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**EVALUATION AND OPTIMIZATION OF NEW
THERAPEUTIC SOURCES OF NATURAL COMPOUNDS**

ABSTRACT

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ABSTRACT

The chosen research topic was based on the fact that the health of the population is nowadays a challenge for specialists in the field. Due to the evolutions associated with the modern world on all levels (economic, educational, scientific, etc.), part of the population has access to different information and various resources while another part of the population is deprived of both information and resources. Regardless of the environment and lifestyle, the vast majority of people use plants, plant derivatives or herbal preparations to prevent or combat certain diseases. As the aging process takes place, the population becomes more susceptible to diseases and disabilities due to various factors such as low immunity, low cell function, DNA damage, higher incidence of inflammation, etc. Therefore, healthy aging plays an important role in public health policies.

The medicinal properties of plants are always credited for their therapeutic effects and effectiveness in treating various diseases without side effects or with insignificant side effects as an important component in addressing the health of the population from the past to the present. The role of phytomedicine in reducing aging processes is very crucial due to its bioactive compounds and important constituents (e.g., polyphenols, triterpenes) which are considered to exert anti-aging properties, but also contribute to reducing age-related problems. Moreover, oxidative stress and inflammation triggered by increased oxidative stress are the cause of many chronic diseases. Plant-derived compounds provide potential new biologically active molecules capable of counteracting these processes. Natural products are structurally optimized by evolution to fulfill certain biological functions, including the regulation of endogenous defense mechanisms and interaction with other organisms, a property that explains their relevance especially for infectious diseases and cancer. Many clinical and population studies highlight important data on the consistent and effective protection resulting from prolonged intake of various natural products (such as olive oil, teas, tinctures, fresh fruits and vegetables) against the onset of aging-related pathologies, such as neurodegeneration, cardiovascular disease, metabolic diseases and cancer.

The use of natural resources / plants for the prevention and treatment of diseases is almost universal, being in line with the concerns of different research areas from local to international level. The use of medicinal plants is growing worldwide, given the extraordinary expansion of traditional medicine and a significant interest in herbal

treatments. Plants are used in medicine to maintain and enhance health (physical, mental and spiritual) but also to treat diseases from simple to complex forms. Traditional medicine has maintained its popularity in all regions or in the developing world and its use is spreading rapidly in industrialized countries and the global market for herbal medicines amounts to tens of billions of euros and is constantly growing.

Taking into account the fact that natural resources are an important part of daily life but also that the occurrence of many diseases is increasing (for various reasons starting from lifestyle, genetic inheritance, environment, etc.) scientific objectives proposed for resolution in the doctoral research focused mainly on the study of certain plant extracts (rich in triterpenes), biologically active natural molecules (betulinic acid) and biocompatible polymers loaded with biomolecules, in parallel with population health.

The doctoral thesis is structured according to the methodological norms in four main parts: (i) the general part, (ii) the special part, (iii) personal conclusions and contributions and (iv) the bibliography. The general part comprises three chapters describing the current notions related to: (a) population health related to natural resources, herbal products and biologically active compounds, (b) pharmacological applications of triterpenes and (c) biomedical applications of polymers. The subject is a very wide one and it is developed and updated daily through the prism of new and new data related to natural resources and their role on health.

The research directions associated with the study topic are very varied considering the interdisciplinary involvement for scientific success. Within the special part of the paper, three major objectives were established: (a) *in vitro* and *in ovo* preclinical testing of extracts / triterpenes of therapeutic interest; (b) analyzes related to plant extracts associated with public health; and (c) biocompatible polymers with biocompounds - appropriate *in vitro* tests, preclinical procurement and evaluation. The documentary and experimental activities carried out to achieve the mentioned objectives aimed at deepening the data related to the mechanisms exercised, the importance and safety for human health, selecting the appropriate methods for evaluating the compounds and presenting in a clear and concise way the results obtained in line with existing data in the literature.

