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DOCTORAL THESYS

Phytocompounds with antitumor activity

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Hedera helix L.; phytochemical constituents; α-hederin; hederagenin; hederacoside C; flash-chromatography; saponins mixture optimization; *in vitro* cytotoxicity; antiproliferative activity.

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INTRODUCTION

History of human use of plants starts from the earliest times, initially being used as food, however after the discovery of their healing properties, the plants have become a source of treatment of the wounds and suffering and also of improvement health in different human communities. Evidence of the therapeutic use of plants has been mentioned since the time of the Sumerian and Akkadian civilizations in the third millennium BC. Plant products in ancient times formed part of the specific traditional medicine systems of China, India (ayurvedic medicine) and Egypt. Egyptian papyrus scrolls contained thousands of herbal recipes with therapeutic applications, for cosmetic use or for preservation and embalming. In ancient Greece, scholars such as Hippocrates described in medical treatises already theories of tumor formation and their potential therapeutic methods. Popular herbal healing methods have been passed down in our country since ancient times, so Herodotus (5th century BC) mentioned in his writings that *Leonurus cardiaca* (Goosefoot) plant was used to treat heart disorders by people living in north of the Danube.

For a long time the plant extracts and their naturally occurring compounds have been used in traditional medicine in various empirically treatments, but recently as a result of their scientific research has shown that they contain active principles able to treat many diseases including cancer. In studies conducted on specific natural compounds in ayurvedic Indian medicine, the obtained results have showed that many of this compounds have strong anti-inflammatory and anti-tumor properties.

In past time the Turmeric (*Curcuma longa*) has been widely used in ayurvedic medicine for its therapeutic benefic attributed today to the presence of curcumin, the active ingredient in turmeric, which has the ability to suppress the development of tumors in a wide variety of cancers, a fact confirmed by studies intense effects of this compound.

Extract Mayapple, *Podophyllum peltatum* , traditionally used by Native Americans in the fight against skin cancer, on malignant neoplasms and of other diseases, is based on single major component podophyllotoxin which became the first in a series of effective antitumor agents called podophyllins.

Camptothecin, identified in *Camptotheca acuminate* extracts used in traditional Chinese medicine, has been found to have antitumor activity, and its topotecan and irinotecan derivatives are frequently used to treat ovarian and colon cancers.

Preliminary studies of Alternative and Complementary Medicine have allowed more and more naturally occurring therapeutic compounds used in traditional medicine to become a source for new drugs at the forefront of modern medicine.

The use of plants as anti-tumor agents has started with the discovery and development of vinca alkaloids (vinblastine and vincristine, 1950), and isolating the podophyllotoxins. Vinca alkaloids have been responsible for curing some forms of leukemia, and vincristine inhibits the microtubules assembly, inducing their association into spiral aggregates.

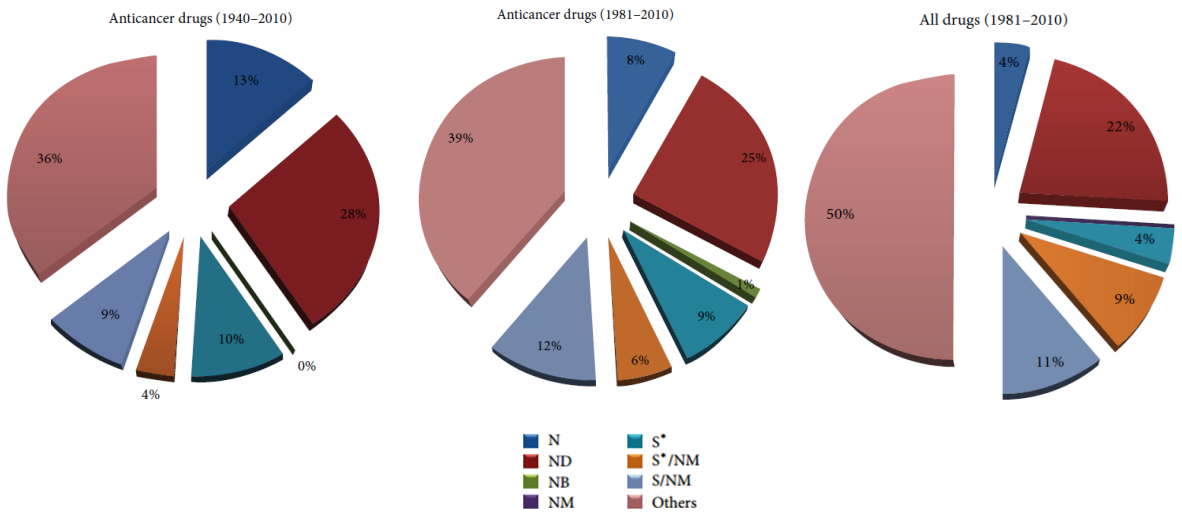


Figure 1. Figure 1: Sources of anticancer drugs from the 1940s to 2010. ∗Natural product (N), derived from a natural product, usually a synthetic derivative (ND). Natural product "Botanical" (NB); natural product mimic (NM); totally synthetic drug (S) made by total synthesis, but the pharmacophore is/was from a natural product (S∗).

Since 1955, at international level antitumor agents have been widely registered and verified following standardized testing protocols. Since the 1980s, research on the chemotherapeutic effects of natural compounds has yielded many promising results. The discovering of antitumor activity of many traditional herbal medical remedies has been supported and validated by the results of numerous scientific studies.

Between 1940 and 2010, a total number of 27 antitumor drugs (**Figure 1.)** [1] were obtained from natural sources. Examples are: actinomycin D, paclitaxel and vincristine which are currently some of the most widely used chemotherapeutic agents in the treatment of cancer, while topotecan, dexamethasone, etoposide and even tamoxifen are compounds with antitumor action similar to natural products. A further certain example is resveratrol, polyphenol present in grapes, has been shown to have potential both as a preventive agent and as an antitumor agent. Similarly, piperlongumine, an extract from *Piper longum* , selectively induce reactive oxygen species in cancer cells, causing cell death by apoptosis.

In the 1980s, Bagshawe et al. developed a new use of natural compounds, Targeted Antibody Precursor Enzyme Therapy (ADEPT). This technique uses tumor specific antibodies bound to an enzyme that causes conversion of an inactive drug (prodrug) in the form of active toxic when it comes into contact with the tumor . Many natural compounds have been used successfully as prodrugs, including doxorubicin and taxol. Scientific validation of these natural compounds with therapeutic action in terms of effectiveness, safety in their use and their mechanism of action, will provide an important position in modern medicine, particularly in research and cancer therapy.

I. BIBLIOGRAPHICAL RESEARCH

CHAPTER 1. PHYTOCOMPOUNDS WITH ANTITUMOR ACTIVITY

The research in the field of cell biology has led to a huge increase in information over the last two decades about the molecular events that lead to the development of cancer. The interference of specific molecular pathways through which cancer cells survive offers the attractive advantage of specific anticancer therapies without chemotherapy toxicity on normal tissues. Targeted molecular therapies for cancer are medications or substances that block the growth and spread of cancer by interfering with specific molecules involved in the growth and progression of cancer. Because these molecules are also called targeted molecules, anticancer therapies of this type are called "targeted molecular therapies" because they target cellular mechanisms that are more specific and less harmful to the normal cell.

1.3. Phytocompounds way of action in cancer therapies

The action mode of therapeutic compounds in plants known to have an antitumor effect can be determined by *in vitro* laboratory studies on tumor cell cultures and respectively *in vivo* on animal models carrying of tumor tissue grafts, in order to identify natural compounds that can target specific molecular pathways of tumor cells survival, to destroy them selectively and effectively. Many of the results confirmed that the various distinct manifestations of natural compounds with antitumor activity on cancer cells mainly include: cell division, immune system, uncontrolled replication, invasion and metastasis, angiogenesis, cell death mechanisms, aberrant metabolism, related tumors and oxidative stress.

Table 1.1. The antitumor activity of phytocompounds originally used in traditional medicine, in present used in tumor therapies.

Class of compounds	The active principle	The plant	Signaling path affected	Dose	Tumor type
Flavonoids	Apigenin (1.2.1.)	Chamomile *(Matricaria chamomilla)*	HER *(ErbB2 protein)* - the receiver epidermal growth factor	50 μM, 48 h	Pharynx (FaDU)
			Invasion and metastasis	40 μM, 16 h	Ovar (A2780)
			Cell death	60 μM, 24 h	Multiple myeloma (U266, RPMI 8226)
	Licochalcone-A (1.2.8.)	Marigold *(Calendula officinalis)*	Invasion and metastasis	250mg / body Kg	Melanoma (BF16 / F10)
		Liquorice *(Glycyrrhizaglabra)*	Cell cycle	25 μM, 48 h	Prostate (PC3)
			Cell death	20 μM, 12 h	Breast (MCF-7)
	Resveratrol (1.2.4.)	Grape *(Vitis vinifera)*	HER *(ErbB2 protein)* - the receiver epidermal growth factor	50 μM, 4 h or 10mM, 24 h	Breast (SKBr, MCF-7)

			Cell cycle	300μM, 24h	Esophagus (HCE7), healthy (MCF-7), colon (SW480)
			HDAC*(histondeacetylase)* - the enzyme involved in gene expression	10 μM, 96 h	San (MCF-7, MDA-MB231)
			Invasion and metastasis	100μM, 24h	Melanoma (KM3)
			Cell death	50 μM, 18 h	Myeloma (RPMI 8226), melanoma (KM3)
			Cellular metabolism	10 μM, 24 h	Liver (HepG2)
	Silimarin (1.2.18.)	Milk thistle *(Silybum marianum)*	*Human Telomerase Reverse Transcriptase* (hTERT) Replication	100μg / mL, 48 h	Leukemia (K562)
			Invasion and metastasis	27 μM, 48 h	Prostate (LNCaP, DU145)
	Silibinin (1.2.14.)		Angiogenesis	100μM, 1h	Breast (MCF-7)
			Cellular metabolism	40 μM, 30min.	Fibroblasts (3T3-L1), Ovar (CHO)
Lignanolide	Arctigenin (1.17.)	Greater burdock *(Arctium lappa)*	Cellular metabolism	10mM, 30min.until 12p.m.	Lung (A549)
Organo-sulfides	Alicin (1.2.19.)	Garlic *(Allium sativum)*	Cell cycle	5–10μM, 8–24 h	Leukemia (U937)
				50μM, 6–12h	Breast (MCF-7)
			HDAC modulator *(histondeacetylase)* - the enzyme involved in gene expression	20 μM - 2mM, 24 h	Colon (HT29)
	Dialyl sulfide (1.2.12.)		Invasion and metastasis	10–25μM or 2–10mM	Colon (colo205), prostate (PC-3, DU145), ovary (Skov-3)
			Angiogenesis	10 μg / body kg	Melanoma (BF16 / F10)
			Cell death	10 μM, 24 h	Leukemia (U937)

Polycyclic Quinones	Hypericin (1.2.2.)	St. John's wort *(Hypericum perforatum)*	HER *(ErbB2 protein)* - the receptor epidermal growth factor	25 nM, 24 h + hγ	Breast (SKBR-3, MCF-7)
			Inflammation	50 μg / mL, 1 h	Lung (A549 / 8), colon (DLD-1)
	Hyperforin (1.2.3.)		Invasion and metastasis	0.5μM, 6h	Fibrosarcom (HT-1080)
			Angiogenesis	27 μM, 48 h	Endotheliu m (HUVEC)
			Cell death	20 μM, 12 h	Breast (MCF-7), leukemia (Jurkat)
Polyphenols	Carnosol (1.2.5.), carnosic acid (1.6.)	Rosemary *(Rosmarinus officinalis)*	Cell cycle	50mM, 24–48h	Colon (Caco-2)
			Invasion and metastasis	5–10μM, 9–12 h	Melanoma (B16 / F10)
			Angiogenesis	25 μM, 7 h	Endotheliu m (HUVEC)
			Cell death	25 μM, 14 h	Leukemia (SEM, RS4 / 11, MV4 / 11, HT1080, HL60)
			Inflammation	10 μM, 1 h	Melanoma B16 / F10
Sesqui-terpene Lactones	Helenalin (1.2.9.)	Mountain Arnica *(Arnica montana)*	Cell cycle	1 μM, 24 h	Leukemia (Jurkat)
			*Human Telomerase Reverse Transcriptase (*hTERT) Replication	1–4μM, 48h or 100μM, 1h	Leukemia (HL-60, Jurkat)
			Cellular metabolism	0.125mg / day	tumor-bearing mice ascites Ehrlich
			Inflammation	1–2μM, 24h or 10 μM, 1 h	Leukemia (Jurkat), ovary (A2780), colon (RKO), breast (MCF-7)
	Parthenolid (1.2.10.)	Feverfew *(Tanacetum parthenium)*	Cell death	2 μM, 24 h	Ovar (A2780), colon (RKO), breast (MCF-7)
			Cell cycle	5–10mM, 24 h	Bladder (5637), cervix (HeLa)

			HDAC (*histondeacetylase) modulator*	15 μM, up to 3 h	Breast (ZR-75–1), colon HCT-116
			Inflammation	30 μM, 1 h	Cervix (HeLa)
Terpenoid	Crocin (1.2.11.)	Saffron *(Crocus sativus)*	*Human Telomerase Reverse Transcriptase* (hTERT) Replication	3 mg / mL	Liver (HepG2)
			Cell death	3 mg / mL	Stomach (AGS)

In **Table 1.1.** are indicated the antitumor activities of some plant phytocompounds used initially in traditional medicine, currently used in antitumor therapies. In **Figure 1.1.** are shown the chemical structures of mentioned antitumor compounds.

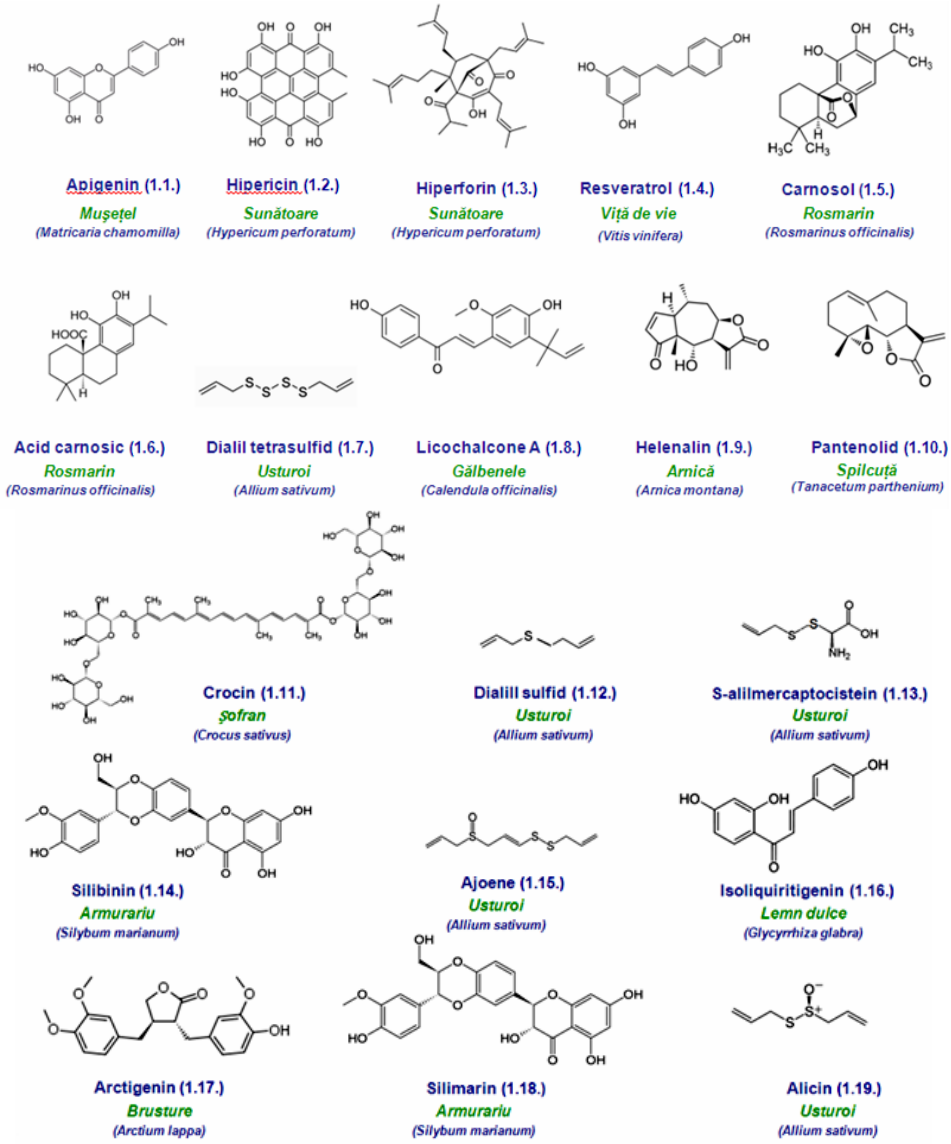


Figure 1.1. Antitumor compounds from plants first used in traditional medicine, presently used in tumor therapies.

