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Development of Innovative Therapies for Menopausal Disorders

PhD Thesis Abstract

Doctoral Supervisor:
PROF. UNIV. DR. DENISA IOANA UDEANU

PhD Student:
OPRESCU (CRIŞAN) SIMONA

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Carol Davila University of Medicine and Pharmacy, Bucharest
37 Dionisie Lupu Street, Bucharest, Sector 2, 020021, Romania
Tax Code: 4192910 | Account: RO57TREZ70220F330500XXXX | Bank: Treasury Sector 2
Tel: +40.21 318.0719; +40.21 318.0721; +40.21 318.0722
www.umfcd.ro

Contained

List of Published Scientific Papers

1. **Crisan** S., Pop A. L., Hentes P., Lacatusu I., Badea N., Zetu C., A. M. Ciobanu, Penes O. N., Varlas V., Ozon E. A., Udeanu, D. I., Pre-formulation, formulation and pilot scale-up studies to establish the qualitative and quantitative composition of an innovative nanoform dietary supplement for menopausal therapy Farmacia, 2024, 72(2), https://doi.org/10.31925/farmacia.2024.2.15, (IF - 1.4 AP – PA/2 equal contribution)
2. **Crisan**, S.; Pop, A.L†.; Lacatusu, I.; Badea, N.; Mustaciosu, C.; Radu, M.; Varlas, V.N*.; Peneş, O.N.*; Ciobanu, A.M.; Ghica, M.; et al. Safety of Innovative Nanotechnology Oral Formulations Loaded with Bioactive Menopause Molecules: Influence of Genotoxicity and Biochemical Parameters on a Menopausal Rat Model. Nutrients 2023, 15, 4951. https://doi.org/10.3390/nu15234951 IF 5,9 AP/2
3. Pop, A.L.; Nasui, B.A.; Bors, R.G.; Penes, O.N.; Prada, A.G.; Clotea, E.; **Crisan**, S.; Cobelschi, C.; Mehedintu, C.; Carstoiu, M.M.; et al. The Current Strategy in Hormonal and Non-Hormonal Therapies in Menopause—A Comprehensive Review. *Life* 2023, *13*, 649. https://doi.org/10.3390/life13030649 AP
4. Pop, A.L., S.**Crisan**, P.Henteș, M.-A. Pali, I. Lacatusu, N. Badea, A. M. Ciobanu, O. N. Penes, B. A. Nasui*, D. I. Udeanu Comparative dissolution study of a solid pharmaceutical form containing nanostructured lipid carrier (NLC), incorporating diosgenin – conventional vs. biorelevant dissolution media. Farmacia, 2023, Vol 71, 1FI 1,433 http://dx.doi.org/10.31925/farmacia.2023.1.15 AP
5. Iordache, T. A., Badea, N., Mihaila, M., **Crisan**, S., Pop, A. L., & Lacatusu, I. (2023). Polygonum cuspidatum Loaded Nanostructured Lipid Carriers for Dual Inhibition of TNF-α-and IL-6 Cytokines and Free Radical Species. Materials, 16(9), 3492.APc IF 3,4 Q2 https://doi.org/10.3390/ma16093492
6. Pop, A. L., Musuc, A. M., Nicoară, A. C., Ozon, E. A., **Crisan**, S., Penes, O. N., & Secăreanu, A. A. (2022). Optimization of the Preformulation and Formulation Parameters in the Development of New Extended-Release Tablets Containing Felodipine. Applied Sciences, 12(11), 5333. (IF2,838) - articol zona galbena Q2 https://doi.org/10.3390/app12115333
7. Pop, A.L., Henteș, P., Pali M-A, Oșanu L., Ciobanu A-M, Nasui B.A., Mititelu M, **Crisan** S., Peneș O.N., Study Regarding A New Extended-Release Calcium Ascorbate And Hesperidin Solid Oral Formulation, Farmacia, 2022, Vol. 70, 1, http://dx.doi.org/10.31925/farmacia.2022.1.22 (IF1,4)
8. Casian T, Iurian S, Gâvan A, Porfire A, Pop AL, **Crisan** S, Pușcaș AM, Tomuță I. In-

Depth Understanding of Granule Compression Behavior under Variable Raw Material and Processing Conditions. Pharmaceutics. 2022; 14(1):177. https://doi.org/10.3390/pharmaceutics14010177 (IF 6,321) - articol zona rosie Q1

9. Iordache, T.-A.; Badea, N.; Mihaila, M.; **Crisan**, S.; Pop, A.L.; Lacatusu, I. Challenges in Coopted Hydrophilic and Lipophilic Herbal Bioactives in the Same Nanostructured Carriers for Effective Bioavailability and Anti-Inflammatory Action. Nanomaterials 2021, 11, 3035. https://doi.org/10.3390/nano11113035

10. Pop, A.L.; **Crisan**, S.; Bârcă, M.; Ciobanu, A.-M.; Varlas, V.N.; Pop, C.; Pali, M.-A.; Cauni, D.; Ozon, E.A.; Udeanu, D.; Trifu, S.; Năsui, B.A. Evaluation of Dissolution Profiles of a Newly Developed Solid Oral Immediate-Release Formula Containing Alpha-Lipoic Acid. *Processes* 2021, 9, 176. doi: 10.3390/pr9010176, https://doi.org/10.3390/pr9010176 (IF 2,753)

11. A.-M.Ciobanu, A. L. Pop, S. **CRISAN**, M. Pali, G.T. A Burcea-Dragomiroiu, D. E. Popa, D. Lupuliasa, M. BÂRCĂ, HPLC studies for assessing the stability of carvedilol tablets, FARMACIA, 2017, Vol. 65, 4, 523 (IF 1,507)

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Introduction

Menopause is a natural physiological stage in a woman's life, but the hormonal changes associated with it can cause a series of unpleasant manifestations that affect the quality of life.

