PhD THESIS

Research on obtaining, characterizing, and valorizing extracts of *Artemisia* spp. for phytotherapeutic purposes

(SUMMARY OF Ph.D. THESIS)

PhD student **Maria-Evelina Bordean**

Scientific Coordinator **Matei-Marcel Duda PhD, Professor**

INTRODUCTION

The use of plants in treating various conditions, both in humans and animals, is a practice as ancient as human existence itself. Currently, global research on plants has burgeoned, with studies showing the immense potential of medicinal plants utilized in various traditional systems.

The urgency for mass production and optimized utilization of secondary metabolites from plants has intensified, particularly in light of diseases stemming from the COVID-19 pandemic. Revolutionary advances in genomics and biotechnologies have ushered in a new era of research, reshaping our comprehension of biosynthesis, regulation, and manipulation of bioactive molecules from medicinal plants (SHI et al., 2024).

The incidence and mortality rates caused by cancer are increasing worldwide. The high costs, side effects, and drug resistance associated with cancer treatment have spurred scientists to invest in the research of new antitumor drugs based on medicinal plants. These plants contain bioactive compounds that act as antitumor agents, often with more favorable side effects and toxicity profiles compared to conventional chemotherapeutic agents (ADICO et al., 2024).

The search for and development of plant-based medicines have been significant priorities in science for centuries, with *Artemisia* species garnering attention from researchers in recent years due to their chemical composition and biological activity. A milestone in this scientific pursuit was the discovery of artemisinin, an antimalarial agent, by the Chinese scientist Youyou Tu, who was awarded the Nobel Prize in Physiology or Medicine in 2015. The source of this drug was *A. annua* L., a species long known in traditional Chinese medicine (SHARIFI-RAD et al., 2022).

Modern pharmacological research has been focused on validating and elucidating the mechanisms underlying the activities described earlier. Recently, it has been demonstrated that extracts of *Artemisia* species exhibit a wide range of biological activities. *A. absinthium* L. has attracted research attention due to its hepatoprotective, neuroprotective, antidepressant, cytotoxic, and digestive stimulant properties (HESHMATI AFSHAR et al., 2021; HAMDOON, 2022; ABDEL-GAWAD et al., 2022).

Medicinal plants constitute an important local heritage, yet their relevance extends globally. Today, phytopharmacology also relies on *Artemisia* species, renowned for their historical medicinal and aromatic applications, encompassing various uses in medicine, cosmetology, and the food industry.

Keywords: *Artemisia absinthium* L.*, Artemisia annua* L.*,* antibacterial effect, tumor cells, migration, Nrf2, apoptosis.

STRUCTURE OF THE THESIS

The first part of the thesis, the ”LITERATURE REVIEW”, comprises 3 chapters.

Chapter 1: *Artemisia absinthium* L. - the proverbial quintessence of bitterness encompasses in 3 subchapters aspects related to the general characteristics of the plant, its chemical composition, and its biological activity.

Chapter 2: *Artemisia annua* L. - natural remedy officially recognised by the Nobel Prize is structured into three subchapters that describe *Artemisia annua* L., discussing the general characteristics of this species, morphological aspects, chemical composition, and biological activities.

Chapter 3. *Artemisia* spp. in cancer management, comprises four subchapters, the entire chapter referring to neoplastic diseases, plant phytochemicals viewed as potential candidates in neoplastic disease therapy, and the mechanisms of action of antitumor agents.

The second part, titled “PERSONAL CONTRIBUTION” comprises 5 chapters and a bibliography.

Chapter 4. The purpose, objectives, and experimental design outline the goals of the study and the experimental methodologies employed. The research aims to quantify, characterize, and evaluate the biological effects of ethanol extracts of *Artemisia* spp. with the goal of identifying an extract with potential antitumor effects. The assessment of possibilities for the valorization of bioactive compounds from Artemisia spp. encompasses three main research directions, each with specific objectives:

I. Study of ethanol extracts of *Artemisia* spp. from the compositional aspect of biologically active compounds of interest.

O1.1. Identification and quantification of the total phenolic content (TPC).
O1.2. Evaluation of antioxidant activity (ABTS and DPPH assays).
O1.3. Quantitative determination of phenolic compounds (HPLC).