CHAPTER 2. *HEDERA HELIX* L. – COMPOSITION AND PHARMACOLOGICAL PROPRIETIES

2.1. General characteristics of the plant

The plant with the genus *Hedera* is one of the 55 genera belonging to the family *Araliaceae* [2]. To date , 12 species, 3 subspecies and one variety *Hedera helix L.* [3], with the common name ivy, have been recognized in the genus *Hedera* . (Figure 2.1.). Ivy is an indigenous species, a climbing liana plant with a length of up to 20 m, it grows hanging with the help of short adventitious roots on tree trunks, fences or house walls, and if it does not have climbing support the plant becomes creeping on the ground. With its evergreen leaves, the ivy is evergreen, able to vegetate continuously, with very good resistance over the winter when it feels good at 10-12 ° C, a temperature that ensures a certain state of rest. The shape of the leaves is of two types, those on the flowering branches are whole or ovate rhombic 3-8 cm in size , while the leaves on the sterile branches have a lobed palmate shape 4-10 cm in size, with 3-5 sharp lobes or rounded.

Division	Spermatophyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Subclass	Asterids
Order	Apiales
Family	Araliaceae
Kind	*Hedera*
Species	*Hedera helix L.*

Figure 2.1. Taxonomic classification of the plant *Hedera helix* L.

The color of the leaves on the surface is dark green with lighter radial ribs and the lower surface is green to gray. The flowers are small and grouped in dense inflorescences 3-5 cm in diameter, greenish-yellow, rich in nectar, bloom from late summer to mid-autumn [4]. The fruits have the shape of globular berries, when mature they become black-purple, toxic to humans (**Figure 2.2.**).

Uses of the ivy plant (*Hedera helix* L.) in traditional medicine

First therapeutic uses of the ivy plant (*Hedera helix* L.) in traditional medicine comes from ancient Greek:

- *for internal use: the* leaves were soaked in wine and used to treat intoxications, while the infusion of young leaves (after drying) was used to treat coughs and for expectoration .
- *for external use:* in inflammation treating heaving antibacterial, antifungal , analgesic, sedative, antiparasitic, antitumor, emmenagogue efects.

Contraindications : ingested fruits cause vomiting or diarrhea; green plant can produce skin and mucous membranes irritations, therefore should not be administrated to persons allergic to its constituents.

Figure 2.2. Aspects of the vegetative organs of the plant.

2.2. Phytocompounds identified in the plant *Hedera helix L.*

In traditional European medicine the plant was used in diseases of the gastrointestinal tract and in calculosis. It was also used for external applications and as an analgesic for neuralgia and rheumatism and as a revulsive for ulcers and burns. It has expectorant and spasmolytic properties and is used in diseases accompanied by inflammation of the bronchi and in bronchial asthma [5 , 6-8]. Saponins are the majority of bioactive compounds that generate these therapeutic effects, but polyphenols and flavonoids also participate.

The plant has vasoprotective properties, derived from inhibition of elastase and hyaluronidase [9]. These enzymes degrade elastin and hyaluronic acid, respectively, and are involved in the formation of the perivascular extracellular matrix, in which elastane and hyaluronic acid together with collagen are the main components. It has been determined that inhibition of elastase and hyaluronidase is mainly due to fragmentation of triterpene saponins such as hederagenin sapogenins and oleanoic acid. The vasoprotective properties make the plant useful in treating venous insufficiency .

α-Hederin has been shown to protect the liver from hepatotoxic compounds such as acetaminophen and carbon tetrachloride [10]. The mechanism of this protective effect may depend on the inhibition of cytochrome P450, a cellular enzyme that can produce oxidative stress by metabolizing xenobiotic compounds and is partly responsible for their hepatotoxic effects [11]. In a study on the hepatotoxicity of cadmium, it was determined that α-hederin protects the liver by inducing the synthesis of metallothioneins, which can bind heavy metals, reducing their toxicity [12].

Other studies have indicated antibiotic action against bacteria and fungi [13]. Anthelmintic activities have also been detected [5 , 14 , 15]. α-Hederin,

hederacoside C, hederacolchisid E and hederacolchisid F showed strong anti-inflammatory activity in rat-induced paw edema [8 , 16].

Table 2.1. Chemical compounds identified in the leaves of *Hedera helix* L .

Triterpene saponins (2.5-6%)	Flavonoids	Coumarine	Polyacetylene	Phenolic acids
• Hederagenin • Aoleanolic acid • Baiogenin *bidesmosides:* • Hederacoside C (mainly) from 1.7 to 4.8% • Hederasaponins A, B, D, E, F, G, H, I • 3-sulfates of oleanolic acid and Equinocystic acid (*α-16-OH-oleanic acid*) • 28-O-β-gentiobiosiloleanate 3-sulfate (*helicoside L-8a*) *monodesmosides:* • α-hederin 0.1-0.3% • Hederagenin 3-O-β-glucoside	• Quercetin • Campferol • Rutin (*quercetin 3-O-rutinoside*) • Isoquercitrin (*quercetin 3-O-glucoside*) • Astragalin (*campferol 3-O-glucoside*) • Campferol 3-O-rutinoside	• Scopolin (*scopoletin 7-O-glycoside*)	• Falcarinon • Falkarinol • 11,12-dehydrophalcarinol	• Caffeic acid • Chlorogenic acid • Neochlorogenic acid • 3,5-O-dicafeoyl-quinic acid • 4,5-O-dicafeoyl-quinic acid • Rosemary acid [(R) - (+) enantiomer] • Dihydroxybenzoic acid • Protocatechic acid • *p* -Cumaric acid

Table 2.2 . Chemical compounds identified in the fruits of ivy .

Triterpene saponins	Fatty acids	Polyacetylene
• H elixoside A (*3-O- β-glucosyl- (1 → 2) -β-glucosyl-28-O-β-glucosyl - (1 → 6) -β-glucosyl hederagenin*) • H elixoside B (*oleanolic acid 3-O-β-glucosyl- (1 → 2) -glucosyl 28- O-β-glucosyl- (1 → 6) -β-glucosyl-3-O-β-glucosyl hederagenin*) • Ac. 3-O-β-glucosyl- (1 → 2) -β-glucosyl oleanolic *(3-O-β-glucosyl- (1 → 2) -β-glucosyl-hederagenin)* • S taunoside A *(3-O-β-glucosyl- 28-O-β-glucosyl- (1 → 6) -β-glucosyl hederagenin)*	• Petroselinic acid • Oleic acid • *cis* -Vaccenic acid • Palmitoleic acid	• Falcarinon • Falkarinol • Panaxidol ((*Z) -9,10-epoxy-1-heptadecen-4,6-diin-3-one*)) • β-lectin

2.3. Saponins present in *Hedera helix* L. and their antitumor activity

Hedera helix has been increasingly studied for its antitumor potential [5] with data from studies showing that the active ingredients present in ivy have antitumor activity against certain types of tumor cultures. In a study [6] by Quetin-Leclercq J., Elias R. and Balansard G. on the triterpene saponins bidesmosides (hederacoside B, C and D) and monodesmosides (α -, β -, δ -hederin and hederagenin) were tested on 4 strains: mouse breast tumor cells, normal 3T3 mouse fibroblasts, normal human Flow 2002 cells, and human HeLa epithelial tumor cells. The results of the study indicated that saponins are about 5 times less active compared to the reference compound strychnopentamine. The most active compounds were α- and β -hederins, which were cytotoxic at a concentration greater than or equal to 10 µg / mL. Hederacoside bidesmosides C, B, D and hederagenin were inactive at concentrations up to 200 µg / mL.

Another study [1 7] mentions *in vitro* experimental results with serum α -hederin testing in serum-free medium, finding its toxic effect by inhibiting the proliferation of B16 mouse melanoma cells and normal 3T3 mouse fibroblast cells at concentrations. less than

5 µg / mL after only 8 hours of treatment. The α-hederin induced alteration of cell membranes and cause cell death and vacuolation of the cytoplasm. The cytotoxicity of the phytocompound was reduced by the presence in the culture medium of FCS (Fetal Calf Serum) or BSA (Bovine Serum Albumin), which indicates that α -hederin as well as other saponins may form chemical bonds with proteins.

Chinese researcher Lin Cheng and colleagues conducted a study [18] in which he purified and identified α -hederin from *Clematis ganpiniana , a* plant with antitumor activity used in traditional Chinese medicine. They determined the potent inhibitory character of α -hederin on the growth of breast tumor cells by inducing their apoptosis. Most antitumor therapies address tumor inhibition by triggering apoptosis of tumor cells. Mitochondria play an important role in the survival of tumor cells, so it is one of the main targets on which antitumor drugs act [19].

To highlight the mitochondrial membrane potential, was used staining with JC-1 (cationic carbocyanin dye) that accumulates in the mitochondria. It was determined that α-hederin induces depolarization of the mitochondrial membrane potential and causes the release of Apaf-1 (factor-1 of apoptotic protease activation) and of cytochrome c (metalloprotein with role in oxygen transport) from the intermembrane space in the cytoplasm, favoring the activation of enzymes caspase-3 and 9 (protease of cistein-aspartic acid) that induce apoptosis.

Table 2.3. The antitumor activity of saponins present in *Hedera helix* L. demonstrated in previous studies.

Phytocompus	Cell cultures	Results	Ref.
• Hederacoside B, C and D, α - , β -, δ –hederin and Hederagenin	4 breast tumor strains : • B16 mouse tumor cells • 3T3 mouse fibroblast • normal human cells • HeLa human tumor cells	• α - and β -hederins was cytotoxic at conc. ≥10µg / mL • bidesmosides: hederacoside B , C , D and hederagenin are inactive at conc. >200 µg / mL • saponins are 5 times less cytotoxic compared to the reference compounds stricnopentamine	[21]
• α -hederin tested *in vitro* in serum free medium	• B16 mouse melanoma cells • 3T3 normal mouse fibroblast cells	• it inhibit the proliferation of both types of cultures at conc.≤ 5 mg / mL after 8 hours by altering the membranes and vacuolation of the cytoplasm • fetal serum present in the culture medium can form a chemical bounds with cellular proteins	[22]
• α - hederin from*Clematis ganpiniana*	• breast tumor cells	• it inhibit the increasing of the tumor cells and cause apoptosis • proposed mechanism : α -hederin induce depolarization of the mitochondrial membrane potential and causes the release of the gene Apaf-1 and the protein cytochrome C with a role in the initiation of apoptosis	[23]
• α-hederin from*Clematis ganpiniana*	• MCF-7 and MDA-MB-231 breast tumor cells	• Inducing apoptosis of both types of cells	[24]
• Triterpenic Saponins	• liver, gastric, esophageal and colorectal human cancer	• inducing apoptosis	[21 – 26]
• α-hederin from *Nigella sativa*	• mouse P388 leukemic cells	• induces apoptosis through mitochondrial disturbances	[27]

Testing of the phytocompound α-hederin (from *Clematis ganpiniana)* on two human breast cancer cell cultures, type: MCF-7 and MDA-MB-231 resulted in the induction of apoptosis in both types of cultures [20]. This is the first report on the effects induced by α -hederin on inhibition of cell proliferation and pro-apoptotic effects on breast tumor cells and pathways related to the apoptosis phenomenon, suggesting that α-hederin (from *Clematis ganpiniana)* is a candidate promising for breast cancer chemotherapy.

Mitochondrial apoptotic pathways have been reported to be frequently approached to highlight the activity of triterpene saponins in other human cancers, including liver [21-23], gastric [24], esophageal [25] and colorectal [26]. α -hederin from *Nigella sativa* has been reported to induce apoptosis by mitochondrial disturbances in mouse P388 leukemic cells [27].

II. EXPERIMENTAL RESEARCH

OBJECTIVES

- Analysis quality of aqueous, ethanolic and etheric *Hedera helix* L. leaves extracts in order to emphasize its characteristic phytocompounds classes.
- Obtaining by various methods of the ethanolic ivy leaves extracts and its total saponins content evaluation.
- Saponins isolation from ethanolic *Hedera helix* leaves extracts.
- Fractionation and physico-chemical and biological characterization of isolated saponins.
- Evaluation of the phytochemical and antiproliferative potential of *Hedera helix* L. leaf extract fractions separated by flash chromatography.
- Optimization by mathematical modeling of a composition of triterpenic saponins with antiproliferative activity.

CHAPTER 4. QUALITATIVE ANALYSIS OF SOME EXTRACTS FROM *HEDERA HELIX* L. LEAVES

The plant material used was leaves of *Hedera helix* L. commercialized as "Ivy Tea" produced by S.C. HYPERICUM IMPEX S.R.L. The dried and crushed leaves were subjected to extraction in water, alcohol and diethyl ether solvents. The identification of the classes of compounds present in ivy extracts was performed using methods corresponding to the physico-

chemical properties of each group of active principles [133, 134]. The results obtained from the qualitative analysis were centralized in **Table 4.11**.

Table 4.2. Centralizer of the results obtained in the qualitative analysis of the classes of compounds in the extracts from the leaves of *Hedera helix*.

Classes of compounds	Aqueous extract			Alcoholic extract			Etheric extract	
	Non-hydrolyzed aqueous extract A1.	Hydrolyzed aqueous extract A2.		Non-hydrolyzed alcoholic extract B1.	Hydrolyzed alcoholic extract B2.		Hydrolyzed etheric extract C2.	
		Etheric hydrolyzed extract A2.1.	*Solution aqueous A2.2.*		*Etheric hydrolyzed extract B2.1.*	*Aqueous hydrolyzed extract B2.2.*	*Etheric hydrolyzed extract C2.1.*	*Aqueous hydrolyzed extract C2.2.*
Reduced carbohydrates	positive	-	-	-	-	-	-	-
Polyosis *(Molish reaction)*	positive	-	-	positive	positive	positive	-	-
Saponins	positive	-	-	-	-	-	-	-
Catechic tannins	positive	-	-	-	-	-	-	-
Proanthocyanidins	positive	-	-	-	-	-	-	-
Alkaloids *(Hager reaction)*	positive	-	-	-	-	positive	-	negative
Alkaloids *(Wagner reaction)*	positive	-	-	-	-	positive	-	negative
Anthracenozides *(Borntrage reaction)*	-	negative	-	negative	negative	negative	negative	-
Coumarins	-	positive	-	positive	positive	positive	negative	-
Flavonoids *(Shibata reaction)*	-	positive	-	-	-	-	negative	-
Hexaatomic lactone of coumarins	-	negative	-	-	-	-	-	-
Amino acids	-	-	negative	-	-	-	-	-
Anthocyanins	-	-	negative	-	-	-	-	-
Polyuronides	positive	negative	-	positive	-	negative	-	-
Reducing compounds *(Fehling reactions I and II)*	-	-	-	positive	positive	positive	-	-
Sterolic glycosides	-	-	-	positive	positive	positive	-	-
Proanthocyanidols	-	-	-	positive	-	-	-	-
Sterols and Triterpenes *(Lieberman Burchard reaction)*	-	-	-	-	-	-	-	positive
Flavonols	-	-	-	positive	positive	-	-	-
Flavones	-	-	-	-	-	positive	-	-
Carotenoids	-	-	-	-	-	-	-	positive

CHAPTER 5. OBTAINING EXTRACTS FROM IVY LEAVES AND EVALUATION OF THE TOTAL SOAP CONTENT

5.1. The objective of the study

In our study, in order to evaluate the content of total saponins present in the extracts of ivy, we obtained ethanolic extracts of leaves of *Hedera* using four extraction methods: refluxing,

ultrasonification, maceration and Soxhlet continuous extraction. Evaluation of total saponins content (CTS) has been carried out according to the adapted Hiai Method [28].