This thesis aims to develop an innovative product based on standardized plant extracts, intended for menopausal women, as a complement to conventional therapies. The proposed solution has the following main attributes:

1. **Using advanced nanotechnology-based technology** to improve the absorption and efficacy of active compounds.
2. **Integrating the product into the eating plan**, providing an optimal intake of essential nutritional principles in the form of a dietary supplement.
3. **Preventive role in health**, by supporting hormonal balance and protection against complications associated with menopause.
4. **Accessibility**, so that the benefits of the product are available to as many people as possible.

The thesis is structured in two main parts: **the general part**, which includes the theoretical foundations and the analysis of the specialized literature, and **the personal contributions part**, which brings together the working hypothesis, the general and specific objectives of the research, the applied methodology and the presentation of the results obtained within the experimental studies carried out, during the 8 chapters.

I. GENERAL PART

1. Current strategy in hormonal and nonhormonal therapies in menopause

1.1. Hormonal and non-hormonal therapy

Most menopausal women experience a range of symptoms, accompanied by physiological changes in the reproductive organs and psycho-affective disorders, with significant negative implications. This change is perceived negatively for a woman's behavior and is associated with the aging process [1].

The present analysis aims to investigate the most recent published research on (a) the evaluation of epidemiological data in menopausal women, through the reports recorded in the last ten years; (b) recent studies on HRT and new emerging medical therapies.

Studies that have determined the risks and benefits of hormone replacement therapy have had inconsistent results. A recent systematic review and meta-analysis, published in 2015, indicates that HRT does not increase all-cause or cause-specific mortality (cardiovascular, stroke, cancer) [11].

1.1.1. Estrogens in Menopausal Hormone Therapy

Sexual dysfunction is common in women with urogenital menopausal syndrome. Thus, vaginal estrogen can be an effective treatment for genitourinary syndrome.

Thus, there are several therapeutic schemes to relieve urogenital symptoms. Current treatment is based on topical estrogens alone [18–22] or combined with testosterone [14,23], which have shown improvements over time in the areas of vaginal trophicity, arousal, orgasm, and satisfaction (Appendix 1-Table 1.). The combined estrogen-progesterone regimen decreased the rate of symptoms of urogenital atrophy and the frequency and severity of hot flashes [31].

The endocrine and metabolic changes that occur in the transition to menopause can accelerate the risk of cardiovascular disease induced by high blood pressure. According to the American Heart Association, initiating menopausal hormone therapy in women under 60 years of age or within 10 years of menopause is the only prophylactic method of reducing the risk of cardiovascular disease [32].

Although data indicate that HRT is associated with positive effects on the cardiovascular system in menopausal women, it remains a question whether these effects vary depending on when treatment is initiated.

The ELITE-Cog study (class I evidence) investigated whether estradiol therapy initiated in the first six years of menopause has a different impact on **the neuropsychological symptoms of menopause** than when initiated ten or more years later.

Participants received oral 17p-estradiol (1 mg/d) or a placebo at a single academic medical center (University of Southern California). In addition, women with a uterus were also assigned to micronized cyclic progesterone (45 mg) as a 4% vaginal gel or placebo gel, a daily application for ten days per 30-day cycle. The study showed that estradiol initiated within six years of menopause does not affect verbal memory, executive functions, or overall cognition differently than treatment that began ten or more years after menopause. In addition, estradiol does not improve or harm these cognitive abilities regardless of how long it has elapsed since menopause [53].

1.1.2. Progestogens in menopausal hormone therapy

Progesterone is usually taken in combination with estrogen in hormonal treatment. Few studies have investigated the effects of progesterone alone on menopausal symptoms. Most of these studies have focused on the effects of progestogens on sleep quality.

1.1.3. Ospemiphene or Bazedoxifene/Estrogen Therapy

Ospemifene is an FDA-approved selective oral estrogen receptor agonist/antagonist. Indications for the use of ospemifene are moderate or severe dyspareunia, and the drug has recently been approved for moderate/severe vaginal dryness, which is a common symptom of vulvovaginal atrophy [72].

Bazedoxifen/estrogen conjugate form (CE/BZA) is the first FDA-approved drug to include estrogen conjugated to an estrogen agonist/antagonist (basedoxifen) [74]. Various studies have investigated how EC/BZA influences both quality of life and health, showing that the drug complex has beneficial effects on various menopause-related symptoms.

1.1.4. Non-hormonal therapy

Bisphosphonates (BPs) are key pharmaceutical agents that act against osteoclast-mediated bone loss. Thus, the most common medical condition for which BP is used is osteoporosis in menopause - a pathology that in Romania has been shown to occur ten years earlier than the internationally accepted limit [78].

Kisspeptins regulate the release of reproductive hormones, peptides encoded by the KISS1 gene [92], a key regulator of hypothalamic gonadotropin-releasing hormone (GnRH) neurons; their discovery led to the investigation of kisspeptin's role in reproductive disorders and the potential for therapeutic targeting of the kisspeptin system (KISS1R) [93]. Currently, the investigated GnRH/gonadotropins are Fezolinetant, MVT-602, Linzagolix.