Olive leaf extract is marketed globally and is considered a natural medicine that has a number of benefits for maintaining human health. It is rich in various classes of compounds with different chemical structures, which are believed to produce the therapeutic effects of the extract. A major problem with regard to products of natural origin

is that related to the variation of the chemical composition due in particular to the origin, which accentuates the composition taking into account the nutrition of the plant, the mode of extraction, the form of storage, etc. The aim of the first study was to determine the cytotoxicity of ethanolic extract from olive leaves on human melanoma cells and to establish the irritant and toxic potential by using the *in vivo* chorioallantoic membrane test employing the HET-CAM method. The extract used is rich in luteolin and rutin, these being the most abundant compounds from the polyphenols class and ursolic and oleanolic acids, from the triterpenes class, were also detected and quantified. In order to evaluate the biological activity, the stimulation of human melanoma cells was performed with different concentrations of the extract, registering a decrease of viable cells at the highest concentration tested (100 µg / mL - ~ 71% viable cells). In the case of the CAM test, the reaction induced by the test extract was classified as follows: 0-0.9 non-irritant effect, 1-4.9 mild irritant effect, 5-8.9 / 9.9 moderate irritant effect, 8.9 / 9.9-21 strong irritant effect. The effects induced by the extract tested at different concentrations, together with the controls were recorded and all three endpoints (hemorrhage, coagulation and lysis). The extract at 100 µg / mL showed late and limited signs of both bleeding and coagulation and early signs, although limited of vasodilation, suggesting a weak irritating effect at this concentration. The present research was performed to evaluate the cytotoxicity of ethanolic extract from olive leaves on human melanoma cells *in vitro* and to establish the irritant and toxic potential *in vivo* by the HET-CAM method. The extract exerts some cytotoxic effects on melanoma cells at the highest concentration tested and *in vivo* 24 hours after treatment expressed a stronger effect both in terms of tumor cell growth and vascularization of melanoma A375 cells.

Agents that target mitochondria or interfere with mitochondrial bioenergetics could be considered an alternative therapy that, in combination with current antimelanoma treatments, increases chemotherapeutic efficacy and delays the occurrence of resistance. One compound that meets these requirements is betulinic acid. It has an extensive pharmacological profile that includes the following effects: anti-cancer, anti-inflammatory, anti-bacterial, anti-HIV, anthelmintic and anti-angiogenic. In addition, betulinic acid also has antidiabetic, antidyslipidemic and other effects. The characteristics that make betulinic acid a very attractive compound for carcinogenesis researchers are the high toxicity to cancer cells and the very low toxicity to normal cells (as demonstrated *in vitro* on skin fibroblasts and peripheral blood lymphocytes and *in vivo* on cells / organs). The present

study aimed to characterize the detailed effects of betulinic acid on mitochondrial bioenergetics and cell behavior in human melanoma cells. The results of previous studies on the non-toxicity induced by betulinic acid treatment in normal cells are debatable. Along these lines, the impact of betulinic acid treatment for 24 hours on the viability of human keratinocytes was verified using the MTT test. Incubation of cells with different concentrations of betulinic acid led to the following results: the lowest concentrations tested (1, 5 and 10 μM) did not affect cell viability, while higher doses (20, 25 and 50 μM) induced a dose-dependent decrease in cell viability, the percentage of viable cells calculated at the highest concentration tested - 50 μM was ~81%. To verify the type of betulinic acid-induced cell death, nuclei were stained using Hoechst 33342. Signs of apoptosis, such as nuclear contraction or nuclear fragmentation, were observed only in keratinocytes treated with the highest concentrations of betulinic acid - 20 and 50 μM , while at 10 μM no impact was observed on the cell nuclei. No signs of necrosis were detected in keratinocytes treated with betulinic acid or DMSO. Taken together, these results indicate that low concentrations of betulinic acid have no impact on the viability and morphology of HaCaT cells, while higher concentrations (20, 25 and 50 μM) reduce cell viability and induce morphological changes (loss of cell contact, cell contraction, nuclear fragmentation) specific to apoptotic death. Because betulinic acid treatment exerted a dose-dependent cytotoxic effect in melanoma cells, its impact on morphological changes was also verified. The presence of several rounded and detached cells, but unchanged adhesion and cell-cell contact were observed at a concentration of 10 μM BA compared to control cells (untreated cells). The highest concentration of betulinic acid tested - 50 μM induced significant morphological changes characterized by the presence of floating round cells, loss of cell-cell adhesions, loss of adhesion, reduced confluence and cell debris, clear signs of cytotoxicity. As concentrations increase, the apoptotic signs become more evident, as follows: nuclear contraction (a morphological hallmark of apoptosis - 10 μM betulinic acid), nuclear fragmentation, the presence of apoptotic bodies and chromatin condensation, characteristics similar to those observed after Staurosporin treatment (5 μM) - positive control for induction of apoptosis. No signs of necrosis were identified in BA-treated cells at the tested concentrations. These results suggest that betulinic acid triggers a dose-dependent cytotoxic effect in melanoma cells by reducing cell viability and inducing apoptotic features: morphological alterations of both the nucleus and the cytoplasm. Because betulinic acid is known to induce apoptosis in cancer cells