Table 5.1. Analytical data on extraction methods applied to fresh ivy leaves

Method	Vegetable mass (g)	Vol. solvent (mL)	Temp. °C	Shake	Time
Reflux	10	50	60	Yes	3h
UAE	10	50	2. 3	Yes	1h
Maceration	10	50	2. 3	Yes	11 days
Soxhlet	10	100	60	-	6h

5.1. Obtaining of the ethanolic extracts from ivy leaves

In **Figure 5.1.** the installations for obtaining ivy leaf extracts are presented: a) refluxation, b) ultrasonication, c) maceration and d) Soxhlet extraction.

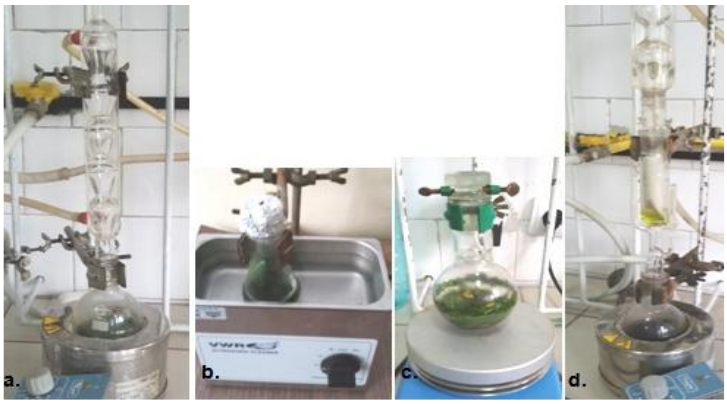


Figure 5.1. Installations for obtaining extracts from ivy leaves :
a) refluxation, b) ultrasonication, c) maceration and d) Soxhlet extraction.

5.3.1. The obtained results at active principles extraction from ivy leaves by the four extraction methods are indicated in **Table 5.3** .

Table 5.3. The obtained results at active principles extraction from ivy leaves by the four extraction methods.

Sample	Vegetable residue (g)	$V_{f.}$ extract (mL)	Extract color	Sample code extract
Ethanolic extract from refluxation	6.98	36	dark green	RTI.4.
Ethanolic extract from UAE	6.64	38	green	RTI.5.
Ethanolic extract from maceration	4.63	36.5	green	RTI.6.
Ethanol extract from Soxhlet extraction	4.64	80.5	dark green	RTI.7.

Determination of total saponin content (TSC)

The method uses a chromogenic reagent system consisting of vanillin and perchloric acid. Saponins are subject to oxidation in strongly acidic medium, followed by dehydrogenation and then by addition of vanillin, resulting in a purple compound absorbance measured spectrophotometrically (Hiai Method).

Standard saponin: ginsenoside Rb1(Sigma, purity ≥ 98%), with a maximum absorption in the visible range at wavelength λ_{max} = 550 nm.

The equation of the calibration curve was: **y = 0.0102 x - 0.0191**, with the correlation coefficient: 0.999.

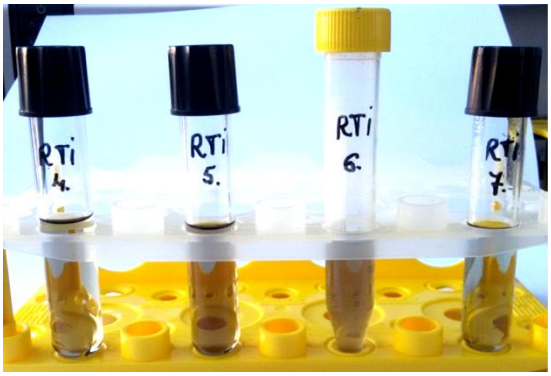


Figure 5.2 . Appearance of the extract samples obtained by the four extraction methods, following the reaction with the chromogenic reagent at the determination of TSC.

Table 5.5. The results obtained at the determining of TSC in tested samples.

Sample	Test code	CTS (µg / mg d.w.)	CTS (%)
Dried ethanolic extract of the refluxation	RTI.4.	30.07	3.01
Dried ethanolic extract. of the UAE	RTI.5.	41.86	4.19
Dried ethanolic extract from maceration	RTI.6.	33.18	3.32
Dried ethanolic extract from Soxhlet extraction	RTI.7.	38.26	3.83

The **Table 5.5.** present the results obtained at the determining of total saponins contained in tested samples.

5.3.3 . Correlation between specific conditions of the four extraction methods and the amount of saponins in the extracts

Results of the total saponins content (**Table 5.5.)** by the method UAE indicated as expected a maximum of 4.19 % in the extract sample. Although the resulting dry extract mass at UAE was only 0.344 g from initial 10 g plant material, the efficiency of UAE was maximal. The results of determining the total saponin content of the extract samples resulting from the other three conventional extraction methods: refluxation, maceration and Soxhlet continuous extraction recorded the following values: 3.01 %, 3.32% and 3.83%, respectively. The total content of

saponification of the extract obtained by Soxhlet method was 3.83 %, representing the highest value recorded from the tested conventional methods. These values were close to 4.19 % and confirm that the Soxhlet extraction method is representative for the category of conventional methods and similar in efficiency to unconventional extraction methods, in this case the UAE. On the other hand, although the extraction time was the longest, 11 days, the extract obtained by maceration had an average saponin content (3.32%) this can be explained by the fact that the operation took place at room temperature, and the efficiency of the method is much lower than UAE or even that performed by Soxhlet refluxation.

CHAPTER 6. SAPONINS ISOLATION FROM *HEDERA HELIX* L. LEAF EXTRACTS

6.1. Study objective

The aim of the study was to isolate saponins from ethanolic extracts from *Hedera helix* leaves, for this, three isolation methods were applied:

- Saponins isolation from ethanolic extract obtained from fresh leaves of *Hedera helix* L. (Method I);
- Hederagenin obtaining from ethanolic extract resulting from repeated maceration of dried ivy leaves (Method II);
- Hederagenin isolation from ethanolic extract obtained by refluxation from dried ivy leaves (Method III).

Plant material source was fresh leaves of *H. helix* L. obtained from "Hofigal S.A.". The plant was authenticated and a voucher specimen (no. 407754) was deposited at the Botanical Garden of Bucharest, Romania.

6.2.1. Saponins isolation from fresh leaves ethanolic extract of *Hedera helix* L. (Method I)

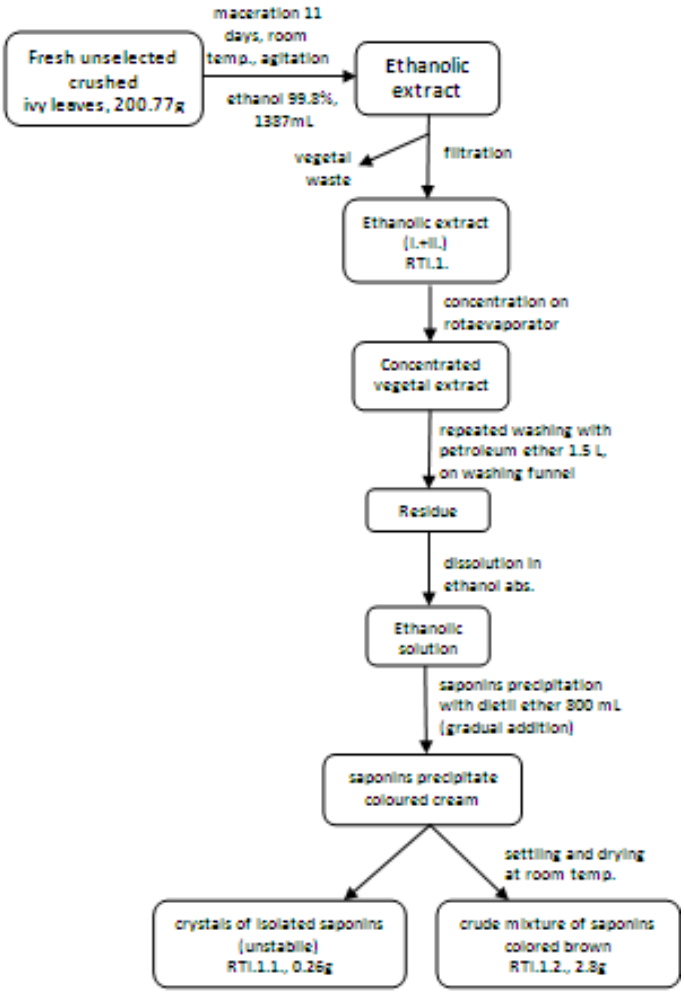
In order to isolate the triterpene saponins from the leaf extracts of *Hedera helix* L. we chose the extraction method by maceration, using two types of plant material: fresh ivy leaves with various shapes; selected fresh ivy leaves of palm shape.

In **Table 6.1.** are centralized the experimental data and the characteristic results of the extracts obtained by maceration of *Hedera helix* L leaves in absolute ethanol.

Table 6.1. Experimental conditions from the maceration of *Hedera helix* L. leaves in ethanol, in order to isolate saponins (Method I).

Extraction	Fresh vegetable mass	Amount (g)	Solvent volume (mL)	Temp. ^{o}C	Shake	Time (days)
Maceration 1.	Ivy leaves I.	100.22	692	2. 3	yes	11
	Ivy leaves II.	100.55	695	2. 3	yes	11
Maceration 2.	Selected ivy leaves	100.63	690	2. 3	yes	8

For hederagenin isolation were used the obtained volumes of extract according to the steps mentioned in **Scheme 6.1.** :



Scheme 6.1. The process of extraction and isolation of crude saponins from ivy leaves (Method I), [29].

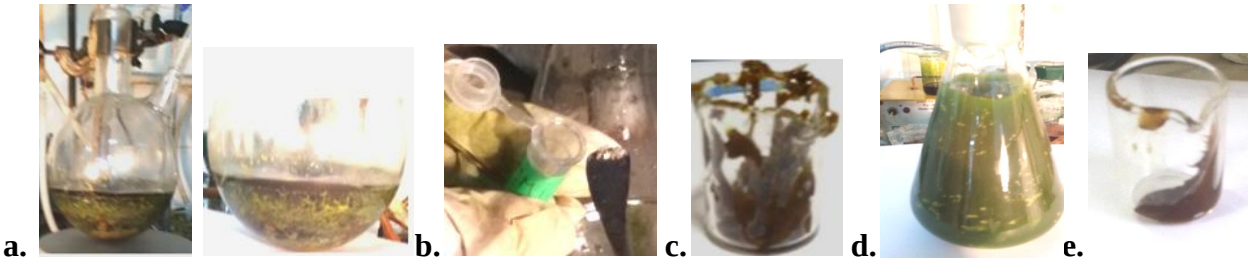


Figure 6.1. a) Precipitation of saponins from unselected ivy leaves extract RTI.1.; b) crystals of isolated saponins RTI.1.1.; c) crude mixture of isolated saponins RTI.1.2. ; d) precipitation of the saponins from selected ivy leaf extract RTI.1.2.; e) crude mixture of saponins isolated RTI.2.1.

The results obtained after saponins isolation are centralized in **Table 6.2.**

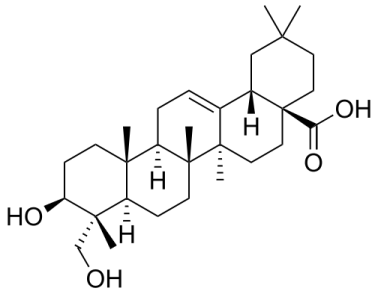
Table 6.2. Results obtained from the saponins isolation method from fresh ivy leaves of various and selected shape (Method I).

<table>
<tr><td rowspan="6">Extraction</td><td colspan="4">a) Extracts of ivy leaves of various shapes I. and II.</td><td colspan="2">b) Extract selected ivy leaves
(palm shape)</td></tr>
<tr><td colspan="2">Maceration I.</td><td colspan="2">Maceration II.</td><td colspan="2">Maceration</td></tr>
<tr><td>$V_{solv.}$
692 mL</td><td>M_{leaves}
100.22 g</td><td>$V_{solv.}$
695 mL</td><td>M_{leaves}
100.55 g</td><td>$V_{solv.}$
100.63 mL</td><td>M_{leaves}
100.63 g</td></tr>
<tr><td colspan="2">$V_{extr.\ I}$ 595 mL</td><td colspan="2">$V_{extr.II}$ 579.5 mL</td><td colspan="2" rowspan="2">$V_{extr.}$
570 mL RTI.2.</td></tr>
<tr><td colspan="4">$V_{extr.\ I+II}$ = 1174 , 5 mL RTI.1.</td></tr>
<tr><td colspan="4">(d.w. = 0.12 g / mL)
$M_{dry\ extract.\ I+II}$ RTI.1.
140.94 g</td><td colspan="2">(d.w. = 0.126g / mL)
$M_{dry\ extract}$ RTI.2.
71.9 g</td></tr>
<tr><td>Isolate saponins</td><td colspan="2">$M_{cryst.\ isol.\ sapon.}$ RTI.1.1.
0.26 g</td><td colspan="2">$M_{crude\ isol.\ sapon..}$ RTI.1.2.
6.475 g</td><td colspan="2">$M_{isol.\ sapon..}$ RTI.2.1.
2.8 g</td></tr>
<tr><td>Total mass of crude isolated saponins</td><td colspan="4">6.735 g</td><td colspan="2">2.8 g</td></tr>
<tr><td>% crude isolated saponins</td><td colspan="4">4.78 %</td><td colspan="2">3.89 %</td></tr>
</table>

Sample RTI.1.2.representing the crude mixture of saponins isolated from ivy extract (I +II) was fractionated by column chromatography (CC). Selected fractions and the samples RTI.1., RTI.1.1., RTI.1.2. and RTI.2.1. have been analyzed by liquid chromatography coupled with mass spectrometry (LC-MS) and their content of total saponins (CTS) was determined.

Isolation of hederagenin from *Hedera helix* L. leaves extracts.

6.2. 2. Hederagenin isolation from dried ivy leaves ethanolic extract obtained by repeated maceration (Method II).



