Fourier transform infrared spectroscopy analysis (ATR-FTIR)

Materials and method

FTIR analysis was performed using the JASCO FTIR-4200 spectrophotometer with ATR-PRO450-S accessory (Tokyo, Japan). The spectra were recorded in the range of wave numbers 4000-400 cm^{-1}, resolution of 4 cm^{-1}[63,67].

Results and discussion

The spectra of the basic components of the formulations are shown in figure 5.1.

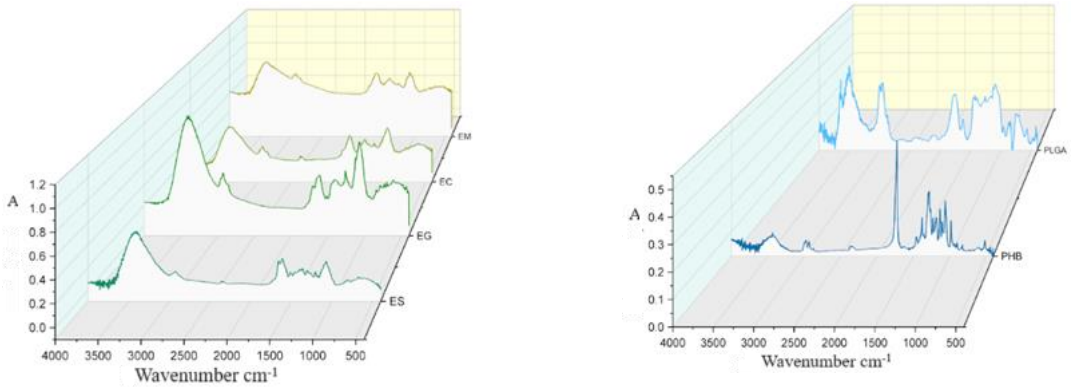
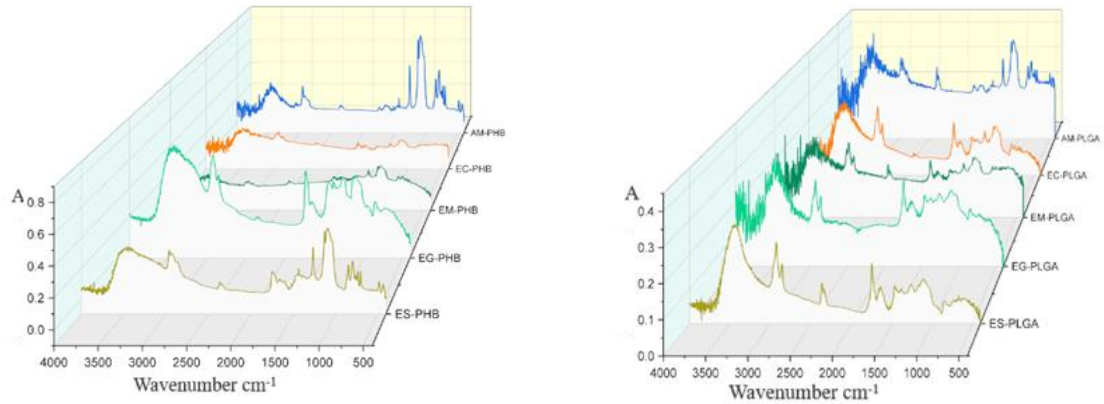


Figure 5.1. Basic components ATR-FTIR spectra. ES = *Sophorae flos* extract; EG = *Ginkgo bilobae folium* extract; EM = *Meliloti herba* extract; EC = *Calendulae flos* extract; AM = extracts mixture 1:1:1:1; PHB = polyhydroxybutirate; PLGA = polylactic co-glycolic acid.

Formulations spectra are shown in figure 5.2.



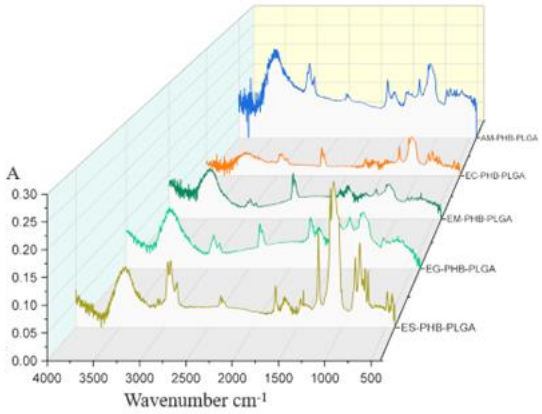


Figure 5.2. Extract-biopolymer formulations' ATR-FTIR spectra. ES = *Sophorae flos* extract; EG = *Ginkgo bilobae folium* extract; EM = *Meliloti herba* extract; EC = *Calendulae flos* extract; AM = extracts mixture 1:1:1:1; PHB = polyhydroxybutirate; PLGA = polylactic co-glycolic acid.