Standardized herbal extracts beneficial in menopausal therapy

Interest in the use of plant extracts in the management of menopausal symptoms has increased significantly in recent decades, amid concerns about the safety of conventional

hormone therapies. Many medicinal plants contain active ingredients, especially phytoestrogens, flavonoids or triterpene glycosides, which can mimic the effects of endogenous estrogens, helping to relieve vasomotor, metabolic or psychoneurological symptoms associated with menopause.

The extracts on which we have focused in the present paper, as well as the active principles underlying their standardization, together with their codification that will be found throughout the paper, are presented in table 2.1. below.

Table 2.1. Standardized plant extracts studied

Cod	Plant extract name	Scientific name	Active ingredient	Standardization (%)
EVeg 1	Black Cohosh  Extract	*Cimicifuga racemosa*	Triterpenoide glicozide	2,5%
EVeg 2	Red clover Extract 	*Trifolium pratense*	Isoflavone (biochanin A, B-formononetin)	40%
EVeg 3	Extract from cane root  asiatică	*Polygonum cuspidatum*	Resveratrol	50%
EVeg 4	Extract of soybeans 	*Glycine max*	Izoflavone (genistein)	40%
EVeg 5	Extract de lemn dulce 	*Glycyrrhiza glabra*	Acid glicirizic	10%
EVeg 6	Extract hops 	*Humulus lupulus*	8-prenilnaringenin (8-PN)	–
DSG	Extract ofwild yam 	*Dioscorea villosa*	Diosgenin	6% / 95% (DSG)

1.2. Conclusions

Menopause is a physiological period during a woman's life, accompanied by a wide spectrum of symptoms that can alter the quality of life of a fully active adult; The use of TSH, the most valuable therapeutic resource for increasing the quality of life in menopausal women, is limited to moderate and severe symptoms. Additionally, many women choose not to use MHT because of fear of cancer or other negative effects. Unfortunately, there is no fully satisfactory therapy, so the future search for better or complementary options is essential. Knowing the latest updates on these topics will help healthcare professionals better get the benefit of therapy, treat women within safe limits, and access all available menopause therapeutic resources to address the still unresolved health problem of the active adult woman.

Early menopausal women with symptoms present can benefit from TSH safely and with clinically proven effectiveness, allowing for improved quality of life. An important role in menopause in terms of therapeutic strategy is represented by confidence in the administration of hormone therapy among both patients and doctors.

1. PERSONAL CONTRIBUTIONS. EXPERIMENTAL PART

2. Working hypothesis and general objectives

In the context of the growing need for natural, safe and effective alternatives for the management of menopausal symptoms, this research starts from the **hypothesis** that the use of nanolipid structures (NLCs) as vectors for plant active ingredients may represent an innovative strategy for the development of an oral dietary supplement with increased bioavailability and optimized pharmacological profile.

The gaps identified in the literature – related to the technological feasibility of simultaneous incorporation of lipophilic and hydrophilic active compounds in solid NLC-based formulations, the scalability of these formulations, the dissolution profile in biorelevant or conventional media and their safety in oral administration – guided the establishment of the main scientific objective:

The general objective of the research is to **develop, characterize, optimize and evaluate in *vitro* and *in vivo* a finished product in oral solid pharmaceutical form, containing a nanolipid matrix loaded with active ingredients of plant origin – diosgenin, glycyrrhizic acid, glycyrzidal triterpenes, polyphenols, isoflavones – intended to support health during menopause.**

3. General research methodology

The research was structured in a logical succession of stages, from the theoretical and conceptual level to the experimental validation of the proposed formulation. The general research plan included the following major components:

1. **Systematic scientific documentation** on menopause, phytogenic therapy, standardized extracts of wild Yam - *Dioscorea villosa*, Licorice - *Glycyrrhiza glabra*, Red clover - *Trifolium pratense,* Black Cohosh - *Cimicifuga racemosa,* Japanese Scrooge - *Polygonum cuspidatum*, Soybean - *Glycine max*, Hops - *Humulus lupulus* as well as modern formulation technologies (especially on solid oral pharmaceutical formulations containing nanolipid systems).
2. **Experimental formulation of nanolipid structures (NLC):** Stage that involved (1) Selection of lipid excipients, emulsifiers and setting of technological conditions, (2) Obtaining colloidal dispersion by high-pressure homogenization/shear homogenization, (3) Simultaneous encapsulation of diosgenin and glycyrrhizinic acid; (4) Stabilization of the formulation by freeze-drying.
3. **Obtaining the oral solid pharmaceutical form: the** main activities focused on (1)Selection of suitable excipients for compression;(2) Evaluation of the technological process of transformation of the lyophilized powder into solid form (tablet/capsule); (3) Testing the scalability of the manufacturing process.
4. **Physicochemical and biopharmaceutical characterization of formulas** by (1) Analysis of particle size, zeta potential, morphology and degree of encapsulation – on NLC; (2) Study of the dissolution profile in conventional and biorelevant media; (3) Evaluation of the stability of the formula.
5. **Evaluation of preclinical safety by:** (1) Genotoxicity tests (e.g. micronucleus test) and (2) Determination of the influence of the product on the biochemical parameters of ovariectomized rats, aiming at:

   Profilul lipidic (TG, Chol, HDL)

   Glycemic markers (Gli)

   Bone markers (Pac, Palc, Ca, phosphorus)

   Markerii renali (Crea, uree, URAC)

   Inflammation (TNF)

   Stealth oxidative (GSH, MDA)

   Profilul hormonal estrogen-progesteron.