Hederagenin
[(3β) -3,23-dihydroxyolean-12-en-28-oic] acid

For hederagenin isolation, were used the extracts volumes obtained according to the steps:

a) Concentration of ethanolic extract RTI.8. at reduced pressure it was carried out using a rotary evaporator at 60° C temperature, until a concentrated dark green vegetal extract was obtained, in amount of 19.30 g, (**Figure 6.6.a**).

b) Acid hydrolysis of the concentrated extract for the carbohydrate removing, was made by adding of 5% v / v, sulfuric acid ethanolic solution (2.7 mL sulfuric acid in 114.8 mL ethanol) and refluxing for 3 h, resulting Mixture 1. with acidic pH.

c) Neutralization of Mixture 1. at pH 6-7 by the addition of potassium hydroxide in ethanol (5% v / v) resulting Mixture 2.

d) The treatment of Mixture 2. with activated carbon (7g) and refluxing for 30 minutes resulting Mixture 3.

e) Filtration of Mixture 3. through Celite filter layer, obtaining Filtrate 1.

f) Concentration at low pressure of Filter 1., up to about 10% of the initial volume, constituting a Residue. To the obtained residue was added 100 mL of hot distilled water. The obtained residual solution was evaporated under reduced pressure.

g) Treatment of the brown residue with acetonitrile to remove impurities.

6.2.3. Isolation of hederagenin from dried ivy leaves ethanolic extract obtained by refluxation (Method III)

To isolate hederagenin, were used extract volumes obtained in accordance with the steps:

a) Concentration of the ethanolic extract RTI.9. at the low pressure was carried out using a rotary evaporator at 60° C with the obtaining of a concentrate vegetable extract of green colored in amount of 32.86 g, noted RTI.9.

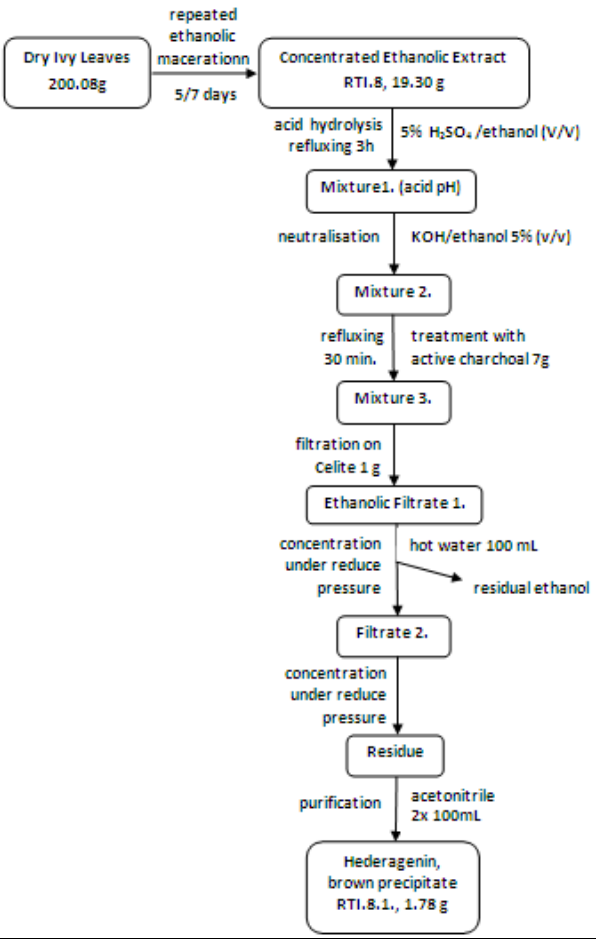
b) Acid hydrolysis of concentrated extract RTI.9., for carbohydrates group removing using a solution of sulfuric acid / ethanol 5% (5.4 mL sulfuric acid / 230 mL ethanol) and refluxation for 3h, it resulting a dark green solution and a white precipitate, with acidic pH.

c) Filtration of the solution obtained by acid hydrolysis, resulting a precipitate RTI.9.1. of brown color, with 4.11 g weight.

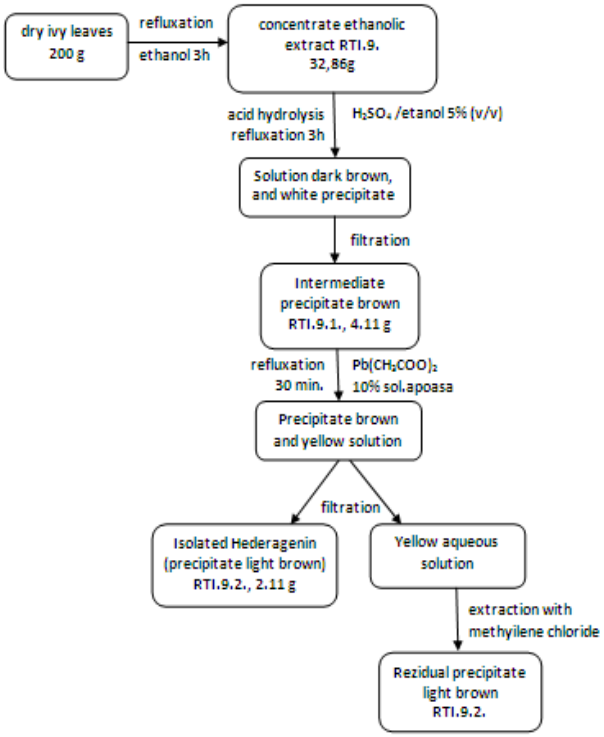
d) Treatment of RTI.9.1. precipitate with the aqueous solution of lead acetate 10% (3 g lead acetate in 27 mL water) and refluxation for 30 minutes with the formation of a brown precipitate in a yellow solution.

e) Filtration of the solution for separation of isolated crude hederagenin precipitate noted RTI.9.2., of brown color with a mass of 2.11 g, and a yellow filtrate.

Analytical data of hederagenin obtaining by the two isolation methods are presented in **Table 6.6.**.



Scheme 2. Hederagenin isolation of the ethanolic extract obtained by repeated maceration of dried ivy leaves (Method II) [30].



Scheme 3. Isolation of hederagenin from ethanolic extract obtained by reflux from dried ivy leaves (Method III) .

Table 6.6. The obtained results at hederagenin isolation from dried leaves of ivy (Methods II and III).

Extraction	Isolation of hederagenin (Method II)		Hederagenin isolation (Method III)	
	Repeated maceration		Refluxation	
	$V_{solv.}$ V_{I+II} = 2911 mL	M_{leaves} 200.08 g	$V_{solv.}$ 2110 mL	M_{leaves} 200 g
Isolation	$M_{dry\ extr.}$ RTI.8 . 19.30 g		$M_{dry\ extr.}$ RTI.9 . 32.86 g	
% crude isolated saponins	9.22 %		6.42 %	

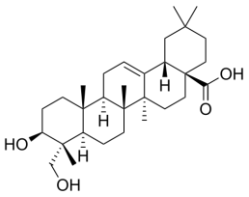
CHAPTER 7. PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF THE SAMPLES RESULTED FROM SAPONIN ISOLATION METHODS

7.1. Objectives

The aim of the study was to fractionate and physico-chemical characterization of the samples resulted from the saponins isolating methods, followed by *in vitro* biological characterization of isolated hederagenin from the ivy leaves ethanolic extract.

7.2. Materials and methods

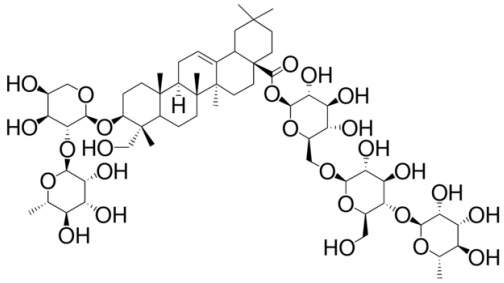
The standard saponins used in CSS, LC-MS analyzes were: α-Hederin, Hederagenin and Hederacoside C. At *in vitro* biological activity determination, the reference standard was Dioscin of purity ≥ 98%, produced by Sigma -Aldrich.



Hederagenin (*saponin*)

[(3β)-3,23-dihydroxyoleane-12-en-28-oic]acid

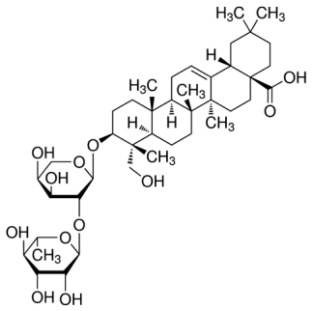
$C_{30}H_{48}O_4$, M = 472,1g/mol



Hederacoside C (*bidesmoidic saponin)*

[(2S,3R,4S,5S,6R)-6-[[(3R,4R,5S,6R)-3,4-dihydroxy-6-(hydroxymethyl)-5-[(2S,3R,4R,5R,6S)-3,4,5-trihidroxy-6-mehyloxane-2-il]oxyoxane-2-il] oxymethyl]-3,4,5-trihydroxyoxan-2-il]

$C_{59}H_{96}O_{26}$, M = 1221,38 g/mol



α-Hederin (*monodesmoidic saponins*)

[(3β,4α)-3-[[2-*O*-(6-deoxy-α-L- 2,6a,6b,9,9,12a -mannopyranosyl)-α--L- arabinopyranosyl]oxy]--23-hidroxyolean-12-en-28-oic] acid

$C_{41}H_{67}O_{12}$, M = 751,4 g/mol

The cell cultures used for testing *in vitro* cytotoxicity were: normal mouse dermal fibroblasts NCTC (clones 929), and human cervical epithelial tumor cells Hep-2 provided by ECACC, Sigma-Aldrich.

Steps:

- ❖ Fractionation of the samples resulting from the saponins isolating methods
- ❖ Physico-chemical characterization of the samples
- ❖ *In vitro* biological characterization of isolated hederagenin from ivy leaves ethanolic extract

Methods for analyzing complicated were applied following signed following:

- ➢ Fractionation by column chromatography (CC)
- ➢ Determination of total saponin content (CTS)
- ➢ Analysis by thin layer chromatography (CSS)
- ➢ Analysis by liquid chromatography coupled with mass spectrometry (LC-MS)

In vitro evaluation of the biological activity of isolated hederagenin.

7.2.1. Physico-chemicals analysis methods

7.2.1.1. Separation by fractionation on a chromatographic column (CC)

Samples selected for fractionation on chromatographic column were: **crude mixture of isolated saponins RTI.1.2.**, and **crude isolated hederagenin RTI.8.1.** (Method II).

Fractionation was performed using a separating column (20 cm x 1.5 cm) filled with a suspension of silica gel granules with particle diameter of 0.6-0.8 mm in ethanol and a elution system consisted of n-butane: glacial acetic acid: water = 4: 1: 5 (v / v).

The sample solubilised in ethanol was passed through the column and the compounds were retention on the stationary phase, followed by theirs solubilisation when the elution system passage through column. The resulted fractions were collected at volumes of about 1 ml each.

The fractions were:

- from the sample crude mixture of isolated saponins RTI.1.2. → 9 fractions noted A1 - A9 ;
- from the sample crude isolated hederagenin RTI.8.1. → 13 fractions noted B1 - B13.

7.2.1.2. Determination of total saponin content (CTS)

The reference standard on TSC determination was saponin ginsenosides Rb1 (Sigma Aldrich) with maximum absorption in the visible range at the wavelength of λ_{max} = 550 nm. The equation of the calibration curve was: **y = 0.0102 x - 0.0191**, with the correlation coefficient: 0.999.

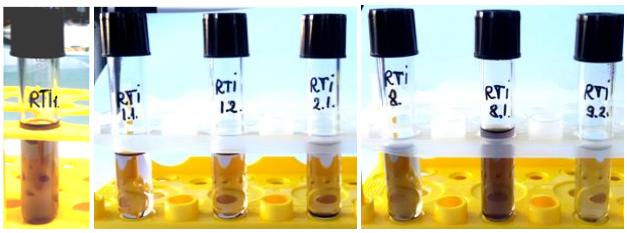


Figure 7.3. Appearance of the analyzed samples, after the reaction with chromogenic reagent at the determining of the total saponin content.

Table 7.3. The results of total saponins content determination.

Sample	Code	Media TSC (µg sapon./ mg .w.)	Mediate CTS (%)
Dry ethanolic extract from maceration (Method I)	RTI.1.	33.25	3.32
Isolated saponin crystal	RTI.1.1.	21.20	2.12
Crude mixture of saponins isolated from ivy leaves of various shapes	RTI.1.2.	25.64	2.56
Crude saponins mixture isolated from selected leaves	RTI.2.1.	27.77	2.78
Dry ethanolic extract from repeated maceration (Method II)	RTI.8.	32.67	3.27
Isolated Hederagenin (Method II)	RTI.8.1.	60.32	6.03
Dry ethanolic extract from refluxation (Method III)	RTI.9.	20.75	2.07
Isolated Hederagenin (Method III)	RTI.9.2.	23.10	2.31

In **Figure 7.3.** is presented the appearance of analyzed samples solutions, resulted from the reaction with the chromogenic reagent at the determination of the total saponin content.

In **Table 7.3.** are shown the results obtained at the determination of the total saponin content in tested samples.

7.2.1.3. Thin layer chromatography (TLC)

The samples analyzed by thin layer chromatography method were:

- crude mixture of isolated saponins RTI.1.2. from ivy extract (I + II), (Method I);
- fractions A2., A7., A8. and A9. of the crude mixture of isolated saponins RTI.1.2. (Method I);
- crude isolated hederagenin RTI.8.1. (Method II);
- fractions B2. and B6. from crude isolated hederagenin RTI.8.1. (from Method II);
- intermediate precipitate RTI.9.1. formed by hydrolysis at the hederagenin isolation process (Method III);
- crude isolated hederagenin RTI.9.2. (Method III).

Table 7.2. The obtained results of the samples analyzed by thin layer chromatography (TLC).

Method	Tested samples	R_f Hederagenin	R_f α-Hederin	R_f Hederacoside C
		0.67	0.46	0.72
Saponins isolation (Method I)	RTI.1.2. crude mixture of isolated saponins	+	+	+
	A2 (RTI.1.2.)	+	-	-
	A7 (RTI.1.2.)	+	-	+
	A8 (RTI.1.2.)	+	+	+
	A9 (RTI.1.2.)	-	+	+
Hederagenin isolation (Method II)	RTI.8.1. crude isolated hederagenin	+	+	-
	B2 (RTI.8.1.)	+	-	-
	B6 (RTI.8.1.)	+	-	-
Hederagenin isolation (Method III)	RTI.9.1. intermediate precipitate	+	-	+
	RTI.9.2. crude isolated hederagenin	+	-	+

In **Table 7.2.** are centralized the results obtained from the analysis by thin layer chromatography (CSS) of the tested samples.

Based on the TLC analysis results, it can be observed that the three saponins of interest were confirmed in the tested samples, as follows:

- Hederagenin, the aglycone of the reference saponins, was present in all tested samples obtained from the three saponins isolation methods, both in crude isolates and in their fractions.
- α-Hederin, which in the ivy leaves can be found in low amounts, was confirmed in crude mixture of isolated saponins RTI.1.2., in his fractions A7 and A9 and in crude isolated hederagenin RTI.8.1. (Method II).
- Hederacoside C, the saponin with highest amount in the ivy leaves, it is a bidesmoside with branched chemical structure and a high molecular mass, which may cause fragmentation of the molecule. The results of TLC analysis confirmed the presence of the hederacoside C in crude izolated saponins mixture RTI.1.2. together with hederagenin, and respectively its fractions A7, A8 and A9. Also the hederacoside C was present in fraction B6 of isolated hederagenin RTI.8.1. (Method II) and in intermediate precipitate RTI.9.1. from hederagenin isolation (Method III).

7.2.1.4. Liquid chromatography coupled with mass spectrometry (LC-MS)

LC-MS analysis was performed using a System LC-MS / MS: HPLC apparatus Agilent type API 3200 (Sciex) coupled with a binary pump Infiniti 1260 (Agilent) and an auto sampler, the analysis software version 1.5 .2 and column: Phenomenex Luna pentaphluoropropionyl (PFP 2) (100 x 2 mm, 3 μm, 100 Å).