through the intrinsic pathway involving mitochondria and data on the role of pro- and anti-apoptotic markers in this process are still established, the effect of betulinic acid on proRNA mRNA expression has been further verified, apoptotic (Bid, Bax, Bak and Bad) and anti-apoptotic (Bcl-2 and Bcl-XL). The sub-cytotoxic concentration of betulinic acid - 10 μ M determined an upward regulation of mRNA expression for most pro-apoptotic markers (Bax, Bad and Bak), except Bid, the most significant increase in expression being calculated for Bak. Betulinic acid treatment had no impact on the anti-apoptotic marker Bcl-XL, while in the case of Bcl-2 an upward regulation was also observed. Furthermore, the impact of betulinic acid on caspase 3, caspase 8 and Apaf 1 mRNA expression was verified and a downward regulation of these marker expressions was observed in betulinic acid-treated cells. It was shown here that both global OCR and ECAR (measured at the end of each experiment) were decreased in a concentration-dependent manner when melanoma cells were treated with betulinic acid for 24 hours, however the concentration was 5 μ M. did not show a significant change in ECAR. Regarding the highest concentration of BA used in the experiments (50 μ M), melanoma cells were found to have the lowest basal respiration and no longer responded to the protocol used to modulate both glycolytic and mitochondrial pathways for production of ATP; as a result, overall OCR and ECAR showed the largest decline compared to untreated cells. Finally, proton production rates (PPR) underwent very similar changes to ECAR in betulinic acid-treated cells. This study aimed in particular at analyzing new perspectives on the effects of betulinic acid on mitochondrial bioenergetics in human melanoma cells, as a metabolism-based approach to fully understand the antitumor property of this bioactive phytochemical. The main working hypothesis was that the antimelanomic mechanism of action of betulinic acid involves the modulation of mitochondrial energy metabolism. The main findings in this regard are the following: (i) a dose-dependent inhibitory effect on both the rate of oxygen consumption (OCR) and the rate of extracellular acidification (ECAR) (classical markers of oxidative phosphorylation and glycolysis, respectively) and (ii) a significant decrease due to a sub-toxic concentration (10 μ M) of both respiratory parameters supported by complex I and II (in particular, active respiration - OXPHOS and maximum respiratory capacity of the respiratory system, electron transfer - respectively ETS). In addition, betulinic acid has been shown to cause a dose-dependent cytotoxic effect characterized by apoptotic traits: morphological alterations (nuclear fragmentation, apoptotic bodies), decreased mitochondrial membrane potential, up-regulation of mRNA expression of pro-apoptotic

markers (Bax, Bad and Bak) and changes in the morphology and location of mitochondria in the melanoma cell line. The results indicate that betulinic acid caused a dose-dependent inhibitory effect on both mitochondrial respiration and glycolysis in human melanoma cells. Mitochondrial bioenergetic dysfunction has been associated with cytoskeleton reorganization (actin fibers), changes in mitochondrial morphology, a decrease in mitochondrial membrane potential, and upward regulation of pro-apoptotic markers (Bax, Bad, and Bax). Therefore, these findings suggest that molecular targeting of mitochondrial bioenergetics with betulinic acid could be a valid strategy for advanced melanoma and provide a new perspective in underestimating the antimelanoma mechanism of action of betulinic acid.