II. Study of phytochemical and antibacterial activity of ethanol extracts of *Artemisia* spp.

O2.1. Evaluation of the antibacterial activity of ethanol extracts of *Artemisia* spp. against standardized bacterial strains: Determination of MIC and MBC values.
O2.2. Antibacterial effects of ethanol extracts of *Artemisia* spp. against 15 strains of multidrug-resistant gram-negative bacilli isolated from the hospital.

III. Study of the in vitro antitumor effect of ethanol extracts of *Artemisia* spp.

O3.1. Evaluation of the effects of extracts on the viability of melanoma and colorectal cancer cells;

O3.1.1. Differentiation between the effect of alcohol contained in extracts and the effect of alcohol administered at similar concentrations as the extracts in various dilutions;
O3.1.2. Determination of IC50 values;
O3.2. Evaluation of the effects of extracts on the migration and metastasis capacity of tumor cells;
O3.3. Analysis of the mechanisms of action of extracts on tumor cells;
O3.3.1. Study of the oxidative stress effects induced by extracts with evaluation of cellular antioxidant systems;
O3.3.2. Evaluation of the induction of programmed cell death (apoptosis).

In this chapter, organic material derived from the wild flora, specifically the species of wormwood (*Artemisia absinthium* L.) and mugwort (*Artemisia annua* L.), collected from the vicinity of the city of Blaj, Alba County, Romania, is presented. The general experimental model encompasses both the characterization of ethanol extracts from these plants and the assessment of their phytotherapeutic applicability. Based on the tested biochemical profile, these extracts demonstrate attributes indicative of a positive response in the treatment of tumor cells.

Chapter 5. Materials and Methods is structured into 8 subchapters, each corresponding to the established research directions. These subchapters present the materials used and the methodologies applied, including experimental methods for determining the classes of biochemical compounds from different *Artemisia* spp., specifically *A. absinthium* L. and *A. annua* L. (indices of antioxidant properties, determination and quantification of total phenolic and flavonoid content, determination of antioxidant capacity); methods for determining antibacterial activity (minimum inhibitory concentration and minimum bactericidal concentration); and experimental methods used to evaluate the *in vitro* antitumor effect (B16F10, DLD-1, HCT116 tumor cell lines) of ethanol extracts of *Artemisia* spp. The working protocol is presented in Fig. 1.

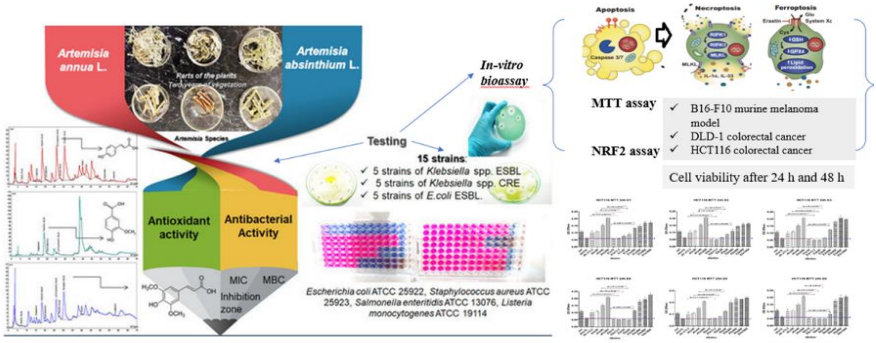

Fig. 1. **The experimental protocol for studying the phytochemical, antibacterial, and cytotoxic activity of *Artemisia* spp. extracts**

Chapter 6. Results and discussions elaborated following the determinations made, are structured according to the three research directions.

Results and discussions for the first research direction

The quantification of total polyphenols, through UV-VIS spectroscopic analysis, denotes the following aspects: The morphological part of the plant and the species influenced the total content of phenolic compounds in the studied wormwood extracts. The highest level was obtained for the leaf sample in the second harvest year. Regarding the species, *A. absinthium* L. recorded the highest content of phenolic compounds, with the leaf extract having a higher content compared to the stem extract. Sinapic acid was present in significant amounts in the leaf and stem extracts of *A. annua* L. In all ethanol leaf extracts, vanillic acid was detected in significant amounts.