The mobile phase was consisting of: A) water with 0.1% formic acid, and B) acetonitrile with 0.1% formic acid. The flow rate was 0.275 mL / min and injection volume of 15 μL.

The reference solutions: α-Hederin, Hederagenin and Hederacoside C (Sigma-Aldrich) of purity ≥ 98% and the samples, were dissolved in MeOH at conc. 1 mg / mL and then were diluted to 5 μg / mL methanol / water (1: 1, v / v) and analyzed LC-MS.

The ESI interface of the mass spectrometer was operated in negative ionization mode, and the data acquisitions were performed in the Q1 full scan on the mass / load range between 250 and 1500 Da.

➢ **The results obtained in liquid chromatography coupled with mass spectrometry (LC-MS)**

The samples selected to be analyzed by LC-MS method were:

- ivy extract RTI.1. obtained by maceration used for the saponins isolation (Method I);
- crystal isolated saponins (unstable) RTI.1.1. (Method I);
- crude mixture of isolated saponins RTI.1.2. (Method I);
- fractions A2., A7., A8. and A9. from crude mixture of isolated saponins RTI.1.2. (Method I);
- ivy extract RTI.8. obtained by repeated maceration from dried ivy leaves used to the hederagenin isolation (Method II);
- isolated crude hederagenin RTI.8.1. (Method II);
- fractions B2., B6. and B11. from crude isolated hederagenin RTI.8.1. (Method II);
- ivy extract RTI.9. obtained by hydrodistillation from dried ivy leaves used to the hederagenin isolation (Method III);
- intermediate precipitate RTI.9.1. (Method III);
- crude isolated hederagenin RTI.9.2. (Method III).

7.3.1. Identification of Hederacoside C

The compound Hederacoside C was quantified by LC-MS analyze, according to the resulting chromatograms, in samples: ethanolic ivy extract RTI.1., in the crude mixture of isolated saponins mixture RTI.1.2., in A8 and A9 fractions of isolated saponin mixture RTI.1.2. (**Figure 7.4.** and **Table 7.3.**).

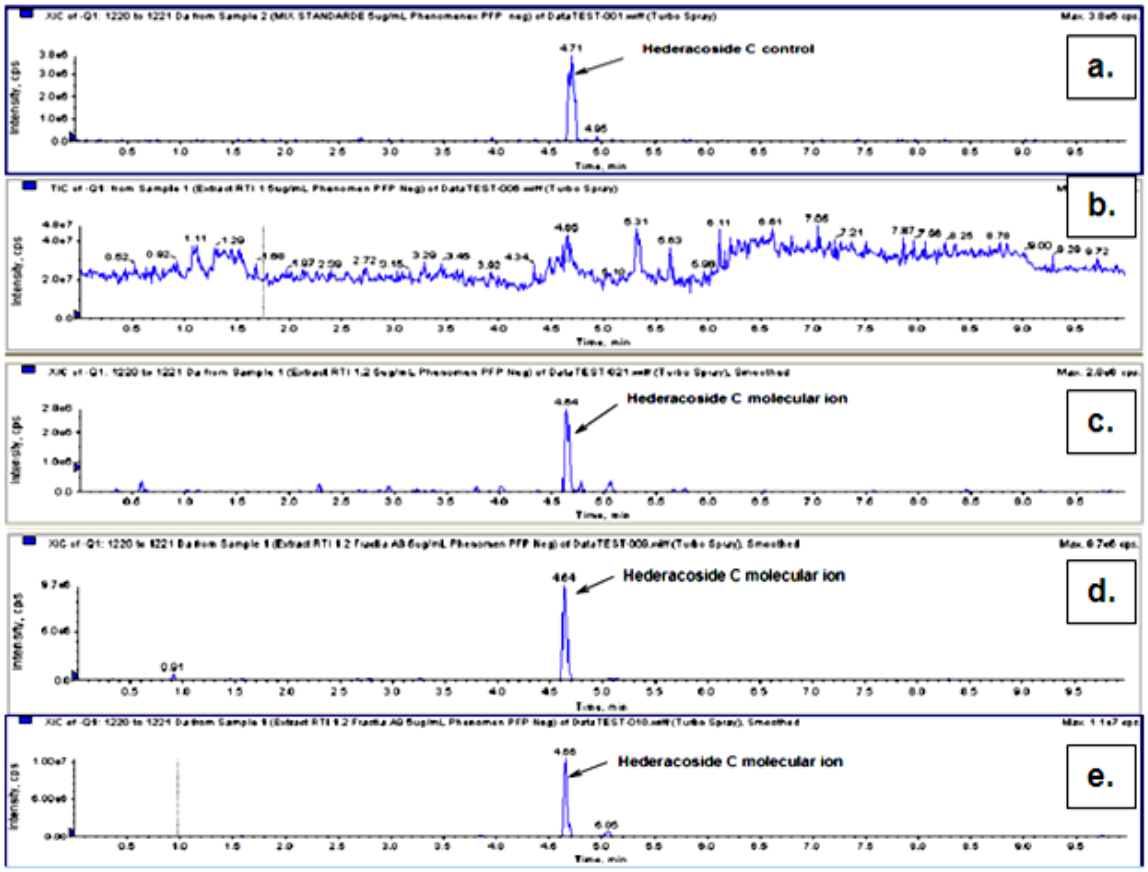


Figure 7.4. LS-MS chromatograms of: a) standard hederacoside C, b) ivy ethanolic extract RTI.1., c) hederacoside C identified in the crude isolated saponin mixture RTI.1.2., d) hederacoside C identified in A8 fraction of RTI.1.2., e) hederacoside C identified in A9fraction of RTI.1.2.

Table 7.3. Hederacoside C presence in samples derived from the saponins isolation (Method I), analyzed by LC-MS (in negative ionization).

Standard saponin	M.W.	Retention time (min)	Signal Intensity (cps)	Sample (5 µg / mL)	Signal intensity of Hederacoside C / Molecular ion in sample (cps)
Hederacoside C 2 mg (5µg / mL)	1221.4	4.71	$3.8 \cdot 10^{6}$	RTI.1.	$3.2 \cdot 10^{6}$
				RTI.1.1.	$6.0 \cdot 10^{5}$
				RTI.1.2.	$2.8 \cdot 10^{6}$
				A2 (RTI.1.2.)	$2.6 \cdot 10^{5}$
				A7 (RTI.1.2.)	$1.9 \cdot 10^{6}$
				A8 (RTI.1.2.)	$9.7 \cdot 10^{6}$
				A9 (RTI.1.2.)	$1.1 \cdot 10^{7}$

7.3.2. Identification of Hederagenin

Hederagenin was predominantly quantified, according to LC-MS chromatograms, in the samples: a) ivy ethanolic extract RTI.1., b) in ivy extract used for isolation of hederagenin RTI.8., c) in the ivy extract RTI.8., d) in the crude isolated hederagenin RTI.8.1. (**Figure 7.5.** and **Table 7.4.**).

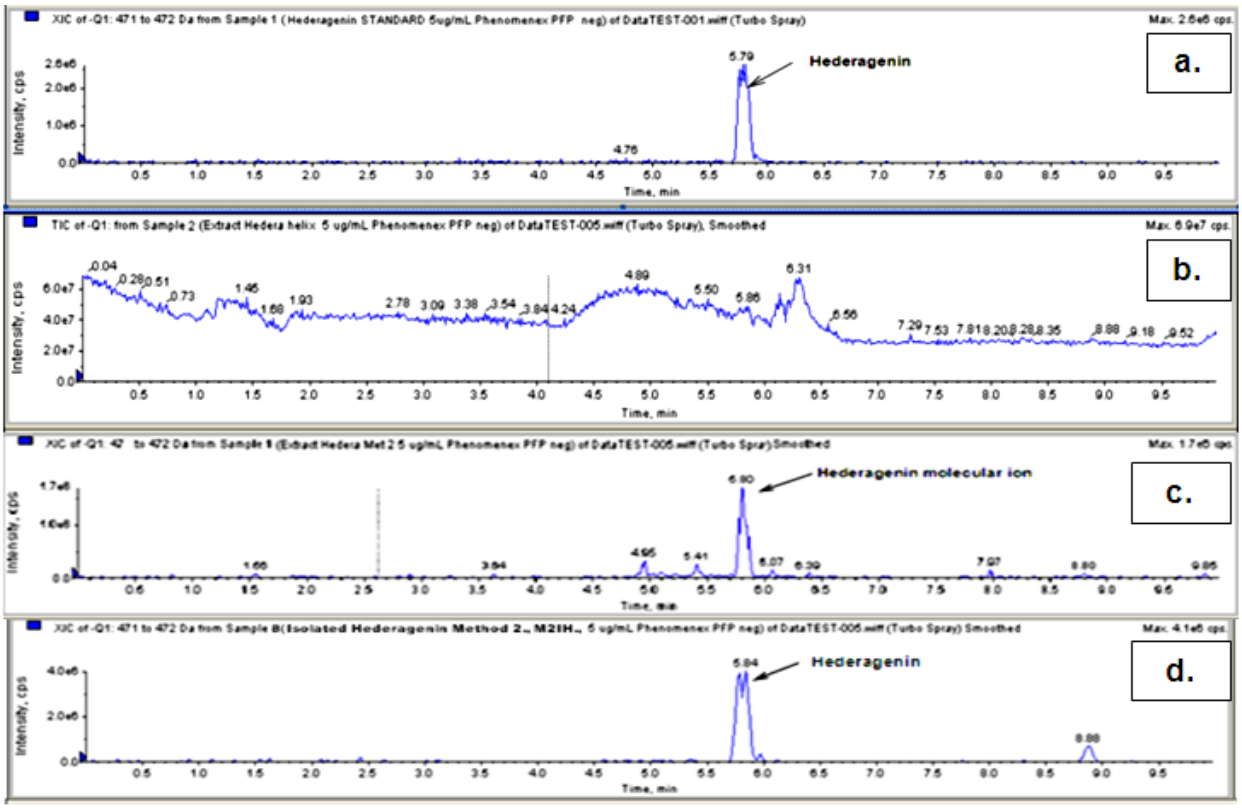


Figure 7.5. LC-MS chromatograms for: a) standard Hederagenin, b) the ivy extract used for isolation of hederagenin RTI.8., c) hederagenin identified in the ivy extract RTI.8., d) hederagenin identified in the sample of crude isolated hederagenin RTI.8.1.

Table 7.4. Hederagenin preponderant presence in the samples derived from the hederagenin isolation – Method II, analyzed by LC-MS (in negative ionization).

Standard saponin	M.W.	Retention time (min)	Signal Intensity (cps)	Sample (5 µg / mL)	Signal Intensity of Hederagenin / Molecular ion in the sample (cps)
Hederagenin 3.09 mg (5µg / mL)	471.7	5.79	$2 \cdot 10^6$	RTI.8.	$1.7 \cdot 10^6$
				RTI.8.1.	$8.2 \cdot 10^6$
				B2 (RTI.8.1.)	$1.7 \cdot 10^6$ *
				B6 (RTI.8.1.)	$2.5 \cdot 10^6$ *
				B11 (RTI.8.1.)	$2.2 \cdot 10^6$ *

7.3.3. Identification of α-Hederin

Based on the specific signal intensity value of α -Hederin standard, the compound could be quantified using LC-MS chromatograms (in negative ionization), in the samples: ethanolic extract RTI.1., fractions A8, A9 of RTI.1.2. (Method I), respectively in the ivy extract RTI.8. and in the isolated crude hederagenin RTI.8.1. (Method II). (**Figure 7.6, Table 7.5.**).

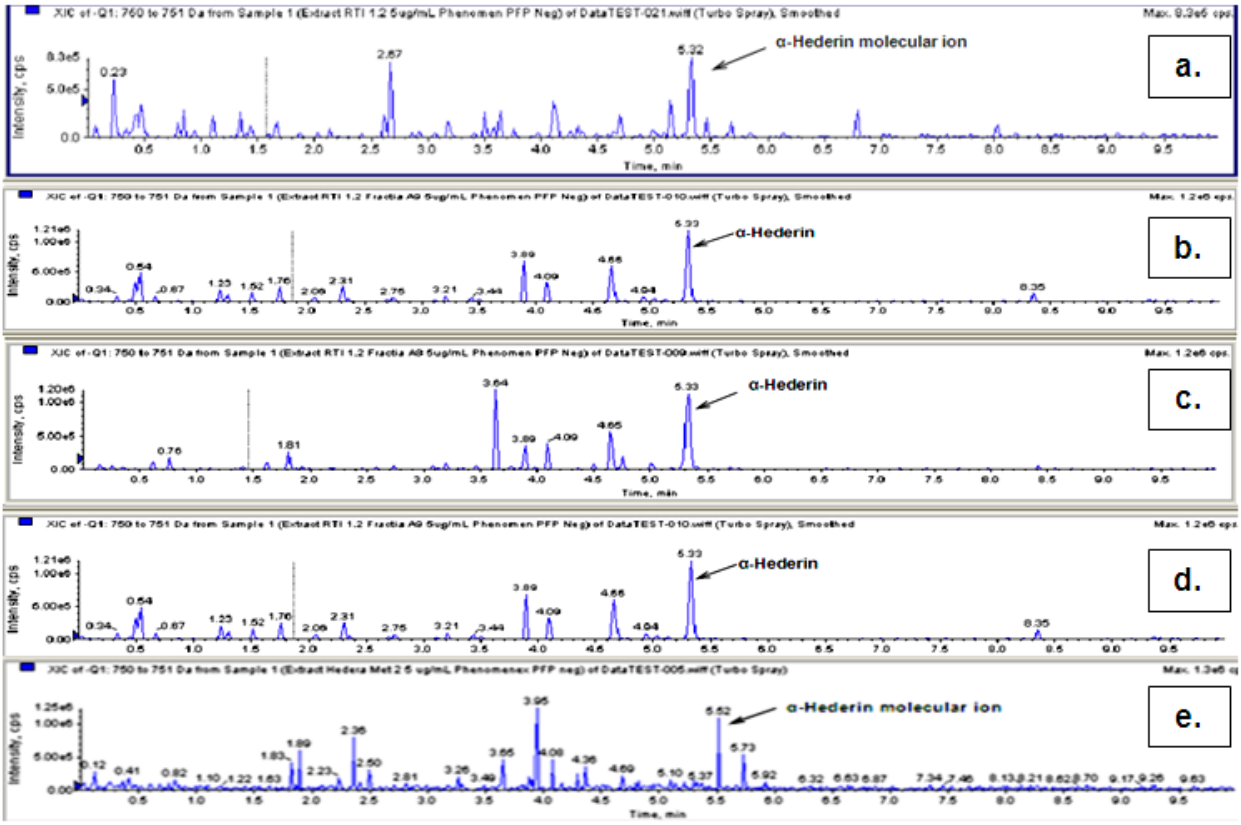


Figure 7.6 . LC-MS chromatograms for: a) standard α – Hederin, b) α-hederin identified in ivy extract RTI.1., c) and d) α -hederin identified in fractions A8 and A9 of the crude isolated saponin mixture RTI.1.2. e) α - hederin identified in the ivy extract RTI.8.

Table 7.5. Presence of α -Hederin samples analyzed by LC- MS (in negative ionization).