Aromatic and medicinal plants have been used since ancient times for the treatment of certain diseases (being the first known form of medical care) and are the basis for the development of the drug industry, but also for prevention having an important role in public health. A medicinal plant abounds in bioactive components that exert a series of pharmacological effects, when consumed in its natural form or after a special preparation, in a form of therapeutic administration to address certain diseases. Various studies have analyzed the beneficial biological activity of plants, and the antioxidant, anti-inflammatory, antidiabetic and antitumor effects have been among the most frequently observed. Moreover, more than half of the medicines approved for human use are of plant origin. The beneficial therapeutic effects of plants are based on the chemical composition that most often abounds in phenolic compounds, terpenes, alkaloids, flavonoids and others. Data from the World Health Organization show that almost 80% of the world's population still uses traditional herbal medicine. Olive has been used for various actions related to human health since ancient times. Following the consumption of different parts of the plant, it has been observed that it shows beneficial biological activity against diabetes, inflammation, hypertension, infections, intestinal diseases, asthma, rheumatism, etc. Consumption of olive oil has been shown to be beneficial for human health, being highly valued in the Mediterranean diet, recognized for the prevention of cardiovascular disease, certain cancers or type 2 diabetes (chronic non-communicable diseases). Olive leaves have a varied chemical composition that includes: iridoid monoterpenes (predominantly oleuropein), triterpenes, flavonoids, chalcone, phenolic acids and coumarins. The second study presents the assessment of the transition from preclinical studies to clinical evaluations of plant extract and the importance of olive leaf extract in public health.

Regarding the metabolism of the biologically active compounds predominant in the olive leaf extract, the data from the literature show the presence of conjugated metabolites in the biological fluids *in vivo*. The study's areas of interest, both for the present and for the future, focus on the bioactivity of major metabolites, which in some cases is superior to parent compounds (e.g., hydroxytyrosol glucuronate is a five-fold free radical scavenger, stronger than hydroxytyrosol). The mechanisms involved in the biological activity exerted by the olive leaf extract are not fully understood. Regarding antioxidant activity, the problems that arise are related to the selection of reactive oxygen species responsible for oxidative stress, but taking into account that the association of reactive oxygen species with the progressive development of systemic diseases is not entirely true if they are taken into account, considering the essential need for the presence of superoxides for normal metabolome and physiological function. The steps required to obtain a finite form that reflects clinical activity are complex, and the transition from *in vitro* studies to clinical evaluation is based on complex resources, both human and material. *In vitro* studies, which predominate in the literature, despite the variety of methods used, provide only a small part of the information about the possible beneficial effects obtained with *in vivo* administration. As research on these effects progresses to *in vivo* studies, the results in the literature are considerably reduced and, finally, clinical trials provide a limited amount of information. Sulfate and glucuronide (conjugate) hydroxytyrosol metabolites appear to be the major metabolites of oleuropein found in biological fluids (plasma, urine) after administration of olive leaf extract. Among the main factors influencing the absorption and metabolism of biologically active compounds in the extract are the type of preparation administered (solid, liquid, syrup, capsules, etc.), the method of release or the frequency of administration. The individual phenolic compounds in the olive leaf extract are known for their potent biological activities tested by *in vitro* methods. However, most reports highlight the benefits of using extracts due to combinations of classes of biologically active compounds that based on synergistic effects have antioxidant and antimicrobial activities at least similar to those of individual compounds, but often superior to them. Natural compounds derived from olive leaf extract are considered a valuable source of models as a basis for new chemopreventive or therapeutic agents for various human diseases. Despite the fact that a significant number of phytochemicals are effective in preclinical studies (*in vitro* or *in vivo* in an animal model), the therapeutic efficacy is considerably reduced when they reach clinical trials. Finding the optimal formulations for capitalizing on the

therapeutic efficacy of olive leaf extract remains a challenge for researchers primarily involved in preclinical studies and selecting patients for clinical trials based on current population health issues remains a challenge for healthcare professionals. The study of biocompatible polymers is a topical one due to the remarkable properties they show. A recent search of several scientific databases has highlighted a large number of published papers based on biocompatible polymers. the use of natural compounds has been restricted due to a low stability related to various factors - light, pH, temperature conditions, etc. and low solubility. An alternative to correct these shortcomings is encapsulation in biocompatible polymers, which provide both protection and preservation of biological properties.