4. Limits of research and future directions

The current research has demonstrated the technological feasibility of formulating a solid oral dosage form containing a nanolipid structure (NLC) based on plant extracts, as well as their therapeutic potential in the context of menopause. However, certain limitations should be noted:

1. Lack of restoration of hormonal homeostasis: Although the treatments tested positively influenced inflammatory and metabolic markers, they failed to normalize hormone levels
2. Limited assessment of biological markers: The study focused on a small number of biomarkers, which provides partial insight into systemic effects.
3. Reduced duration of the in vivo study, without capturing possible cumulative effects, or long-term adverse effects.
4. Lack of detailed histopathological analyses:

Prospects for further research

1. Based on the results obtained and the mentioned limitations, the following directions can be outlined for future research:
2. Optimization of nanolipid formulations
3. Extension of preclinical assessments
4. Exploring a broader spectrum of biomarkers, including: IL-6, CRP, IL-10 for the complete assessment of the inflammatory response; additional oxidative stress markers (e.g. SOD, catalase, GPx); advanced metabolic markers (e.g. leptin, adiponectin, HOMA).
5. Study of the impact on quality of life, by extending research to human clinical models

5. Pharmaceutical studies on the development of a dietary supplement with a nanolipid structure, beneficial in menopausal therapy.

5.1. Working case and specific objectives

Working hypothesis

The formulation of diosgenin in a nanostructured lipid matrix (NLC) and its conversion into an oral solid pharmaceutical form (tablets or capsules) can lead to a significant improvement in bioavailability and therapeutic efficacy, thus representing a promising alternative for addressing the specific symptoms of menopause.

Specific objectives of the study

1. **Formulation objective:**

   To incorporate the diosgenin-containing NLC lipid matrix into a powdery mixture suitable for compression or filling in capsules, in order to obtain a stable oral solid pharmaceutical form (tablets or capsules).

2. **Technological objective:**

   To carry out the technological transfer of the selected solid NLC forms, from the laboratory stage to pilot scale, in order to assess the industrial feasibility.

3. **Validation objective:**

   To perform process validation and quality control for the selected final formulas in order to ensure their compliance for subsequent inclusion in *in vivo studies.*

5.2. Results and conclusions

Solid finished pharmaceuticals containing nanoforms of plant active ingredients - diosgenin - manufactured after the preformulation and formulation studies presented in the study were evaluated according to the critical parameters of the physicochemical analysis. All tested parameters were in accordance with the requirements. The diosgenin content/pharmaceutical form was within limits, demonstrating that the manufacturing process (from obtaining NLCs to finished products in the form of tablets/capsules) is well controlled, reliable and reproducible. The dissolution results were above the imposed limit of 70%, and this quality attribute was achieved in terms of the release of the active ingredients, respectively in *vitro testing*

This process illustrates a complete and efficient innovative approach and process for the transformation of lipid-based nanoforms into solid pharmaceutical forms to obtain tablets or capsules with nanostructured lipid carriers, ensuring the quality, consistency and efficacy

of the final product. The preformulation and formulation studies described were monitored in accordance with quality requirements, including active ingredient testing, dissolution, microbiological quality and stability.

The findings of our study demonstrate significant advances in the formulation and development of solid dosage forms using nanolipid 'carriers' (NLCs). The successful transformation of NLCs that encapsulate active plant ingredients into a stable powder form is an essential step in pharmaceutical technology.

The physicochemical analysis of the finished products concluded that (a) uncoated, round, biconvex shaped tablets with uniform appearance, compact and homogeneous structure, intact edges, d = 13 mm - figure 5.1 were obtained. and (b) Suitable hard, hard, cylindrical gelatinous capsules with hemispherical ends, uniform on the surface, transparent, size "0" – Figure 5.2.

Final product S1 (lot: S081021)	Final product S2 (lot: S071021)	Final product S3 (lot: P261022)	Final product S4 (lot: P331122)
			

Fig.5.1. Final product in the form of tablets.

Produs finit care conține S1 (Lot: S060521)	Final product S 2 (Lot: S050521)	Final product S 3 (Lot: P251022)	Final product S 4 (Lot: P321122)
			

Fig.5.2. Final product in the form of hard gelatinous capsules.

6. Safety of nanoencapsulated oral formulations with bioactive molecules used in the treatment of menopause

6.1. Working case and specific objectives

Working hypothesis

Pharmaceutical formulations based on nanolipid structures (NLC) loaded with natural active molecules can represent an effective and safe pharmaceutical alternative for relieving menopausal symptoms, with increased bioavailability and beneficial effects on biochemical, metabolic and hormonal parameters.

Research objectives

1. Evaluation of cytotoxicity and genotoxicity of pharmaceutical products tested by *in vitro toxicity studies*

2. Evaluation of the influence of treatment with pharmaceutical formulations based on nanolipid structures loaded with active principles from plants, on the biochemical parameters of ovarian rat females ovaricantized – *in vivo study* , aiming at:

- profilul lipidic (TG, Chol, HDL)
- glycemic markers (Gli)
- bone markers (Pac, Palc, Ca, phosphorus)
- renal markerii (creatinine, ureas, URAC)
- inflammation (TNF)
- stressful oxidative (GSH, MDA)
- hormonal profile

6.2. Results and conclusions

During the present study, as part of a larger research, we investigated during the first stage the cytotoxicity (cell viability) and genotoxicity (micronucleus assay) of NLCs tested using culture cells. In the second part, we tested (1) the influence of NLCs on the biochemical parameters of surgically induced Wistar rat females, following the lipid profile (TG, Chol, HDL), bone markers (Pac, Palc, Ca, phosphorus), glycemic markers (Gli), renal markers (Crea, urea, URAC), general condition and body weight, inflammation parameters (TNF), oxidative stress (GSH, MDA) and, since the formulas target hormonal load, (2) hormonal markers-progesterone and serum estradiol.