Standard saponin	M.W.	Retention time (min)	Signal Intensity (cps)	Sample (5 µg / mL)	Signal intensity. of α-Hederin / Molecular ion in sample (cps)
α-Hederin 2 mg (5µg / mL)	751.4	5.34	$5.5 \cdot 10^6$	RTI.1.	$4.0 \cdot 10^6$
				RTI.1.2.	$7.0 \cdot 10^5$
				A8 (RTI.1.2.)	$1.2 \cdot 10^6$ *
				A9 (RTI.1.2.)	1.2 1· 0^6 *
				RTI.8.	$1.1 \cdot 10^6$
				RTI.8.1.	$1.1 \cdot 10^6$

The quantitative data of the three saponins of interest, identified in the samples analyzed by LC-MS are centralized in **Table.7.6.**,, and the estimation of their concentration in the analyzed compounds is represented in the graph in **Figure 7.7.**

Table 7.6. Estimating the content of the three reference saponins in the analyzed samples LC-M

Sample	Hederagenin (µg/mL)	α-Hederin (µg/mL)	Hederacoside C (µg/mL)
Reference	5,00	5,00	5,00
RTI1.	2,50	3,64	4,21
RTI.1.1.	2,75	0,54	0,79
RTI1.2.	3,25	0,92	3,68
A2 (RTI.1.2.)	8,50	0,14	0,34
A7 (RTI.1.2.)	3,25	0,92	2,50
A8 (RTI.1.2.)	3,50	1,09	12,76
A9 (RTI.1.2.)	0,19	1,09	14,47
RTI.8.	4,25	1,00	0,53
RTI.8.1.	20,50	1,00	0,33
B2 (RTI.8.1.)	4,25	< 0,10	< 0,10
B6 (RTI.8.1.)	6,25	< 0,10	< 0,20
B11 (RTI.8.1.)	5,50	< 0,10	< 0,10
RTI.9.	2,00	0,09	0,21
RTI.9.1.	7,00	< 0,10	0,79
RTI.9.2.	5,75	< 0,10	0,84

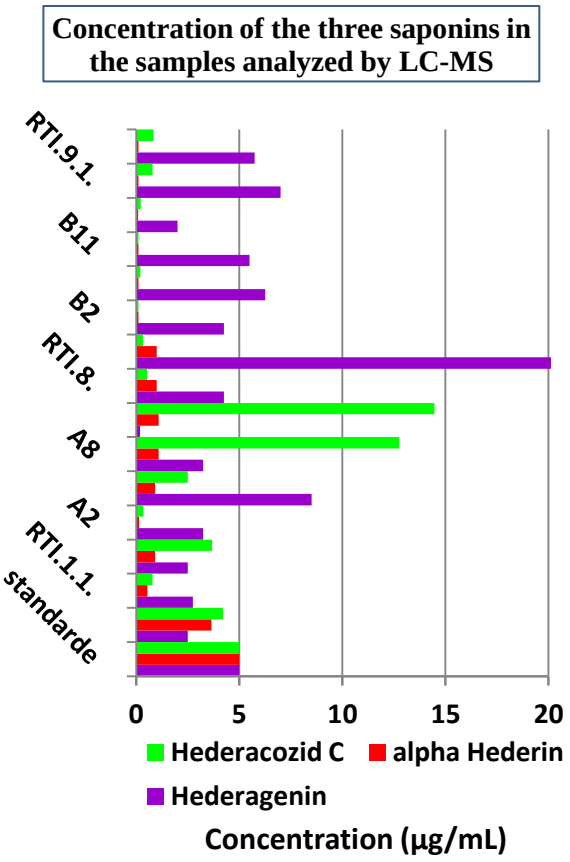


Figure. 7.7. The amount of the three reference saponins in the samples derived from the three saponins isolation methods, according to the LC-MS chromatograms.

7.3.2. *In vitro* biological activity evaluation of isolated hederagenin (RTI.8.1.)

7.3.2.1. *In vitro* biocompatibility testing (according to SR EN ISO 10993-5)

Cell viability was determined using the MTT assay, based on the reduction of the tetrazolium salt under the action of mitochondrial dehydrogenase enzymes from active metabolic cells with the formation of insoluble crystals of purple formazan. Thus, the amount of formazan generated is directly proportional to the number of viable cells. For the experiment, at 24 h and 48 h of incubation the culture medium was replaced with 50 µg / mL MTT solution and the plates were incubated for 3 hours. After incubation, isopropanol was added to solubilize the formazan crystals and the plates were placed on an orbital shaker for 15 min. The optical density (OD) of

the colored solution was measured at a wavelength of 570 nm, using the Mithras LB 940 microplate reader (Berthold Technologies). The results were calculated using the following formula:

.

Cell cultures used for *in vitro* testing were:

- NCTC (clones 929) normal mouse fibroblasts with cultivation density of 4 x 10^4 cells / mL;
- Hep-2 human cervix epithelial tumor cells, with cultivation density of 6 x 10^4 cells / mL;

Culture medium: Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS) and 1% antibiotics penicillin, streptomycin and neomycin (PSN).

Incubation conditions: humidified atmosphere with 5% CO_2 and 37 ° C.

The samples preparation: the sample was solubilized in a small amount of DMSO followed by preparation of 400µg / mL stock solutions in MEM, sterile filtration through Millipore filter 0,22µm.

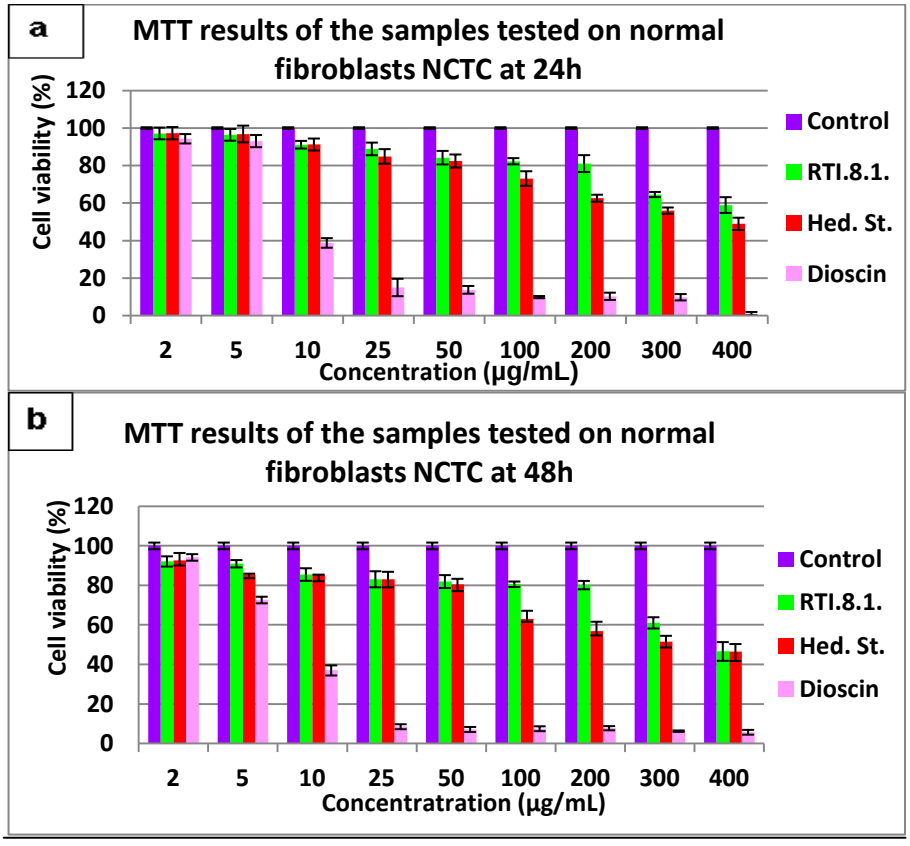


Figure 7.8. Cell viability variation of viability of fibroblast cells normal in the test sample hederagenin NCTC isolated RTI.8.1., The standard hederagenin and the dioscin at 24 h (a) and 48h (b) the experiment as determined by the MTT assay.

Untreated cells were used as controls and considered 100% viable. The experiments were performed in triplicate and the results were expressed as mean ± SD. Dioscin acted as a positive witness [31].

7.3.2.2. Antiproliferative activity determination

Evaluation of antiproliferative activity was performed on human cervical Hep-2 tumor cells, tested in culture medium MEM + / - fetal bovine serum (FBS).

The graphs show the variation of cell viability in Hep-2 cervical tumor culture (in MEM with and without fetal serum, respectively) at the testing of isolated hederagenin sample, standard hederagenin and dioscin, at 24 h and 48 h of culture, determined by the MTT assay.

Untreated cells were used as a control and considered 100% viable. The experiments were performed in triplicate and the results were expressed as mean ± SD.

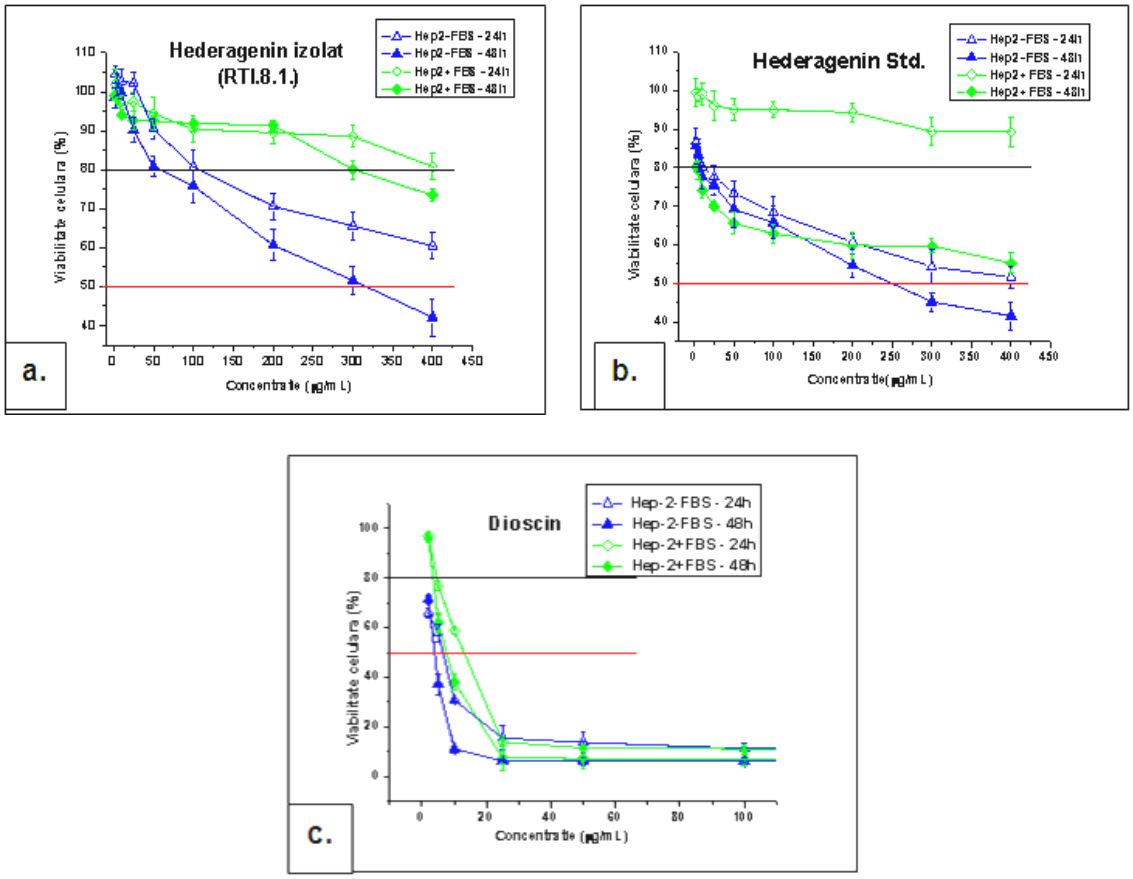


Figure 7.9. Antitumor activity of isolated hederagenin RTI.8.1. (a), standard hederagenin (b) and dioscin (c) determined in a culture of Hep-2 human carcinoma cells by MTT assay, at 24 h and 48 h of cultivation in MEM +/- FBS.

Table 7.7. IC $_{50}$ values determined for isolated hederagenin RTI.8.1., hederagenin standard and dioscin control.

Sample	IC50 (µg / mL)	
	Hep-2-FBS	Hep-2 + FBS
Isolated Hederagenin, RTI.8.1.	320	> 400
Standard hederagenin	250	> 400
Dioscin	3	6

7.3.2.3. Cell morphology evaluation

NCTC and Hep-2 cells morphology treated with isolated hederagenin sample (RTI8.1.)and the two controls, were highlighted by Giemsa staining. Images of treated cultures were taken using a Zeiss microscope.

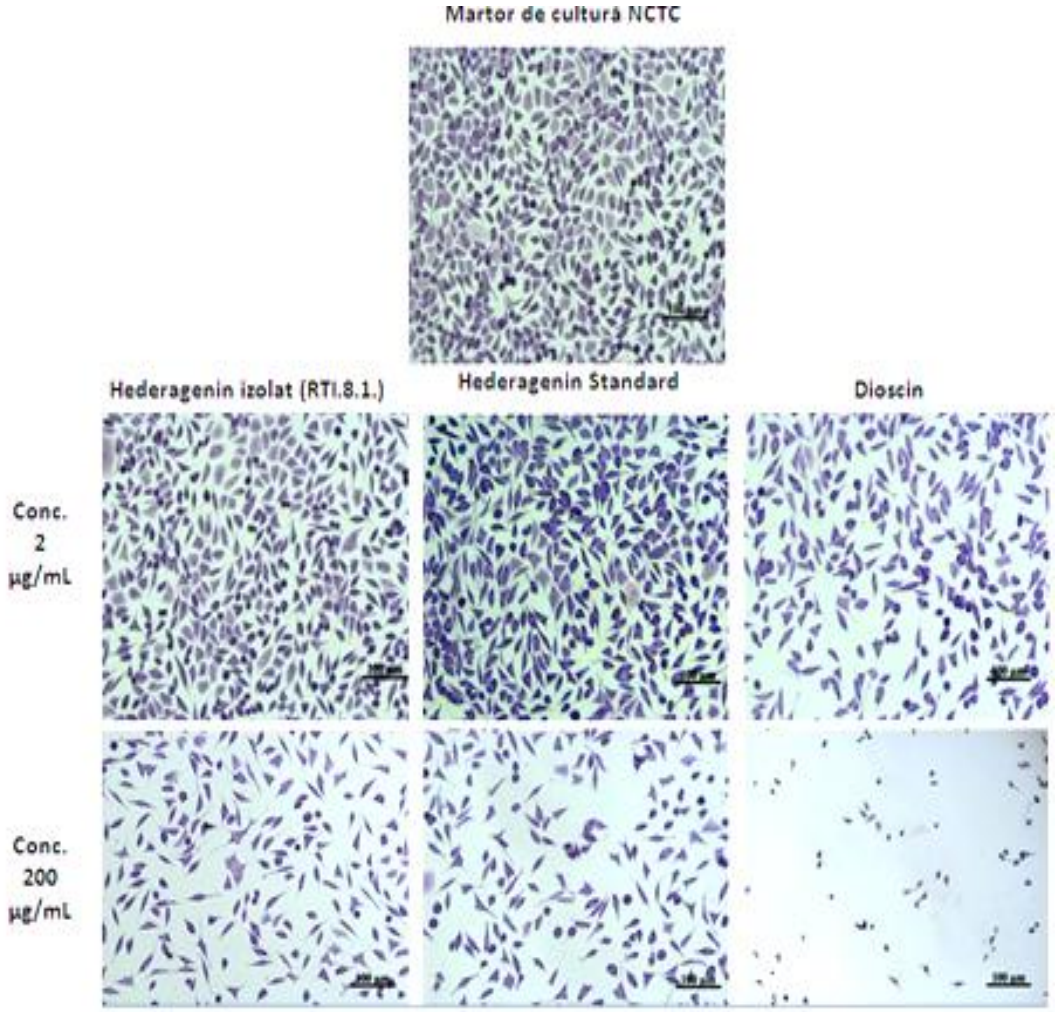


Figure 7.10. Images present the normal NCTC fibroblasts morphology highlighted by Giemsa staining, at 48 h of experiment.