Results and discussions for the second research direction

The following standard strains were tested: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, and *Listeria monocytogenes* ATCC 19114, alongside 15 other multidrug-resistant strains isolated from the hospital: *Klebsiella* spp. ESBL, *Klebsiella* spp. CRE, and *E. coli* ESBL.

The ethanol extract from the leaf of *A. annua* L. exhibited antibacterial activity against all tested bacterial strains. The MIC of the ethanol extract from AnL ranged from <2.00±0.014 mg/ml (against *S. aureus* ATCC 25923) to 375.00±0.014 mg/ml AbS1 (against *E. coli* ATCC 25922 and *S. enteritidis* ATCC 25922 and *S. enteritidis* ATCC 25923). The MBC of the ethanol extract from *A. annua* L. ranged from 5.00±0.014 mg/ml (against *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 19114) to 375.00±0.014 mg/ml AbS1 (against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, and *L. monocytogenes* ATCC 19114). AnL, AbL1, and AbS1 from the first harvest year showed significant activity against *Klebsiella* ESBL (10.863±0.308 mm for AnL; 10.110±1.68 mm for AbL1; 11.246±1.71 mm for AbS1). Significant antibacterial activity against *Klebsiella* CRE was identified in samples of *A. annua* L., both in leaf and stem, with sinapic acid being predominant in both extracts (12.756±0.993 mm for AnL; 9.843±0.945 mm for AnS). Extracts AnL and AnS exhibited the strongest effect against *E. coli* ESBL (8.610±1.861 mm, respectively 5.67±0.682 mm).

Results and discussions for the third research direction

Results and discussions regarding the evaluation of extract effects on the viability of melanoma and colorectal cancer cells

A comparative synthesis of the IC50 calculation data for the three tumor cell lines was performed for each extract. Overall, the highest IC50 values were observed for the DLD-1 cell line (extracts AnL2, AnS2, AnF2, AbL2, and AbF2), suggesting increased resistance to these treatments. The most sensitive line proved to be the HCT116 colorectal cancer cell line.

In our study, the most sensitive cell line was HCT116 to the alcoholic extracts of *A. absinthium* L. - for AbL2 (0.384mg/ml) and AbF2 (0.297mg/ml) with the exception of the stem extract AbS2, where the IC50 was 1.831mg/ml, in the MTT viability assay. Similar IC50 values at 24h were reported by NAZERI et al., 2020, of 1204µl/ml (1,204mg/ml) in response to HCT116 cell treatment with methanolic leaf and stem extract.

Results and discussions regarding the evaluation of the effects of extracts on the migration and metastasis capacity of tumor cells

The murine melanoma cell line exhibits increased proliferation. In the migration assay, at 20 hours, the control cells show approximately 70% closure of the scratch line.

The DLD-1 cells were characterized by a lower migration capacity, as they failed to repair the scratch line even after 48 hours.

The HCT116 cells exhibited a different behavior, with a very low migration rate of the control cells, both at 20 hours and 48 hours.

Results and discussions regarding the analysis of the mechanisms of action of extracts on tumor cells

To evaluate the expression of the transcription factor Nrf2, immunocytochemical staining of tumor cells (B16F10, DLD-1, and HCT 116 cells) was performed.

In our study, treatments with ethanolic extracts of *A. absinthum* L. and *A. annua* L. induced different expressions of the NRF2 protein depending on the cell line and type of extract. DLD-1 colon carcinoma cells showed intense NRF2 upregulation with all ethanolic extracts, cells that otherwise expressed the protein even without treatment, but at lower levels. DLD-1 cells also exhibited the lowest percentages of apoptotic cells post-therapy among the tested lines, with the mention that for *A. annua* L. extracts (AnL2, AnS2, and AnF2), as well as for AbL2, the percentages of necrotic cells were higher (10-13%, with the highest for AnL2 at 31%). These data suggest the induction of a different type of cell death (ferroptosis or necroptosis), possibly through artemisinin's utilization of iron for generating free radicals. Increased NRF2 expression may be an indicator of the cellular response to oxidative stress induced by plant extracts. In comparison, HCT116 cells, which were the most sensitive to plant extracts, especially those derived from *A. absinthium* L., but also to AnL2, reacted with a slight increase in NRF2 expression (diffuse intracytoplasmic). With these extracts, the response of HCT116 cells was dramatic, with the highest values of both apoptosis (~10%) and high percentages of necrotic cells (51% for AnL2 and between 24-41% for *A. absinthium* L.).