Treating the spectra comparatively: the spectra of the base compounds of the formulations vs. the spectrum of the formulation, changes in the intensity of the peaks can be observed. Interactions between extracts and biopolymers can be varied considering the phytocomplex that defines the extract and associate into changes in vibrational levels. At the same time, both shifts of the peaks at higher and lower wave numbers are noted. For example, in the case of the ES-PHB formulation, for the region 4000-2500 cm^{-1}, the characteristic peaks of the O-H vibration for ES (3407.9 cm^{-1}) and for PHB (3430.1 cm^{-1}) both have a lower value than the peak of the ES-PHB formulation (3461.7 cm^{-1}). Among all the formulations obtained, the most visible reduction in the intensity of the bands is noted for the formulations with EC. It can be explained on the basis of the high content of chlorogenic acid (determined by quantitative UHPLC-HRMS/MS analysis), which through the presence of -COOH groups can be involved in several interactions with polymers resulting in the reduction of the intensity of -OH peaks.

All these peak shifts suggest interactions between extracts and biopolymers with the possibility of complex formation (attributed to changes in peak intensity) or adducts (highlighting of new peaks or major changes of already existing peaks indicating new bonds).

5.4. X-ray diffraction analysis (XRD)

Materials and method

The analysis was performed with the Rigaku Ultima IV diffractometer (Rigaku Co., Tokyo, Japan) in geometric parallel field with CuKα radiation (λ = 1.5406 Å), divergence slit 1.00 mm with limitation to 10 mm, 2θ analysis between 10° and 60°, at a speed of 2°/min with a step of 0.02°. The analysis was carried out on the samples as polymeric film [63,67].

Results and discussion

The obtaining of predominantly amorphous formulations was highlighted. The lack of crystalline molecular organization can be an advantage through better solubilization and faster release of the active component from the structure compared to crystalline formulations [68]. The results are consistent with those reported in the literature, for example it was observed that

both quercetin and catechin, compounds also found in the analyzed extracts, interact with the polymers being dispersed in the polymer matrix in non-crystalline phase [69].

5.5. Atomic force microscopy analysis (AFM)

Materials and method

Analysis was performed using XE-100 atomic force microscope (Park Systems Corporate) with decoupled XY/Z scanner and NSC36B tip (MicroMasch) operated in non-contact mode with the following characteristics: radius of curvature less than 8 nm, cone angle 40°, length 90 μm, width 32 μm, thickness 1 μm, height 15 μm, with the resonance frequency of 130 kHz. Image recording was done with the XEI program (v 1.8.0, Park Systems) [63,70].

Results and discussion

The results confirm the hypothesis of obtaining nanoformulations and outline a complex perspective for their topographic and roughness characteristics.

Among the formulations with PHB, ES-PHB has the most compact and uniform appearance, EM-PHB, EG-PHB, EC-PHB are characterized by larger pores, and for AM-PHB, nanostructures with irregular edges such as aggregates are observed. The roughness varied as follows: EC-PHB>EM-PHB>AM-PHB>ES-PHB>EG-PHB (scale 8×8 μm^2) and AM-PHB>EC-PHB>ES-PHB>EM-PHB>EG-PHB (scale 2×2 μm^2).

For PLGA formulations, ES-PLGA and EM-PLGA show large nanostructures (400-500 nm), and EG-PLGA and EC-PLGA small nanoparticles (30-40 nm). The highest value for roughness belongs to ES-PLGA at both scales, indicating the roughest surface. The roughness variation sequence at 8×8 μm^2 is: ES-PLGA>AM-PLGA>EG-PLGA>EC-PLGA>EM-PLGA, and at 2×2 μm^2 is: ES-PLGA>AM-PLGA>EM- PLGA>EG-PLGA>EC-PLGA, for samples with EM, EG and EC the values being similar. Regarding PHB-PLGA (1:1) formulations, EM-PHB-PLGA presents the least defined porosities, being mainly characterized by small nanoparticles. The roughness variation is: AM-PHB-PLGA>ES-PHB-PLGA>EM-PHB-PLGA>EG-PHB-PLGA>EC-PHB-PLGA>EG-PHB-PLGA (scale 8×8 μm^2) and ES-PHB - PLGA>AM-PHB-PLGA>EG-PHB-PLGA>EC-PHB-PLGA>EM-PHB-PLGA (scale 2×2 μm^2).