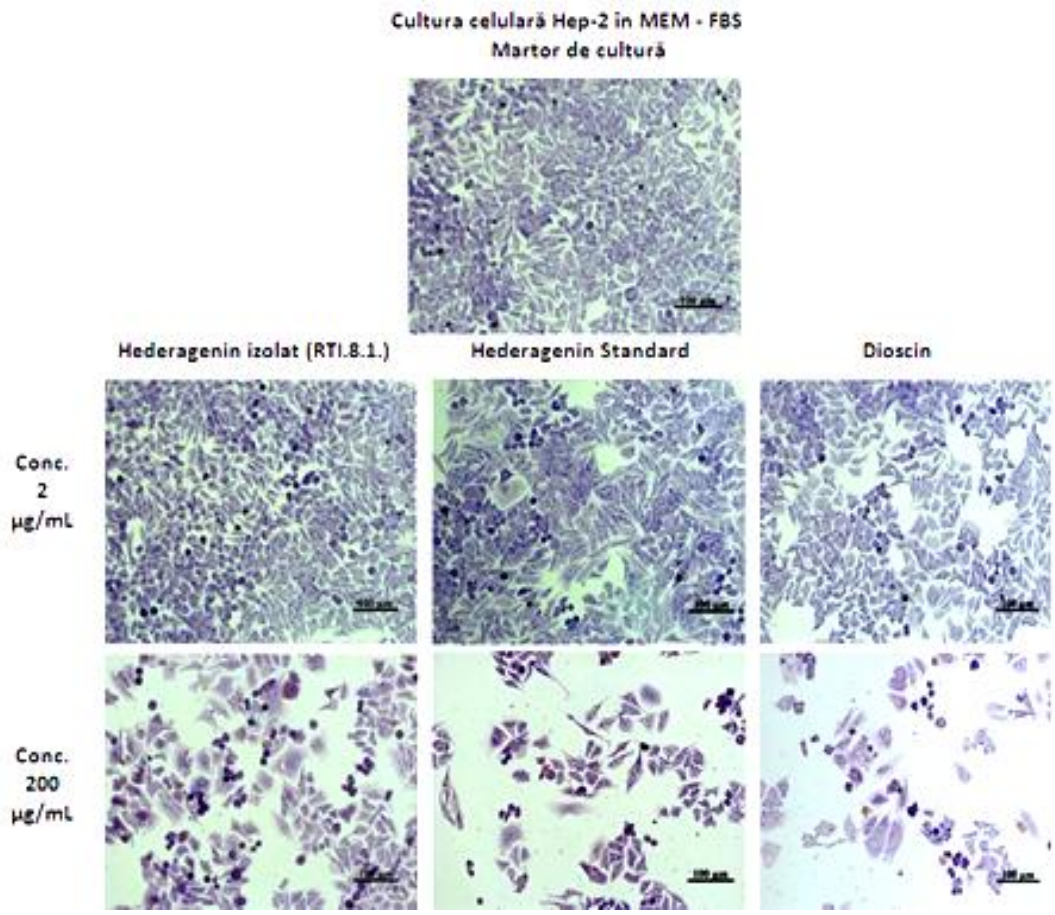


Figure 7.11. Images present Hep-2 tumor epithelial cell morphology at the testing in MEM without FBS, highlighted by Giemsa staining, at 48 hours of experiment.

CHAPTER 8. EVALUATION OF PHYTOCHEMICAL AND ANTIPROLIFERATIVE POTENTIAL OF IVY EXTRACT FRACTIONS SEPARATED BY FLASH CHROMATOGRAPHY

Objectives :

- Obtaining of the ethanolic extract from *Hedera helix* leaves
- Ethanolic extract fractionation
- *In vitro* biological characterization of ethanolic extract and its fractions
- Phytochemical characterization of selected samples

Methods of analysis:

- *In vitro* evaluation of biological activity using MTT test
- Determination of the total flavonoid content
- Determining the total polyphenol content
- Determining the antioxidant activity of ABTS
- Determination of total saponin content (CTS)

8.1. Obtaining of the ethanolic extract from ivy leaves

Dried and crushed *Hedera helix* leaves were extracted by refluxing with ethanol: water (1: 1) at 80° C with stirring for 2 hours. The extract obtained was concentrated under reduced pressure resulting a concentrated extract of syrup consistency.

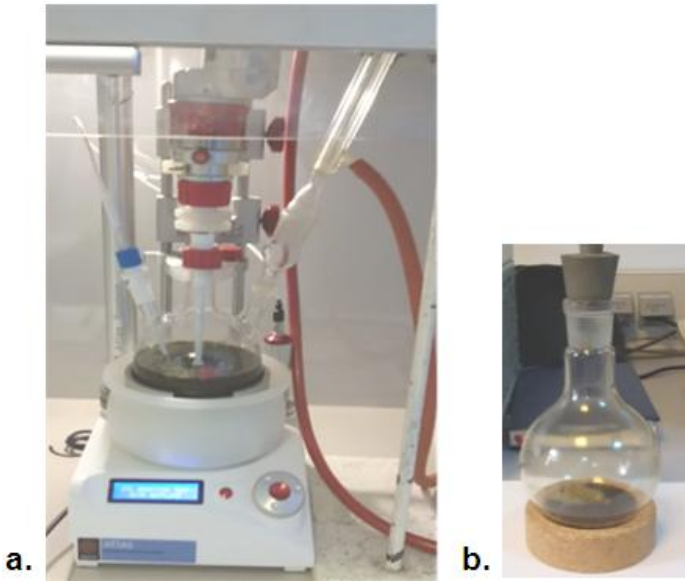


Figure 8.1. a) The installation for obtaining by reflux the ethanolic extract from ivy leaves ; b) concentrated ivy extract, resulted from concentration under reduced pressure.

Table 8.1. Data for the refluxing of ethanolic extract from ivy leaves.

Vegetal mass (g)	Solvent volume EtOH / H_2O (1: 1) (mL)	Residual mass (g)	Ethanolic extract (mL)	Concentrated extract (mL)	Color
100	500	82.4	403	31	dark green

8.2. Flash chromatography fractionation of the ivy leaves ethanolic extract

Test conditions:

Separation system : Flash Chromatograph Biotage (Charlotte) with UV-VIS detector

- Separation Column SNAP Ultra C18 (10 g), filled with granules of silicagel HP-Sphere TM 10 μm.
- Autosampler: Fraction Collector Rack type (16 x100mm).

Solvent system: acetonitrile: water = 30: 100 (with 0.1% phosphoric acid) with volume (1CV),
Flow rate: 10 mL / min,
Detection range: 200-800 nm.

The fractions separated with system software depending on UV absorption spectra, were collected in the collector device. Has result nine fractions noted Fr.1 - Fr.9 with a volume of approx.10 mL, which were brought to dryness [32].

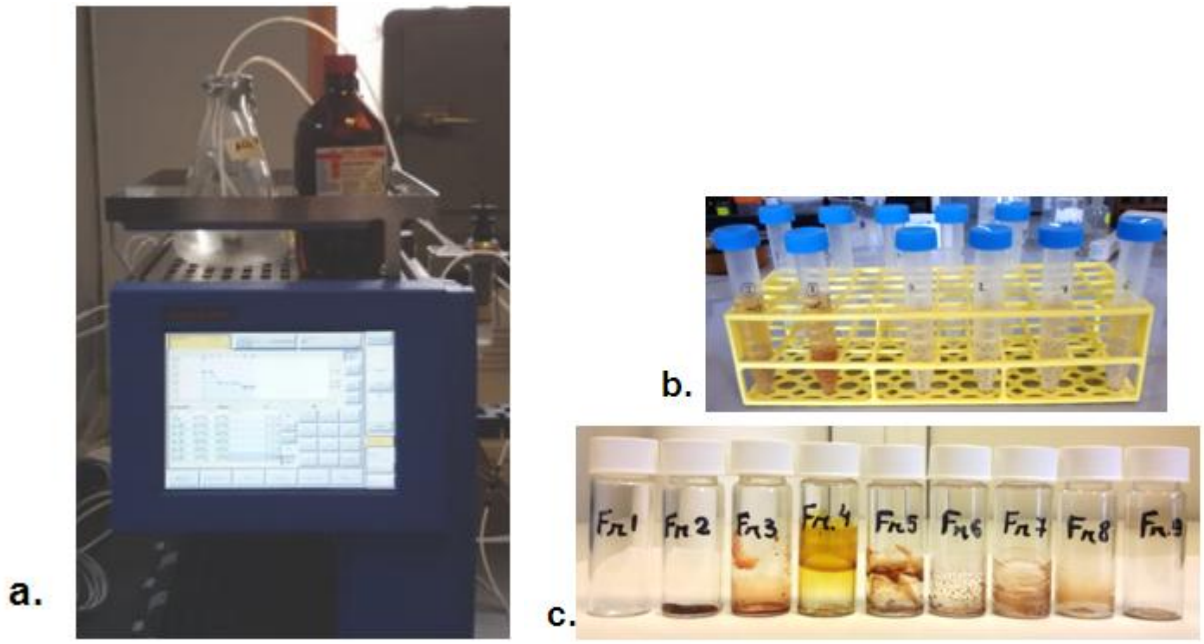


Figure 8.2. a) Flash chromatograph Biotage with UV-VIS detector; b) the fractions collected from ivy leaf extract separation; c) dry fractions.

8.3.1. Biocompatibility and antiproliferative activity evaluation of the ivy extract and its fractions

Hedera helix leaves extract and fractions have been tested *in vitro* on NCTC culture at 24 h, to determine their cytotoxicity. Antiproliferative activity testing of *Hedera helix* leaves extract and its nine fractions was performed on Hep-2 tumor cells, in variants of culture medium with or without fetal bovine serum [12] at 24h.

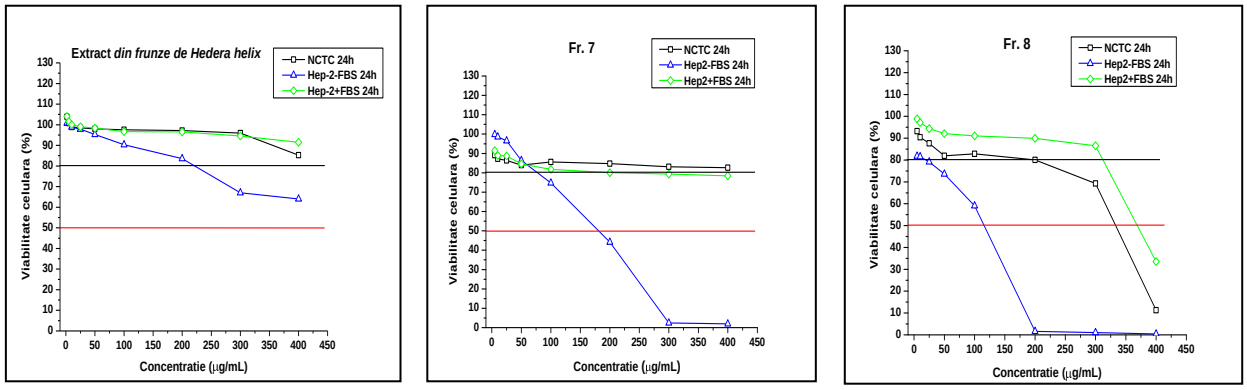


Figure 8.3. Cell viability variation of the NCTC normal fibroblasts and Hep-2 epithelial tumor cells, at the samples testing: a) ivy leaf extract b) fraction 7 and c) fraction 8, determined by MTT assay at 24h.

Table 8.2. IC_{50} values determined for *Hedera helix* extract and its fractions on Hep-2 cell culture.

Sample	IC50 (µg / mL)	
	Hep-2-FBS	Hep-2 + FBS
Extract of *Hedera helix*	> 400	> 400
Fr.1	> 400	> 400
Fr.2	> 400	> 400
Fr.3	> 400	> 400
Fr.4	> 400	> 400
Fr.5	> 400	> 400
Fr.6	> 400	> 400
Fr.7	181	> 400
Fr.8	115.7	368.9
Fr.9	> 400	> 400

8.3.2. Physico-chemical characterization of ivy extract and its fractions 7 and 8

The samples with strong antiproliferative effect, fractions 7 and 8 were biochemically evaluated versus ivy extract in order to reveal the correlation between the amounts of phytocompounds such flavonoids, polyphenols and saponins and their antioxidant and antiproliferative activities.

Determination of total flavonoids content (colorimetric method)

The sample was treated with methanol, $AlCl_3$ 10%, sodium acetate and distilled water, incubated 30 min. at 23° C, with the quercetin as flavonoid standard. The absorbance was read at a wavelength of 415 nm using a Sunrise Tecan plate reader (Austria) .
The equation of calibration curve was y = 0.0038 x + 0.0064, with the correlation coefficient: 0.9993.

8.4.3. Determination of ABTS antioxidant activity (2,2'-azino-bis-3-ethyl-benzo-thiazoline-6-sulfonic acid)

The test sample was mixed with the solution of the diammonium salt ABTS (Fluka) with absorption OD value of 0.7, incubated at room temperature for 6 minutes. The absorbance was measured at 734 nm wavelength in a UV-VIS spectrophotometer (Jasco -530, Japan).

The Trolox equation of calibration curve was made for the concentration range of 0-250 µM . The calculated results of the antioxidant capacity were expressed as Trolox equivalents (TEAC) in mM Trolox / g dry weight, using the formula:

$$TEAC_{proba} = C_{Trolox} \times f \times \frac{OD_{proba} - OD_{blank}}{OD_{Trolox} - OD_{blank}}$$

where: *C Trolox* concentration of mM Trolox / g dry weight and f is sample dilution factor.

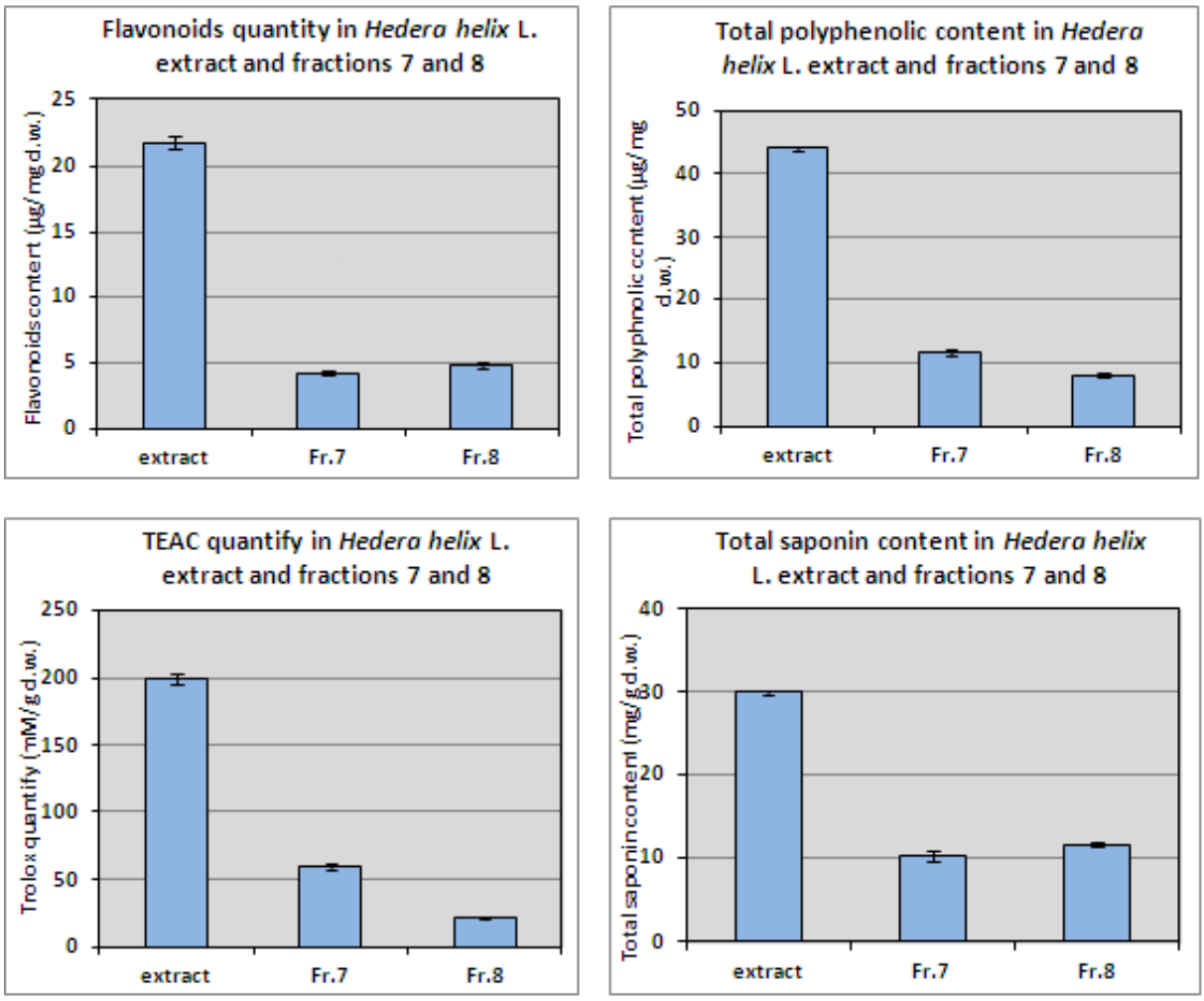


Figure 8.4. The results of total flavonoids content, polyphenols, saponins and the antioxidant activity of the ivy extract and fractions 7 and 8.