The murine melanoma cells also responded to the plant extract treatments regarding NRF2 synthesis, particularly for the AnL2 (most intense). A notable

observation was the alteration in cell morphology. Another observation was an increase in the percentage of apoptotic cells (the highest increase among the three tested cell lines), with values ranging from 16-25%, except for cells treated with AnL2 extract, which exhibited a higher number of necrotic cells (24%).

Chapter 7. Conclusions

Research Direction I

1. Morphological part of the plant, and species influenced the total content of phenolic compounds in the extracts of *A. absinthium* L. and *A. annua* L.
2. The highest TPC values were found in *A. absinthium* L. leaves (487.36±0.08 mg GAE/ml) and in *A. annua* L. leaves (518.09±0.01 mg GAE/ml). Leaf ethanol extracts had higher TPC than ethanol extracts from stems, regardless of species and harvest year.
3. The highest phenolic compound value was obtained for the leaf sample in the second harvest year. Regarding the species, *A. absinthium* L. recorded the highest total phenolic compound content, with leaves having a superior value compared to the stem.
4. Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is the most representative phytochemical hydroxycinnamic acid among the identified flavonoid compounds.

Research Direction II

1. Regarding the activity of wormwood extracts against *S. aureus*, the results showed that leaves, rich in phenolic compounds, exhibited higher antibacterial activity than the stem.
2. *A. annua* L., rich in polyphenolic compounds, especially in leaves, demonstrated antibacterial activity against Salmonella enteritidis.
3. Among all studied *Artemisia* extracts, both leaves and stems of *A. annua* L. showed significant activity against *Klebsiella* spp. CRE and *E. coli* spp. ESBL.
4. The best antibacterial activity against L. monocytogenes ATCC 19114 was found in *A. annua* L. leaves, with MIC = 5.00±0.014 mg/ml and MBC = 5.00±0.014 mg/ml.

Research Direction III

1. The median inhibitory doses (IC50) varied among the 3 cell lines, with the HCT116 line being more sensitive to lower doses of ethanol extracts.
2. Conclusions regarding the 20h migration test:

a. Migration was significantly inhibited for the B16F10 line (in increasing order AbF2 ► AbS2 ► AnL2 ► AnS2 ► AnF2 ► AbL2).

b. Migration was moderately inhibited for the DLD-1 line (poor migration/repair, even in untreated control 30%) (in increasing order AnS2 ► AbS2 ► AbL2 ► AnF2 ► AnL2 ► AbF2).

c. Migration was significantly inhibited for the HCT116 line (in increasing order AbL2 ► AbS2 ► AbF2 ► AnS2 ► AnF2 ► AnL2).

3. Conclusions regarding the 48h migration test:

a. Migration was significantly inhibited for the B16F10 line (in increasing order AbF2 ► AbS2 ► AnL2 ► AnS2 ► AnF2 ► AbL2).

b. Migration was slightly inhibited for the DLD-1 line (poor migration/repair, even in untreated control 36%) (in increasing order AbF2 ► AnL2) and exceeded control values for AnF2 (36%) ► AbS2 (36%) ► AnS2 (38%) ► AbL2 (48%).

c. Migration was inhibited for the HCT116 line with poor repair rate at control 47% only for treatments with AbL2 (21%), AnL2 (25%), AbF2 (27%), AnF2 (28.7%) and exceeded control values for AnS2 and AbS2.

4. Treatments with extracts induced by NRF2 protein expression in all 3 cell lines more intensely for the AnS2, AnF2, AbL2, and AbF2 extracts. It is noteworthy that these extracts showed the highest concentrations of antioxidant compounds according to HPLC analysis.
5. Type of cell death induced by extract treatments:

   a. **Apoptosis**. Extracts of *A. absinthium* L. (AbL2, AbS2, and AbF2) induced the highest rates of cell death through apoptosis (except for the DLD-1 line). Higher rates of Annexin/propidium iodide-positive cells were also observed for extracts of *A. annua* L. (AnS2 and AnF2) with DLD-1 cell resistance to extracts.

   b. **Cellular necrosis** was observed in increased proportions especially for AnL2, AbL2, and AbS2 (only for the HCT116 line).

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