The main findings of our study are that (a) diosgenin (Dioscorea villosa L. standardized extract) formulated in various lipid nanocapsule (NLC) structures in combination with glycicric acid (standardized extract Glycyrrhiza glabra, Eveg 1 and Eveg

2), TTG (triterpene glycosides) (standardized extract Actaea racemosa, Eveg 3 and Eveg 4), and resveratrol polyphenols (standardized extract of Polygonum cuspidatum, Eveg 5 and Eveg 6) tested on cell cultures by the micronucleus method **did not show the genotoxicity of the tested compounds,** the values obtained being similar to those of negative control and untreated cell cultures; (b) the NLC pharmaceutical formulations tested (Eveg 1-6) administered orally in the female rat model with surgically induced menopause showed no influence on bone metabolism, carbohydrate metabolism, liver and biliary markers, blood lipid profile, acid phosphatase or inflammatory markers; (c) NLC formulas tested (Eveg 1-6) in the menopausal model also did not influence oxidative stress markers or hormonal markers.

In addition, plasma levels showed the hypoglycemic, hyporicemic and antioxidant potential of a tested pharmaceutical formula containing nano diosgenin and glycyrrhizic acid encapsulated in the lipid phase based on Nightrose Oil. Further studies on models of metabolic diseases could be carried out on the selected formula.

Limitations

The present study has limitations. The induced menopause pattern was surgically induced, which can be considered a trauma compared to physiological menopause.

Another limitation is the duration of the treatment of 30 days, both for the tested formulas and for the positive control treated with the conventional combination of hormone replacement.

7. Comparative study of dissolution of a pharmaceutical form containing a disogenin-based nanostructured lipid carrier (NLC) - conventional versus biorelevant dissolution media

7.1. Working hypothesis and specific objectives

Working hypothesis

The incorporation of diosgenin (DSG) into a nanostructured lipid matrix (NLC) will improve its bioavailability by increasing the rate of dissolution and gastrointestinal absorption. The comparative study of the dissolution process in conventional versus biorelevant media will provide a clearer understanding of the *in vivo behavior* of DSG encapsulated in NLC, allowing the optimization of the final pharmaceutical formula.

The specific objectives of the study were

(1) determination of the optimal place of absorption of diosgenin encapsulated in a nanolipid structure (gastric environment, intestinal or colonic mucosa) and timing: before or after a meal - using dissolution in special media as a tool;

(2) selection of the appropriate dissolution medium to determine the bioavailability of diosgenin using **conventional** *in vitro dissolution media* – on the standardized crude extract, crude nanocapsules and the final form of the oral finished product (nanoproduct-filled capsules)

(3) the development of the analysis method that will be used later in the analysis of the batch of finished product.

7.4. Results and conclusions

The intestinal environment after a meal is the optimal place and time of absorption of the active ingredient diosgenin encapsulated in the nanolipid structure, measured in biorelevant dissolution media that simulate physiological conditions in vivo.

The recommended dissolution media for routine laboratory analyses included in the finished product specification for interphase scaling tests, with a pH close to that of the special media, are 1.0 g/L sodium lauryl sulfate, 2.0 g/L sodium lauryl sulfate and 4.0 g/L cetrimide, the results obtained in these media respecting the minimum limit imposed by 70% dissolved DSG.

The recommended pharmaceutical form for products containing diosgenin encapsulated in a nanolipid structure is the delayed-release (gastroresistant) form, and the optimal absorption of the active ingredient takes place in the upper intestine.

8. Conclusions and personal contributions

The scientific research objectives set by the three studies allowed

(1) the manufacture of a solid pharmaceutical form, with oral administration, based on a nanolipid structure that incorporated in its structure active ingredients from plants beneficial in menopausal therapy, respectively disogenin from wild yam extract and glycyrrhizic acid from Licorice.

The development of the pharmaceutical form went through all the technological and research and development stages, namely the scientific documentation stage, preformulation, formulation and optimization, as well as the scaling stages, by transposing from laboratory scale to pilot scale and physico-chemical and microbiological control.

By examining the results of the pre-formulation tests of the nanolipid mold with evening primrose oil, diosgenin and licorice extract (S1) and the nanolipid matrix with soybean oil, diosgenin and licorice extract (S2), we obtained adequate results by which it became possible to transform an oily structure into a powder suitable for obtaining a solid pharmaceutical form. Thus, excipients such as spray-dried amorphous granular silicon (Fujisil) and magnesium aluminometasilicate (Neusilin) were selected as lead candidates in the formulation of finished products in the form of capsules or tablets.

Depending on the amount of active ingredient in a dosage form required for administration, we can choose either a tablet (for a higher therapeutic dose) or a capsule (for a lower therapeutic dose). In this study, the manufacturing process and quality results were compliant for both shapes.