Samples with mixture of extracts are the most complex, therefore their topographic appearance differs significantly. The larger scale allowed the examination of features over a larger area, uniformity is important especially for the formation of thin films resulting from sample drying. The narrower scale (2×2 μm^2) allowed the analysis in detail with the highlighting of aggregates that suggest information about the stability of the samples and that can cause the change of features on a larger scale by the formation of larger nanostructures. The

topographic aspects of the samples can subsequently impact the possibility of adhesion and impregnation on the compression stocking matrix.

5.6. Analysis of the possibility of impregnation on the compression stocking material

Materials and method

Immersion of the compression stocking matrix (2×2 cm^2 pieces) in the nanoparticle suspension, keeping in contact for different times (30 min, 60 min and 90 min) and evaluation by weighing and stereomicroscopic analysis.

Results and discussion

No important differences in mass were observed between immersion times: 30 min ≅ 60 min ≅ 90 min, therefore a period of 30 minutes is sufficient. Through the stereomicroscope analysis of the dried pieces of material, the arrangement of the nanoformulations as a polymer film on the surface of the compression stocking material was noted.

5.7. Thermogravimetric analysis (TGA)

Materials and method

The Mettler Toledo thermogravimeter TGA-SDTA851e (Mettler-Toledo, Greifensee, Switzerland) operated at the following parameters was used: air flow below 80 mL/min, heating rate of 10°C/min [63].

Results and discussion

The thermogravimetric analysis had bidirectional utility, on the one hand the stability of the nanoformulations was highlighted, on the other hand it allowed the evaluation of the degree of impregnation. Nanoformulations showed gradual mass loss in the range 25-115°C (volatile solvent loss) and in the range 200-650°C (characteristic for organic substances). At the same time, by the difference between the mass losses, the impregnation capacity on the matrix of the compression stocking was evaluated. The variation was in the range of 2.7-12.4%. Among the 15 samples analyzed, only two samples (EM-PHB and EG-PHB-PLGA) had impregnation below 5%, the majority (11 samples) led to a degree of impregnation in the range of 5-10% and two samples (EM -PHB-PLGA and EM-PLGA) exceeded 10%.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

General conclusions

The current research, as a preliminary analysis in the development of a product useful in the treatment of chronic venous disease, aimed in obtaining and phytochemical characterization of some extracts derived from plant sources with therapeutic potential. At the same time, the study aims to evaluate the activity and cytotoxicity as well as the inclusion in complex formulations with biodegradable polymers alongside the investigation of the possibility of functionalizing a compression stocking matrix.

Based on the data from the specialized literature, four plant sources noted for their antioxidant, anti-inflammatory, veno-lymphatic draining, capillary-protective and promoting reepithelialization properties were selected: *Sophorae flos* (known especially for its rutoside content), *Ginkgo bilobae folium*, *Meliloti herba* and *Calendulae flos*. Four dry extracts (ES, EG, EM, EC) were obtained by ethanol extraction and then lyophilization. The phytochemical screening carried out on the extracts led to the successful identification of compounds of therapeutic interest, some being common (e.g. rutoside, quercetin, isoquercetin) and others being particular to certain extracts: sophoricoside (ES), bilobalide, bilobetin, ginkgolide (EG) , melilotic acid (EM) and calendulosides (EC). From a quantitative point of view, all the extracts present important amounts of flavones, phenolcarboxylic acids and polyphenols, but the *Sophorae flos* extract stands out for the highest values, especially for the flavone content (37.45%). Compared to the active principles identified (UHPLC-HRMS/MS), for ES, the most important quantities are found for: rutoside, isorhamnetin and quercetin. The other extracts are also not to be neglected as they have important amounts of active principles.

From stability's point of view, over a period of 12 months, all the analyzed extracts showed a relatively good stability, with the flavonoid content recording the least variations.

