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PHD THESIS

**CONTRIBUTIONS RELATED TO THE EFFECT OF PLANTS
EXTRACTS WITH THERAPEUTIC POTENTIAL ON CELL
CULTURES AND CHORIOALLANTOID MEMBRANE**

– R E S U M E –

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CONTENTS

List of scientific published articles	V
List of abbreviations and symbols	VI
List of figures	VII
List of tables.....	XI
Dedication	XII
Acknowledgment.....	XIII
INTRODUCTION	XV

GENERAL PART

CHAPTER 1. CURRENT NOTIONS RELATED TO THE BIOLOGY AND EVOLUTION OF MALIGNANT DISEASES	1
1.1 Introduction	1
1.2 Angiogenesis associated with malignant tumors	6
1.3 Current angiogenic therapy	10
CHAPTER 2. PLANT EXTRACTS WITH ANGIOGENIC EFFECTS	14
2.1 Introduction	14
2.2 Biological effects attributed to medicinal plants and active constituents.....	15
2.3 Angiogenesis and medicinal plants.....	20
CHAPTER 3. MODELS FOR TESTING COMPOUNDS WITH AN ANGIOGENIC POTENTIAL.....	23
3.1 Introduction	23
3.1 Models for testing therapy efficacy in tumor-associated angiogenesis.....	28

EXPERIMENTAL PART

CHAPTER 4. EVALUATION OF THE BIOLOGICAL ACTIVITY BY <i>IN VITRO</i> , <i>IN OVO</i> AND <i>IN VIVO</i> METHODS OF THE EXTRACT OF <i>OCIMUM BASILICUM</i> L.	34
4.1 Introduction	34
4.2 Materials and methods	35
4.2.1 Materials.....	35
4.2.2 Methods.....	36
4.3 Results and discussions	41

4.3.1 Chemical composition of <i>Ocimum basilicum</i> L. extracts.....	41
4.3.2 Bioactivity of <i>Ocimum basilicum</i> L extracts.	45
4.4 Conclusions.....	62
CHAPTER 5. ASSESSMENT OF BIOLOGICAL ACTIVITY BY <i>IN VITRO</i> METHODS OF <i>HELLEBORUS PURPURESCENS</i> EXTRACT.....	63
5.1 Introduction	63
5.2 Materials and methods	65
5.3 Results and discussions	68
5.3.1 Chemical composition of <i>Helleborus purpurescens</i> extract.....	68
5.3.2 Bioactivity of <i>Helleborus purpurescens</i> extract.....	69
5.4 Conclusions.....	79
CHAPTER 6. THE EFFECT OF PLANT EXTRACTS ON THE CHORIOALLANTOID MEMBRANE (MCA) OF THE EMBRYONED EGG	80
6.1 Introduction	80
6.2 Materials and methods	85
6.3 Results and discussions	87
6.4 Conclusions.....	103
CONCLUSIONS AND PERSONAL CONTRIBUTIONS.....	104
REFERENCES	108

RESUME

Nowadays, phytotherapy has become a modern science, a branch of medical sciences. This was possible only to the extent of the development of other sciences, such as: histology, histopathology, cellular pharmacology, biochemistry, and others.

Without underestimating the role of chemotherapy (the treatment of diseases with the help of synthetic drugs), phytotherapy in particular, and natural therapy in general, come to complete the set of preventive and curative methods currently practiced. But not only to complement them, because numerous active plant substances, those with strong action, are considered basic treatments and not just adjuncts, in modern therapy. Many of the synthetic drugs are harmful, either through unwanted side effects of toxic, often unpredictable causes, or through their allergic nature, because synthetic substances behave like a foreign body introduced into the body, while medicinal plants and biosynthesis substances are valued as harmless, which the body recognizes as "friends".

The use of different plants in medicine is not new, it is not a surprise, but a necessity. Plant extracts are not only useful as remedies, but also as models to produce drugs or biologically active substances. The use of phytotherapy encompasses a wide spectrum of human ailments, for many of which the mechanisms of action and the effects on symptoms are known. Less known is the morphological and molecular impact of these substances. Data on the effect of plant extracts on pre-existing or newly formed blood vessels are sparse, insufficiently demonstrated or, in some cases, missing.