All tested parameters met the requirements. The content of diosgenin/pharmaceutical form was within limits, proving that the manufacturing process (from obtaining NLC to finished products in the form of tablets/capsules) is well controlled, reliable and reproducible. The dissolution results were above the imposed limit of 70%, and this quality attribute was achieved in terms of the release of the active ingredients, respectively, *in vitro testing*.

(2) study of the dissolution profile on three categories of samples:

- extract de yam sălbatic standardizat la 95% DSG,
- NLCs containing wild yam extract standardized to 6%DSG
- finished product formulated in the form of a hard gelatin capsule containing NLC with standardized extract of wild yam DSG, tested in two types of dissolution media: (a) conventional and (b) special (biorelevant) media, achieving the scientific objectives of the

study, respectively 1) determining the optimal site of absorption of diosgenin encapsulated in a nanolipid structure (gastric environment, intestinal or colonic mucosa) and the timing: before or after a meal - using as a tool dissolving in special media that reproduce real conditions in the human body; (2) the establishment of routine dissolution tests (QC control) of diosgenin encapsulated in a nanolipid structure, which are the recommended conventional dissolution media and which have a pH closer to that of the special media in which consistent results have been obtained.

With the help of biorelevant dissolution media simulating physiological conditions *in vivo*, it has been demonstrated that the intestinal environment after a meal is the optimal place and time for the absorption of the active ingredient diosgenin encapsulated in the nanolipid structure.

The recommended dissolution media for routine laboratory analyses included in the finished product specification for interphase scaling tests, with a pH close to that of the special media, are 1.0 g/L sodium lauryl sulfate, 2.0 g/L sodium lauryl sulfate and 4.0 g/L cetrimide, the results obtained in these media respecting the minimum limit imposed by 70% dissolved DSG.

The recommended pharmaceutical form for products containing diosgenin encapsulated in a nanolipid structure is the delayed-release (gastroresistant) form, and the optimal absorption of the active ingredient takes place in the upper intestine.

(3) Establishing the safety profile

Through *in vitro* testing on **the cytotoxicity and genotoxicity** of the developed dosage forms, we have shown that the cytotoxic effect occurs from doses of 1 mg/ml for some compounds (NN control) or even higher amounts for others (NLC-Eveg 3). Given that the body expects those substances to reach a maximum dose of 10 mg/kg, which would correspond *in vitro* to 10 μg/ml, **the therapeutic dose can be considered to be far from producing cytotoxic effects.**

Also, the micronucleus method did not highlight **the genotoxicity** of the test compounds, the values obtained being similar to those of the negative control and untreated cell cultures.

Biochemical determinations from the *in vivo* study on a female rat model with induced menopause showed that of the six nanolipid structure-based dosage forms studied, **NLC-Eveg 1**, formulated with DSG as the active ingredient and licorice extract encapsulated in Nightlight oil, showed the best effects in maintaining biochemical parameters close to the

non-operated control batch, different from Eveg 2, which contained the same active ingredients, but encapsulated in soybean oil.

The treatments administered failed to restore hormone levels, but they influenced metabolic and inflammatory markers, which makes them interesting candidates for adjuvant therapies.

These results provide a solid basis for the development of new therapeutic strategies aimed at managing the effects of menopause, with a focus on reducing oxidative stress and optimizing carbohydrate and lipid metabolism.

Recommendations for future research

1. Optimization of formulations to improve bioavailability and therapeutic effects.
2. Further long-term studies, including histopathological analyses, to confirm the safety of treatments.
3. Evaluation of other inflammatory and oxidative stress markers to better understand the effects on physiological balance.

References (selective)

[1] Henes M, Hübner S. Hormontherapie in der Peri- und Postmenopause. Internist (Berl) 2020;61:558–64. https://doi.org/10.1007/s00108-020-00789-x.

[2] Burger HG, MacLennan AH, Huang K-E, Castelo-Branco C. Evidence-based assessment of the impact of the WHI on women's health. Climacteric 2012;15:281–7. https://doi.org/10.3109/13697137.2012.655564.

[3] Cagnacci A, Venier M. The Controversial History of Hormone Replacement Therapy. Medicina (B Aires) 2019;55:602. https://doi.org/10.3390/medicina55090602.

[4] Utian WH, Schiff I. NAMS-gallup survey on women's knowledge, information sources, and attitudes to menopause and hormone replacement therapy. Menopause 2018;25:1172–9. https://doi.org/10.1097/GME.0000000000001213.

[5] Langer RD, Hodis HN, Lobo RA, Allison MA. Hormone replacement therapy – where are we now? Climacteric 2021;24:3–10. https://doi.org/10.1080/13697137.2020.1851183

[22] Golmakani N, Parnan Emamverdikhan A, Zarifian A, Sajadi Tabassi SA, Hassanzadeh M. Vitamin E as alternative local treatment in genitourinary syndrome of menopause: a randomized controlled trial. Int Urogynecol J 2019;30:831–7. https://doi.org/10.1007/s00192-018-3698-z.

[23] Marko KI, Simon JA. Androgen therapy for women after menopause. Best Pract Res Clin Endocrinol Metab 2021;35:101592. https://doi.org/10.1016/j.beem.2021.101592.

[24] Archer DF, Labrie F, Bouchard C, Portman DJ, Koltun W, Cusan L, Labrie C, Côté I, Lavoie L, Martel C, Balser J. Treatment of pain at sexual activity (dyspareunia) with intravaginal dehydroepiandrosterone (prasterone). Menopause 2015;22:950–63. https://doi.org/10.1097/GME.0000000000000428.