The third study was performed to observe the behavior of healthy cells in the presence of extracts of raw birch bark, apple pomace and olive leaf extracts, formulated as poly-lactic-co-glycolic nanoparticles (PLGA). These plants are known for their rich triterpene content, and the study is proposed to use the data obtained in evaluating the potential of such formulations in conditions such as inflammation and malignant tumor environment. The aim of the present study was to encapsulate three different crude extracts rich in triterpenes in a biocompatible polymer and to evaluate their properties *in vitro* for other applications as alternative anti-inflammatory and / or anti-tumor therapeutic agents. Particle size is an important parameter in the biomedical use of nanoparticles. In this study, the polydispersity index was determined together with the particle size. Index values differ significantly between control nanoparticles and those loaded with extracts. The DLS results revealed that the average diameter of the biopolymer samples was between 216-264 nm with a polydispersity index between 0.32-0.41. The values of the zeta potentials were negative, between - 28.9 and - 32 mV, which indicates the stability of the formulations obtained. The solutions prepared for evaluation - different concentrations of biocompatible formulations of poly-lactic-co-glycolic acid with extracts of birch bark, apple pomace and olive obtained by the method of evaporation of the emulsion - are safe in doses below 15 µg / mL and has a cytotoxic effect against normal cells, keratinocytes and fibroblasts, only at the highest concentration tested in certain cases. Activity on these cells was observed by microscopy. Stimulation of the crude extract causes various transformations in the shape of the cells. *In vitro* evaluations are essential to elucidate the behavior of various compounds as potential candidates in the treatment of various pathologies involving inflammatory and tumor processes. However, *in silico*, *in vitro*, *ex vivo* and *in vivo* studies need to be

developed and correlated in order to obtain a complete picture of a certain direction regarding the approach and treatment of the pathology. The originality of this research is to elucidate the behavior of different healthy human cells, both immortalized and primary, in the presence of biopolymer formulations by using PLGA together with raw birch bark, apple pomace and ethanolic olive leaf extract, in terms of viability. These cell types are commonly used in *in vitro* studies to test new compounds with anti-inflammatory and carcinogenic potential, and assessing normal cellular toxicity is a key step in the study of new curative compounds.

This doctoral thesis, in terms of experimental research, had three main objectives which involved: (i) preclinical *in vitro* and *in ovo* testing of extracts / triterpenes of therapeutic interest, (b) analysis of the association of plant extracts with population health and (c) preclinical *in vitro* testing of biocompatible polymers loaded with natural extracts. All these objectives have been fully achieved, which is mainly apparent from the published scientific articles.

The original contributions from all experiments performed and disseminated show on the one hand the cytotoxic activity of certain extracts (against malignant skin cells), elucidation of certain mechanisms of action in tumor processes of biologically active compounds (e.g., molecular targeting of mitochondrial bioenergetics with betulinic acid could be a valid strategy for advanced melanoma and offer a new perspective in underestimating the antitumor mechanism of action of betulinic acid) and on the other hand provide the starting point for preliminary cytotoxic safety (not affected by biocompatible polymeric formulations loaded with natural extracts of healthy cells).

The elucidation of the mode of action and synergism must be deepened in future research. The economic implications of the proposed research are substantial and often require a financial effort to obtain conclusive results, taking into account that most experiments are carried out at least in triplicate to obtain statistically significant and reproducible results.

Due to the complexity and interdisciplinary typical of the presented topic, the future research directions are multiple: the evaluation of extracts can be analyzed in both observational and interventional clinical trials, the mechanisms of action of betulinic acid can also be evaluated on reconstructed human tissue model (in case for preventive use) or on an animal model (in case of therapeutic use), biocompatible polymers extremely useful for the protection and transport of active substances require long-term studies to complete the toxicological profile.