The field of phytotherapy is one of great interest in the current period, and the aim of European and national health and social policies is to improve the quality of life, including by ensuring the superior quality of pharmaceutical and parapharmaceutical products. In the future, the competitiveness of the European parapharmaceutical industry containing plant compounds will depend on the *in vitro* and later *in vivo* study of new plant extracts with multitargeted effects (for example on angiogenesis), which can subsequently be incorporated into various therapeutic products.

The main purpose of this paper is to evaluate the biological activity and the safety profile of certain types of medicinal plant products (basil, thyme, hellebore, thuja, mistletoe), their influence in angiogenesis by promoting efficacy and safety. The motivation for choosing these substances resides in the fact that they are all used in one form or another in phytotherapy, but the mechanisms of action and the effects at the tissue level are insufficiently studied or unknown. Therefore, while maintaining properties similar to synthetic active substances for the plant extracts under study, the mechanisms of action and safety profiles were evaluated, and the effectiveness of these products was also tried to be highlighted. Plant products that meet current standards of quality, safety and efficacy were used, and their effectiveness was analyzed in studies *in vitro* (on healthy and tumor cell cultures), *in ovo* (on angiogenesis, on embryonated egg), and where it was case, *in vivo* (on the skin of healthy volunteers). The present research is innovative because within it, for the first time, the *in ovo* evaluation, on embryonated egg, of some macerated plant products with an effect on angiogenesis is carried out.

The general part contains three main chapters. The first chapter deals with the biology and evolution of malignant diseases – angiogenesis associated with malignant tumors and aspects related to current angiogenic therapy; in the second chapter, aspects related to plant extracts with angiogenic effects are presented – the biological effects attributed to medicinal plants and active constituents as well as angiogenesis and medicinal plants; and in the last chapter, models for testing compounds with an angiogenic role are discussed.

The special part comprises three main directions. The first direction is related to the evaluation of the potential activity of two hydroalcoholic extracts, obtained from the plant *Ocimum basilicum* L. Experimental studies were carried out that focused on: (a) the characterization of the extracts to establish the chemical composition (in terms of the total content of compounds phenolics, the total content of flavonoids and flavonols and, respectively, condensed tannins, the content in predominant polyphenols), (b) the analysis of the antioxidant activity (through the prism of the free radical scavenging effect), (c) the correlated impact on cell viability (cytotoxic potential on four lines normal human and murine cells and on a tumor cell line - human melanoma), (d) antiangiogenic potential *in ovo* (embryonic egg chorioallantoic membrane study) and (e) effect on healthy skin (non-invasive *in vivo* measurement of skin biophysical parameters). The second direction of

research was represented by the evaluation of the biological activity by *in vitro* methods of the extract of *Helleborus purpureus* which renders: (a) the evaluation of the chemical composition of the hydroalcoholic extract of *H. purpureus*, (b) the determination of the antioxidant activity, (c) evaluation of the cytotoxic effect on four tumor cell lines (squamous carcinoma, murine melanoma and two breast cancer lines) and on two healthy cell lines (human keratinocytes and murine epidermal cells) and (d) apoptotic gene expression analysis. The last research direction involved the analysis of the effect of standardized plant extracts on the chorioallantoic membrane (MCA) of the embryonated egg. An *in ovo* experiment was realized in which the angiogenic potential of five different types of medicinal plants (*Ocimum basilicum*, *Thymus vulgaris*, *Helleborus purpureus*, *Thuja Occidentalis* and *Viscum album*) was evaluated, in order to establish the toxic or non-toxic character. On the one hand, the potential effectiveness of the samples (macerated prepared in a specialized company – Favisan Laboratories) was taken into account, and on the other hand, the harmful effects they can induce on the chorioallantoic membrane and the embryo were analyzed.