In order to highlight the implications in the treatment of chronic venous disease of the obtained extracts and to evaluate the cytotoxicity, *in vitro*, *in vivo* and *in silico* analyzes were carried out. Three *in vitro* methods were applied to determine the antioxidant activity, revealing the best results for *Sophorae flos* extract. For the other extracts, the antioxidant activity follows the sequences: EC>EG>EM (FRAP), EG>EC>EM (DPPH) and EG>EM>EC (ABTS). The value of the radical inhibitory concentration (IC_{50} or EC_{50}) is lower as the extract has a higher concentration of active principles (inverse correlation assessed by negative Pearson coefficient). *In silico*, interference with the zinc-catalytic area of MMP (isoforms MMP1, MMP2, MMP3, MMP9) was noted for some active constituents in the extracts (eg.

isoquercitrin, narcissin, catechin, sophoricoside). The inhibition of these enzymes can be considered a complementary mechanism to the antioxidant activity for the annihilation of pathological processes in chronic venous disease.

In vitro cytotoxicity analysis, by MTT assay on endothelial cell lines (HPAEC type), revealed that, after 72 hours of exposure to the analyzed extracts, cell viability was affected in the following order: EG>ES>EC>EM. *In silico*, the possibility of intracellular penetration, of reaching intracellular targets and of interaction of some phytoconstituents from the extracts with possible apoptotic pathways was studied. Thus, the best cellular permeability belongs to the following compounds: calendulosides, isorhamnetin, ginkgolide, bilobalide, bilobetin and quercetin. At the same time, mitochondrial penetration and interactions with caspase-3, caspase-8 and the c-Myc-Max heterocomplex were highlighted, which could explain the cytotoxic implications. *In vivo*, by BSLA test (*Artemia salina*) cytological changes were observed at 24h: body deformations, loss of cuticle adhesion (ES), cellular inclusions (EG, EC, EM), but for none of the extracts were revealed lethal effects on larvae at concentrations below 1200 μg/mL, concluding that they are non-toxic.

The extracts were integrated into formulations with biopolymers (PHB, PLGA), resulting in 15 suspension-type samples which, after drying, formed a polymeric film. The extensibility test (Ojeda-Arbussa method) revealed a high spreading capacity (especially for the samples with PHB, and among the extracts, the samples with EC and EG). All formulations have a pH of 5.5, concluding that they are suitable for topic-transdermal administration (compatible with skin). Through comparative ATR-FTIR analysis between the spectra of the basic components and the formulations, shifts of the bands at higher wave numbers were observed, as well as changes in their intensity, confirming the formation of adducts or complexes between extracts and biopolymers. These changes were best represented for the EC samples. Through XRD analysis, it was noted that the obtained formulations are amorphous. AFM analysis confirmed the obtaining of nanoparticles and outlined the topographical profiles of the nanoformulations by evaluation at two scales (2×2 μm^2, 8×8 μm^2). Both polymer-dependent and extract-dependent differences were observed. For formulations with PHB, the lowest roughness (assessed by Rq, root mean square roughness) belongs to EG-PHB. For those with PLGA, the highest roughness is observed for ES-PLGA. Among the samples with the polymer mixture, the dual topography of the sample with EM was noted, which at the larger scale has an intermediate roughness, the roughness decreases at the smaller scale, outlining the smoothest surface from all the analyzed samples. Samples with mixed extracts are the most complex and show

significant differences in topographic appearance. Aggregate formation was also observed for some samples.

The functionalization potential of a compression stocking matrix was investigated. Immersion of the compression stocking in nanoformulation for 30 minutes and drying for 12 hours at room temperature leads to the formation of a polymer film on the surface of the compression material. Through thermogravimetric analysis, the degradation profile of the nanoformulations was outlined, observing gradual mass loss for volatile solvents in the range of 25-115°C and mass loss of organic substances in the range of 200-650°C. The impregnation capacity of the extracts on the compression stocking matrix was also determined. Most of the samples showed a degree of impregnation between 5-10%.

In conclusion, the results of this research represent a point of orientation towards the development of new pharmaceutical products based on plant extracts, biopolymers and compressive matrices, revealing new therapeutic opportunities for the treatment of chronic venous disease. The obtained results were disseminated in the specialized literature through three original articles.