[60] Pinkerton J V., Joffe H, Kazempour K, Mekonnen H, Bhaskar S, Lippman J. Low-dose paroxetine (7.5 mg) improves sleep in women with vasomotor symptoms associated with menopause. Menopause 2015;22:50–8. https://doi.org/10.1097/GME.0000000000000311.

[106] Varlas VN, Bors RG, Albu D, Penes ON, Nasui BA, Mehedintu C, Pop AL. Social Freezing: Pressing Pause on Fertility. Int J Environ Res Public Health 2021;18:8088. https://doi.org/10.3390/ijerph18158088.

[107] Peneş NO, Weber B, Păun SD. Role of genetic polymorphism in nutritional supplementation therapy in personalized medicine. Rom J Morphol Embryol 2017;58:53–8. https://doi.org/http://dx.doi.org/10.23977/medsc.2021.020103.

[108] Pisoschi AM, Pop A, Cimpeanu C, Turcuş V, Predoi G, Iordache F. Nanoencapsulation techniques for compounds and products with antioxidant and antimicrobial activity - A critical view. Eur J Med Chem 2018;157:1326–45. https://doi.org/10.1016/j.ejmech.2018.08.076.

[109] Lasoń E, Sikora E, Ogonowski J. Influence of process parameters on properties of Nanostructured Lipid Carriers (NLC) formulation. Acta Biochim Pol 1970;60. https://doi.org/10.18388/abp.2013_2056.

[113] Lacatusu I, Iordache TA, Mihaila M, Mihaiescu DE, Pop AL, Badea N. Multifaced Role of Dual Herbal Principles Loaded-Lipid Nanocarriers in Providing High Therapeutic Efficacity. Pharmaceutics 2021;13:1511. https://doi.org/10.3390/pharmaceutics13091511.

[114] COC LMC. CURCUMIN CO-LOADED WITH A LIPID MEDIATOR IN THE SAME NANOSTRUCTURED LIPID DELIVERY SYSTEM. Farmacia 2022;70:932–43. https://doi.org/10.31925/farmacia.2022.5.20.