In the first study, which involved the evaluation of the potential biological activity of two hydroalcoholic extracts obtained from the aerial part (without leaves) and from the leaves of *Ocimum basilicum* L., *in vitro*, *in ovo* and *in vivo* experiments were carried out which were completed each other. The hydroalcoholic extracts were investigated by liquid chromatography coupled with mass spectrometry, evaluated spectrophotometrically from the point of view of chemical composition for the total content of phenolic compounds, the total content of flavonoids and flavonols and respectively condensed tannins. Both hydroalcoholic extracts were found to be rich in polyphenolic compounds, of which kaempferol and quercetin predominate. The obtained *in vitro* results on healthy cells were compared with the antitumor activity *in vitro* against the A375 tumor cell line. In addition, the *in ovo* antiangiogenic potential induced by hydroalcoholic extracts of basil was also investigated. Female volunteers with healthy normal skin were selected for the *in vivo* study, and the assessment was made by non-invasive *in vivo* measurement of skin biophysical parameters. The hydroalcoholic extract of *Ocimum basilicum* L. obtained from the leaves induced a significant antioxidant activity, the results being close to the value of ascorbic acid, used as a standard reference. The results of the current study corroborate the traditional use of

Ocimum basilicum L. as an antioxidant and suggest that the high antioxidant effect is due to the high amount of polyphenolic compounds. The potential cytotoxicity of the hydroalcoholic extracts of basil was evaluated by the established MTT method, following the cell viability, in the presence of the extracts, of different types of healthy and tumor cells - immortalized human keratinocytes (HaCaT), human skin fibroblasts (1BR3), epidermis of mice (JB6Cl41-5a), primary human melanocytes (HEMa), human melanoma A375 cells. *In vitro* evaluations have shown that *Ocimum basilicum* extract is a source of non-toxic active compounds on skin-related cells and may act as a possible protective agent against melanoma. The extracts did not induce a significant cytotoxic effect on any selected normal cell lines, but showed relevant activity on A375 cells. The study of the toxicological profile continued by evaluating the activity of the extracts on the chorioallantoic membrane. Considering the low values obtained regarding the irritant effects in the chorionallantoid membrane of the egg on the blood vessels, we can emphasize that both extracts can be considered as biocompatible ingredients. Both hydroalcoholic extracts of basil show good biocompatibility and tolerance on the developing vascular plexus as indicated by the HET-CAM assay. Hydroalcoholic extracts applied in the tested concentration range appear to be optimal beneficial ingredients for wound healing and regenerative medicine and can even be included in nutraceutical formulations if the plant extracts are characterized in terms of active compounds and biological profile. Regarding the potential activity of the hydroalcoholic extracts on the human skin, the biophysical parameters of the skin were evaluated by non-invasive methods. The decrease in erythema values after the application of the extracts indicates the anti-inflammatory potential of *Ocimum basilicum* L. The results obtained from the *in vivo* evaluations and biophysical parameters of the skin indicated that *Ocimum basilicum* L. exhibits anti-inflammatory activity and promising wound healing properties.

Apoptosis is the programmed cell death that under physiological conditions contributes to the maintenance of homeostasis and the elimination of unwanted cells. In cancer, apoptosis plays an important role in preventing tumor formation. Losing apoptotic control, cancer cells become more resistant to treatment and therefore survive longer, becoming more invasive and aggressive. *H. purpurascens*, is a medicinal plant with therapeutic effects used in traditional medicine since ancient times. In the second study, the evaluation of the

composition of a hydroalcoholic extract of *Helleborus purpureus* (HPex) was considered in terms of the composition of polyphenols, the antioxidant activity was determined, the potential cytotoxic effect on four different cancer cell lines was evaluated (squamous cell carcinoma-A431; murine melanoma - B164A5; and breast cancer - MCF-7 and MDA-MB-231) compared to the effect on two healthy cell lines (human keratinocyte cell lines - HaCaT and murine epidermal cells - JB6) and the expression of the main genes involved in the apoptosis process was determined, thus providing a possible explanation for the cytotoxic effect of *H. purpureus* extract. LC-MS analysis showed that the extract contained high levels of flavonoids, especially quercetin, kaempferol and epicatechin. First of all, the hydroalcoholic extract exerted a strong antioxidant effect, close to that of ascorbic acid. Next, starting from the beneficial role played by antioxidants in antitumor therapy, the hydroalcoholic extract proved its selective cytotoxic effects in the four tumor cell lines used, the proliferation of healthy cells not being affected by stimulation with *H. purpureus* extract. Finally, the influence of HPex on increasing the expression of pro-apoptotic genes (Bax and Bad) and decreasing the expression of anti-apoptotic genes (Bcl-2) was highlighted in the breast cancer cell line - MCF-7. The extract has important therapeutic value for the future of oncology therapy, however, further studies are needed to elucidate the mechanism of action and identify the key phytoconstituents that explain these biological effects.