Originality

In the context of this research project, the degree of originality can be found in the following elements:

- the selection of vegetal products for the most comprehensive coverage of the manifestations of vascular damage induced by chronic venous disease: *Sophorae flos* (noted for its high content of antioxidant, anti-inflammatory and capillary-protective flavonoids), *Ginkgo bilobae folium* (content of ginkgolides with antithrombotic effects), *Meliloti herba* (known as veno-lymphatic drainer) and *Calendulae flos* (known for its wound healing and reepithelialization effect) and highlighting the active phytocompounds of therapeutic interest in the extracts obtained from these plant materials (Chapter 3);
- the application of *in vitro*, *in vivo* and *in silico* methods for the detailed characterization of the extracts and their main active constituents, on one hand from therapeutic implications (Chapter 4 – 4.1., 4.2.) and on the other hand, from cytotoxicity point of view (Chapter 4 – 4.3.);
- the integration of the four extracts into nanoformulations with biopolymers, including nanoformulations with the mixture of extracts to obtain their cumulative therapeutic benefits (Chapter 5 – 5.1.);

- physico-chemical characterization by advanced methods and extensive comparisons between the obtained formulations (Chapter 5 – 5.2., 5.3., 5.4., 5.5.);
- investigating the possibility of simultaneous administration of pharmacological and compressive therapy through the impregnation analysis of nanoformulations on the matrix of compression stockings in order to obtain functionalized compression stockings, which can improve patient's treatment adherance (Chapter 5 – 5.6., 5.7.).

Research perspectives

Since the present research approach constitutes a preliminary analysis as a first step towards the development of complex pharmaceutical products, the research perspectives are extensive. As a consecutive step to the current study, the stability of the nanoformulations over time will be analyzed and the possibility of their optimization in order to acquire very well-defined physico-chemical properties that would allow an optimal reproductibility for a future technological flow. At the same time, the evaluation (*in vitro*) for release of the active principles from these nanoformulations represents a direction of further analysis, as well as the study of the transcutaneous permeability of both the nanoformulations as a whole and the released active substances.

Also, an optimization possibility for the impregnation method could be considered, like a spray form with extract-biopolymer nanoparticles and the analysis of the pharmacotechnical particularities for this type of product. The polymeric film (resulted after the nanoformulations' drying) can be integrated into a transdermal patch-type matrix as a direction to be investigated.

Isolation of nanoparticles from nanoformulations and their reintegration into other pharmaceutical forms is a promising research field. The possibility of developing various pharmaceutical products, based on these nanoformulations, comes additively with the perspectives of their characterization from biopharmaceutical, pharmacokinetic, pharmacodynamic and pharmacotoxicological point of view.

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List of published works

Articles published in ISI rated journals

1. **Andreea Roxana Ungureanu**, Carmen Lidia Chițescu, Emanuela Alice Luță, Alina Moroșan, Dan Eduard Mihaiescu, Dragoș Paul Mihai, Liliana Costea, Emma Adriana Ozon, Ancuța Cătălina Fița, Teodora Dalila Balaci, Rica Boscencu, Cerasela Elena Gîrd. Outlook on Chronic Venous Disease Treatment: Phytochemical Screening, In Vitro Antioxidant Activity and In Silico Studies for Three Vegetal Extracts (2023). *Molecules*, 28(9), 3668, ISSN: 1420-3049, https://doi.org/10.3390/molecules28093668
2. **Andreea Roxana Ungureanu**, Violeta Popovici, Camelia Oprean, Corina Danciu, Verginica Schröder, Octavian Tudorel Olaru, Dragoș Paul Mihai, Liliana Popescu, Emanuela-Alice Luță, Carmen Lidia Chițescu, Cerasela Elena Gîrd. Cytotoxicity Analysis and In Silico Studies of Three Plant Extracts with Potential Application in Treatment of Endothelial Dysfunction (2023). *Pharmaceutics*, 15(8), 2125, ISSN: 1999-4923, https://doi.org/10.3390/pharmaceutics15082125
3. **Andreea Roxana Ungureanu**, Emma Adriana Ozon, Adina Magdalena Musuc, Mihai Anastasescu, Irina Atkinson, Raul-Augustin Mitran, Adriana Rusu, Liliana Popescu, Cerasela Elena Gîrd. Preparation and Preliminary Analysis of Several Nanoformulations Based on Plant Extracts and Biodegradable Polymers as a Possible Application for Chronic Venous Disease Therapy (2024). *Polymers*, 16(10), 1362, ISSN: 2073-4360, https://doi.org/10.3390/polym16101362