[122] POP AL. COMPARATIVE DISSOLUTION STUDY OF A SOLID PHARMACEUTICAL FORM CONTAINING NANOSTRUCTURED LIPID CARRIER (NLC) INCORPORATING DIOSGENIN – CONVENTIONAL VERSUS BIORELEVANT DISSOLUTION MEDIA. Farmacia 2023;71:116–29. https://doi.org/10.31925/farmacia.2023.1.15.
[126] Pop A, Nasui B, Bors R, Penes O, Prada A, Clotea E, **Crisan S**, Cobelschi C, Mehedintu C, Carstoiu M, Varlas V. The Current Strategy in Hormonal and Non-Hormonal Therapies in Menopause—A Comprehensive Review. Life 2023;13:649. https://doi.org/10.3390/life13030649.
[127] VARLAS VN. KEY POINTS IN FERTILITY PRESERVATION TREATMENT STRATEGIES DURING COVID-19 PANDEMIC. AN UPDATE ON PHARMACOLOGICAL THERAPIES. Farmacia 2021;69:189–99. https://doi.org/10.31925/farmacia.2021.2.1.
[130] Iordache T-A, Badea N, Mihaila M, **Crisan S**, Pop AL, Lacatusu I. Challenges in Coopted Hydrophilic and Lipophilic Herbal Bioactives in the Same Nanostructured Carriers for Effective Bioavailability and Anti-Inflammatory Action. Nanomaterials 2021;11:3035. https://doi.org/10.3390/nano11113035.
[131] Iordache T-A, Badea N, Mihaila M, **Crisan S**, Pop AL, Lacatusu I. Polygonum cuspidatum Loaded Nanostructured Lipid Carriers for Dual Inhibition of TNF-α- and IL-6 Cytokines and Free Radical Species. Materials (Basel) 2023;16:3492. https://doi.org/10.3390/ma16093492.
[132] Pop A, **Crișan S**, Bârcă M, Ciobanu A-M, Varlas V, Pop C, Pali M-A, Cauni D, Ozon E, Udeanu D, Trifu S, Năsui B. Evaluation of Dissolution Profiles of a Newly Developed Solid Oral Immediate-Release Formula Containing Alpha-Lipoic Acid. Processes 2021;9:176. https://doi.org/10.3390/pr9010176.
[133] Fița AC, Secăreanu AA, Musuc AM, Ozon EA, Sarbu I, Atkinson I, Rusu A, Mati E, Anuta V, Pop AL. The Influence of the Polymer Type on the Quality of Newly Developed Oral Immediate-Release Tablets Containing Amiodarone Solid Dispersions Obtained by Hot-Melt Extrusion. Molecules 2022;27:6600. https://doi.org/10.3390/molecules27196600.
[161] Coc LMC, Lacatusu I, Badea N, Barbinta-Patrascu ME, Meghea A. Effective Lipid Nanocarriers Based on Linseed Oil for Delivery of Natural Polyphenolic Active. J Nanomater 2021;2021:1–9. https://doi.org/10.1155/2021/8853941.
[162] Parhizkar S, Latiff LA, Parsa A. Effect of Nigella sativa on reproductive system in experimental menopause rat model. Avicenna J Phytomedicine 2016;6:95–103.
[163] Yin W, Borniger JC, Wang X, Maguire SM, Munselle ML, Bezner KS, Tesfamariam HM, Garcia AN, Hofmann HA, Nelson RJ, Gore AC. Estradiol treatment improves biological rhythms in a preclinical rat model of menopause. Neurobiol Aging 2019;83:1–10. https://doi.org/10.1016/j.neurobiolaging.2019.08.029.
[164] Koebele S V., Bimonte-Nelson HA. Modeling menopause: The utility of rodents in translational behavioral endocrinology research. Maturitas 2016;87:5–17. https://doi.org/10.1016/j.maturitas.2016.01.015.
[165] Hsu K-H, Chang C-C, Tsai H-D, Tsai F-J, Hsieh Y-Y. Effects of Yam and Diosgenin on Calpain Systems in Skeletal Muscle of Ovariectomized Rats. Taiwan J Obstet Gynecol 2008;47:180–6. https://doi.org/10.1016/S1028-4559(08)60077-7.
[166] Høegh-Andersen P, Tankó LB, Andersen TL, Lundberg C V, Mo JA, Heegaard A-M, Delaissé J-M, Christgau S. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. Arthritis Res Ther 2004;6:R169. https://doi.org/10.1186/ar1152.
[167] Bradford MM. A rapid and sensitive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54. https://doi.org/10.1016/0003-2697(76)90527-3.
[168] Del Rio D, Pellegrini N, Colombi B, Bianchi M, Serafini M, Torta F, Tegoni M, Musci M, Brighenti F. Rapid Fluorimetric Method to Detect Total Plasma Malondialdehyde with Mild Derivatization Conditions. Clin Chem 2003;49:690–2. https://doi.org/10.1373/49.4.690.
[169] Zuccari G, Alfei S. Development of Phytochemical Delivery Systems by Nano-Suspension and Nano-Emulsion Techniques. Int J Mol Sci 2023;24:9824. https://doi.org/10.3390/ijms24129824.
[170] Cypriyana P J J, S S, Angalene J LA, Samrot A V., Kumar S S, Ponniah P, Chakravarthi S. Overview on toxicity of nanoparticles, it's mechanism, models used in toxicity studies and disposal methods – A review. Biocatal Agric Biotechnol 2021;36:102117. https://doi.org/10.1016/j.bcab.2021.102117.
[171] Idrees H, Zaidi SZJ, Sabir A, Khan RU, Zhang X, Hassan S. A Review of Biodegradable Natural Polymer-Based Nanoparticles for Drug Delivery Applications. Nanomaterials 2020;10:1970. https://doi.org/10.3390/nano10101970.
[172] Yuan D, He H, Wu Y, Fan J, Cao Y. Physiologically Based Pharmacokinetic Modeling of Nanoparticles. J Pharm Sci 2019;108:58–72. https://doi.org/10.1016/j.xphs.2018.10.037.
[184] Liu K, Zhao W, Gao X, Huang F, Kou J, Liu B. Diosgenin ameliorates palmitate-induced endothelial dysfunction and insulin resistance via blocking IKKβ and IRS-1 pathways. Atherosclerosis 2012;223:350–8. https://doi.org/10.1016/j.atherosclerosis.2012.06.012.
[192] Lacatusu I, Badea N, Udeanu D, Coc L, Pop A, Cioates Negut C, Tanase C, Stan R, Meghea A. Improved anti-obesity effect of herbal active and endogenous lipids co-loaded lipid nanocarriers: Preparation, in vitro and in vivo evaluation. Mater Sci Eng C 2019;99:12–24. https://doi.org/10.1016/j.msec.2019.01.071.
[200] Mishra P, Mandlik D, Arulmozhi S, Mahadik K. Nephroprotective role of diosgenin in gentamicin-induced renal toxicity: biochemical, antioxidant, immunological and histopathological approach. Futur J Pharm Sci 2021;7:169. https://doi.org/10.1186/s43094-021-00318-z.
[202] Nagamma T, Konuri A, Bhat KMR, Maheshwari R, Udupa P, Nayak Y. Modulation of inflammatory markers by petroleum ether fraction of Trigonella foenum-graecum L. seed extract in ovariectomized rats. J Food Biochem 2021;45. https://doi.org/10.1111/jfbc.13690.
[203] Michalak O, Krzeczyński P, Cieślak M, Cmoch P, Cybulski M, Królewska-Golińska K, Kaźmierczak-Barańska J, Trzaskowski B, Ostrowska K. Synthesis and anti–tumour, immunomodulating activity of diosgenin and tigogenin conjugates. J Steroid Biochem Mol Biol 2020;198:105573. https://doi.org/10.1016/j.jsbmb.2019.105573.
[204] Li X, Liu S, Qu L, Chen Y, Yuan C, Qin A, Liang J, Huang Q, Jiang M, Zou W. Dioscin and diosgenin: Insights into their potential protective effects in cardiac diseases. J Ethnopharmacol 2021;274:114018. https://doi.org/10.1016/j.jep.2021.114018.
[205] Choi K-O, Choe J, Suh S, Ko S. Positively Charged Nanostructured Lipid Carriers and Their Effect on the Dissolution of Poorly Soluble Drugs. Molecules 2016;21:672. https://doi.org/10.3390/molecules21050672.
[206] CASIAN T. INFLUENCE OF DISSOLUTION CONDITIONS ON THE DISSOLUTION RATE AND RELEASE MECHANISM OF KETOPROFEN FROM EXTENDED RELEASE HYDROPHILIC MATRIX SYSTEMS. Farmacia 2022;70:331–43. https://doi.org/10.31925/farmacia.2022.2.20.