The method of studying the effect of different substances or cells on the chorioallantoic membrane has a number of advantages. The vast majority of animal models for the study of tissues and cells involve the use of immunosuppressed animals in order to achieve viable heterotopic transplants. On the other hand, these models are difficult to approach especially when using human cells, due to limited inter-species compatibility. The chorioallantoic membrane is naturally immunodeficient, so the implantation of cells, tissues or tumors from different species is easily accepted without generating an immune response. The MCA is a favorable environment for normal and/or tumor cell growth, being very well vascularized. The close vascular connections with the embryo make the experiments refer to the whole ensemble of structures. All the changes induced by the implantation can be observed with the naked eye, or even better, and preferably with the stereomicroscope specially built for this purpose, which allows even the localization and evolution of the implanted cells. In addition, the survival

rate of implanted cells is much higher on MCA than on murine model without significant damage. For these advantages, in addition to the low cost price and practically indefinite repeatability, this method was considered for the present study.

Like any other study method in medicine and biology, the MCA method also has certain limitations. The experiment may (rarely) show non-specific superinfections, but which rarely occur when the implant is made early after incubation. For this reason, in the present experiments, the fifth day was chosen for implantation on the MCA surface of the test substance. This is because early in the embryonated hen's egg, the immune system is immature and the host reaction against the graft does not develop, regardless of the type of implanted substance. The actual duration of the experiment is relatively short, approximately 7-10 days, so it is unlikely that the tumor implanted on the MCA will produce distant dissemination before the end of the experiment. There is a relatively small pool of avian tissue-specific molecular markers available so that it is sometimes difficult to characterize the developmental process of cells or implanted substances. Virtually any substance, cell or tissue can be implanted on or into the MCA during the first week of natural development. These particularities were used for the present research, considering the accuracy of the method, the repeatability and the sequential application of short-term experiments. This method has been extensively applied to the study of angiogenesis under normal and pathological conditions, as mentioned above. There are very few articles on the effect of substances extracted from plants with apparent utility in non-conventional medical therapy.

Starting from the results obtained in the studies by biochemical, pharmacological and toxicity methods, an *in ovo* experiment was set up based on the application of five types of preparations from different medicinal plants (*Ocimum basilicum*, *Thymus vulgaris*, *Helleborus purpureus*, *Thuja Occidentalis* and *Viscum album*) used in the present study, in order to establish the toxic or non-toxic nature of these substances in an *in vivo* experiment. On the one hand, the potential effectiveness of five types of samples (macerated prepared within a profile company - Favisan Laboratories) was taken into account, and on the other hand, the harmful effects they can induce on the chorioallantoic membrane and the embryo. The study of the behavior of the chorioallantoic membrane on which

standardized extracts of basil, hellebore, mistletoe, thyme and thuja were placed allow the following conclusions to be drawn: (a) the survival of the specimens subjected to the experiment is strictly dependent on the substance used for stimulation; (b) the strongest toxic effect was noted for the rosehip extract; (c) the least toxic to the chorioallantoic membrane was the basil extract, followed by the specimens treated with thyme extract; (d) the study method using the chorioallantoic membrane has been shown to be a faithful indicator of the toxicity of the extracts with which the stimulation was carried out; (e) we believe that this method can be used as a preliminary step for testing the potentially toxic activity of a large number of substances with possible therapeutic effects.

Future studies that should be conducted are related to the deepening of the mechanisms of action of the plants presented in the present study along with finding optimal formulations that show increased efficacy, with the preservation of biological effects and without exerting toxic effects. A potential experiment on *in vitro* and *in vivo* models to study the effect of plant extracts on tumor cell implantation could be an interesting continuation of this study. This methodology could constitute the experimental bases for the introduction of a wide variety of substances with antitumor effect in oncological medical practice.