Table 8.3. Phytochemical characteristics of *H. helix* extract and its fractions 7 and 8.

Sample	Total polyphenol content * (mg Gallic acid / g d.w.)	Total flavonoid content * (mg Quercetin / g d.w.)	ABTS Antioxidant capacity * (mM Trolox / g d.w.)	Total saponin content * (mg Ginsenoside / g d.w.)
Extract *H. helix*	44.02 ± 0.26	21.75 ± 0.51	199.27 ± 4.44	30.02 ± 0.68
Fr.7	11.60 ± 0.50	4.18 ± 0.14	59.37 ± 2.63	41.30 ± 0.26
Fr.8	8.03 ± 0.25	4.75 ± 0.21	22.11 ± 1.31	44.90 ± 0.19

9. OPTIMIZATION BY MATHEMATICAL MODELING OF A TRITERPENIC SAPONINE COMPOSITION WITH ANTIPROLIFERATIVE ACTIVITY

The objective of the study

The aim of the study was to evaluate *in vitro* cytotoxic and antiproliferative activity induced by three standard triterpene saponins: α-hederin, hederagenin and hederacoside C, and to optimize a mixture of the three saponins, to produce a maximum antiproliferative effect.

Stages of the study:

1. *In vitro* evaluation of cytotoxic and antiproliferative activity induced by three standard triterpene saponins: α-hederin, hederagenin and hederacoside C

2. Mathematical modeling of mixtures using RSM by Design Expert 11 software.

3. Determination of the influence of mixture variants of three saponins generated by mathematical modeling, on cell viability

4. Statistical analysis

9.1. The influence of standard saponins on cell viability

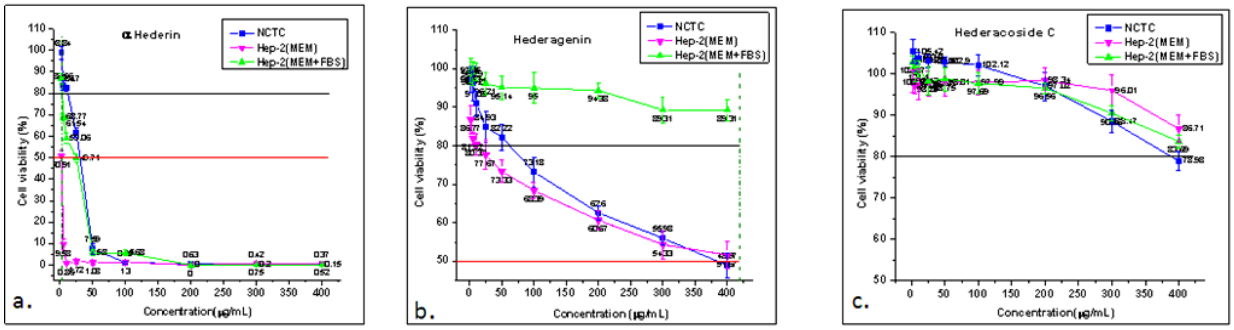


Figure 9.1. Cytotoxicity and *in vitro* antiproliferative activity of standard saponins: a) α-hederin, b) hederagenin and c) hederacoside C, tested on NCTC fibroblast cells and Hep-2 tumor cells in MEM without FBS or MEM with FBS, determined by the MTT assay. The values represent mean ± SD (n = 3).

Table 9.1. Saponins cytotoxicity results on NCTC cells.

Induced effect on NCTC cells	α-Hederin		Hederagenin		Hederacoside C	
	Conc. (µg/mL)	Cell Viability(%)	Conc. (µg/mL)	Cell viability (%)	Conc. (µg/mL)	Cell viability (%)
Biocompatible	≤10	≥ 80	≤50	≥ 80	2 - 300	105,42 – 88,47
Slightly cytotoxic	25	61,54	100 – 300	73 – 55		
Cytotoxic	50-400	≥ 0,37	400	48,97	400	78,98

Table 9.2. IC50 values of saponins cultured on Hep-2 tumors cells in MEM - / + FBS.

Standard saponin	IC_{50} (µg / mL)	
	Hep-2 MEM	Hep-2 MEM + FBS
α-Hederin	2.00	24.85
Hederagenin	413.36	> 500
Hederacoside C	> 400	> 400

9.2. Saponin mixtures optimization with antiproliferative activity.

9.2.2. Mathematical modeling of the saponins mixtures using RSM with Design Expert 11. software

Table 9.3.The independent factors taken into account consideration and their limits of variation in µg / mL.

Component	Name	Conc. min. (µg / mL)	Conc.max. (µg / mL)
A	α-Hederin	2	8
B	Hederagenin	100	498
C	Hederacoside C	200	598
Total			700.00

The experimental matrix used in mixture design has the following features:

- Type model: Scheffe
- Absence of matching points: 5
- Replicated points: 3
- Additional model points: 0
- Additional central points: 0
- Blocks: 1
- Total runs: 14

9.2.2. Influence of the three saponins mixtures in variants generated by mathematical modeling, on the cellular viability

Table 9.4. *In vitro* results of cell viability determined by the MTT test of the 14 mixtures variants of the three saponins, generated by mathematical modeling according to the experimental matrix.

Mixture Variant	Mixture Composition (µg/mL)			Cell viability (%)		
	A: α-Hederin	B: Hederagenin	C: Hederacoside C	NCTC	Hep-2 (MEM)	Hep-2 (MEM + FBS)
1	8.000	381.029	310.971	43.94	4.44	49.39
2	3.824	100.000	596.176	77.28	37.13	77.00
3	2.000	279.100	418.900	72.08	59.84	66.10
4	3.514	496.486	200.000	63.43	37.54	68.92
5	8.000	139.800	552.200	68.85	1.82	61.50
6	2.000	343.630	354.370	72.91	49.04	63.66
7	2.000	188.104	509.896	79.46	57.11	73.15
8	5.305	301.292	393.403	60.27	2.02	63.00
9	3.824	100.000	596.176	80.89	35.52	76.53
10	5.305	301.292	393.403	64.71	2.12	66.57
11	2.000	424.557	273.443	70.58	63.37	68.54
12	8.000	462.150	229.850	49.81	16.75	44.32
13	8.000	230.898	461.102	65.16	1.61	69.58
14	5.305	301.292	393.403	63.21	2.32	62.07
Answer range				**from 43.94 to 80.89**	**from 1.61 to 63.37**	**from 44.32 to 76.99**
The ratio from max. to min.				**1.84**	**39.25**	**1.73**
Transformation				**-**	**Natural log**	**-**

9.3. Statistical analysis

Using the analysis of variance, the software proposed the models presented in **Tables 9.6. and 9.7.** .

Table 9.6. Variability of parameters *F* and *p* according to the analysis of variance.

Answer	Model type	Model		Lack of fit		R^2
		F value	p value	F value	p value	
NCTC	Quadratic Model	22.77	0.0002	2.79	0.2142	0.9343
Hep-2 (MEM)	Cubic Model	457.91	<0.0001	5.18	0.1072	0.9990
Hep-2 (MEM + FBS)	Special Quartic Model	52.48	0.0002	0.191	0.8355	0.9882

- *F* and *p* values showed that the models were significant
- Precision of the proposed model was evaluated with the correlation coefficient value

The values represent mean. ± SD (n = 3).
The limits of variation were very large, therefore as transformation average was used ln (Y).

Table 9.7. Coefficients of the model and associated *p* values.

Model	Coefficients (of coded equation) and p value										
	A	B	C	AB	AC	BC	ABC	AB(AB)	AC(A-C)	ABC^2	A
NCTC	18786.6	70.9735	8.3454	−20.7322	−19.5965	15.8615	-	-	-	-	18.7866
P value	<0.0001	<0.0001	<0.0001	0.5880	0.6085	0.1430	-	-	-	-	<0.0001
ln Hep-2 (MEM)	5.65968 E + 06	4,51276	3.99676	8.5363 E + 06	8,552 E + 06	1.0551	5.76906 E + 06	2.87669 E + 06	2.89269 E + 06	-	5.65968 E + 06
P value	<0.0001	<0.0001	<0.0001	0.0052	0.0052	0.3829	0.0052	0.0053	0.0052	-	<0.0001
Hep-2 (MEM + FBS)	- 9343.46	76.5045	88.5913	7533.95	6852.24	71.2811	-	-	-	16.7169	- 9343.46
P value	<0.0001	<0.0001	<0.0001	0.9173	0.9248	0.0152	-	-	-	0.0391	<0.0001

- The relationship between the response and the significant variables represented by the equation are correct
- The condition that the model to be considered statistically significant was accomplished, the *F* value was biger, and *p* value was < 0.05.

Using the models generated by software, it was possible to describe the response surface for each variant.

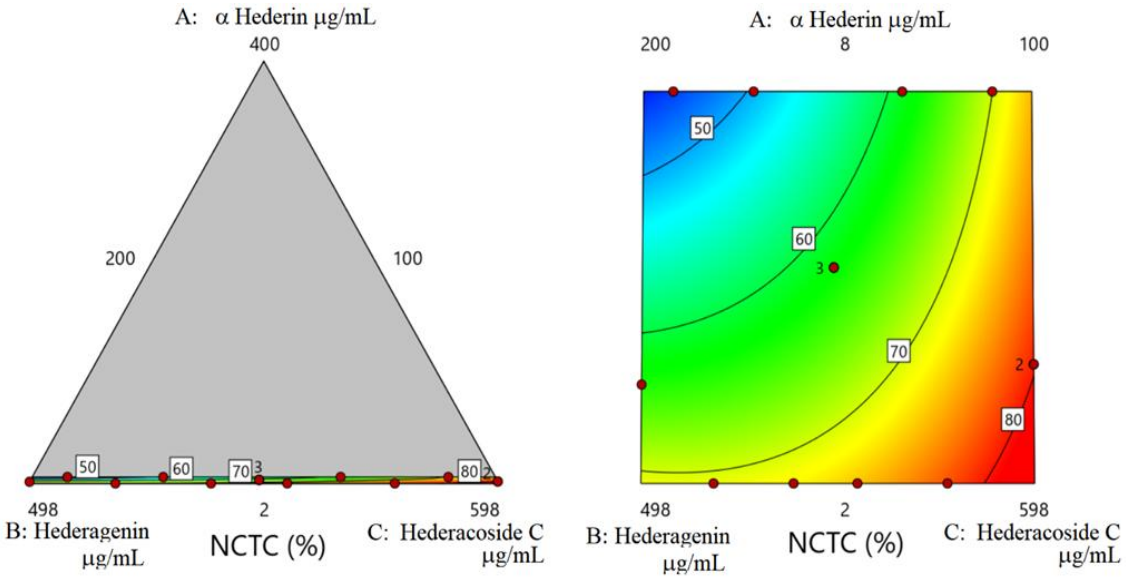


Figure 9.2. The response surface of ternary cell viability of fibroblasts NCTC and the concentration zone of the tested compounds.

Ternary graph indicated a large difference between the variations fields between α-hederin and the other two components, requiring presentation of the enlarge diagram for the relevation of the concentration area.

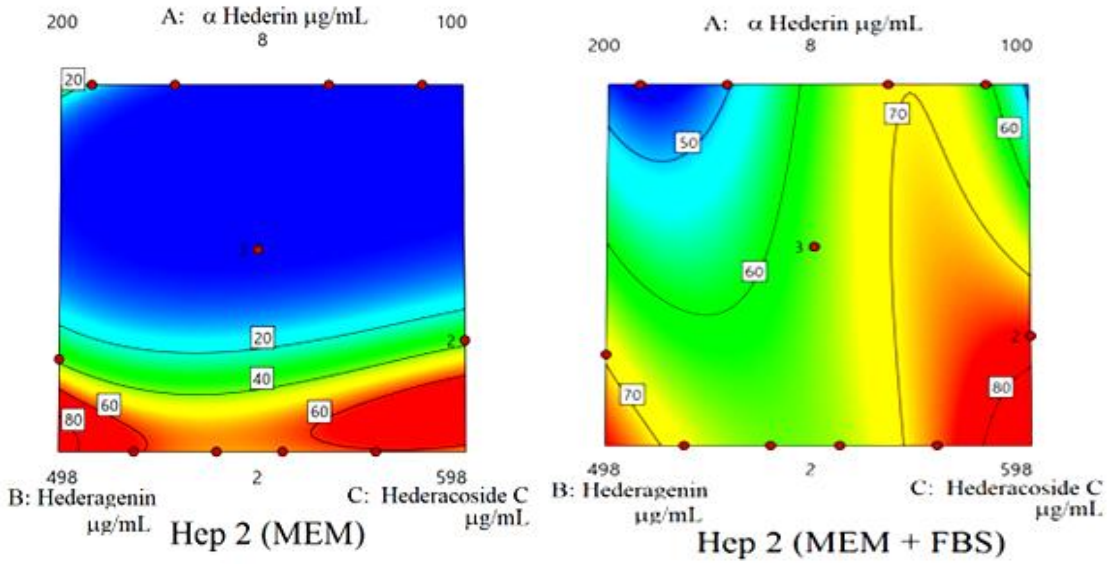


Figure 9.3. Cell viability response surfaces for concentration surfaces of test compounds on: a) Hep-2 cells in MEM (without FBS); b) Hep -2 in MEM + FBS .

Cell viability of Hep-2 cells was smaller in the presence of MEM without FBS than in MEM with FBS.

Areas with cell viability below 50% were determined in particular by α-hederin concentration for Hep-2 (MEM), while for the Hep-2 (MEM + FSB) involved factors were more numerous, area with viability under 50% being much more restricted [33].

Table 9.8. Restrictions for the response surfaces.

Name	Objective	Minimum limit	Maximum limit
A: α-Hederin	to be in the range	2.00	8.00
B: Hederagenin	to be in the range	100.00	498.00
C: Hederacoside C	to be in the range	200.00	598.00
NCTC	maximization	79.00	80,89
Hep-2 (MEM)	minimization	1.61	50.00
Hep-2 (MEM + FBS)	without restrictions	44.319	76.99

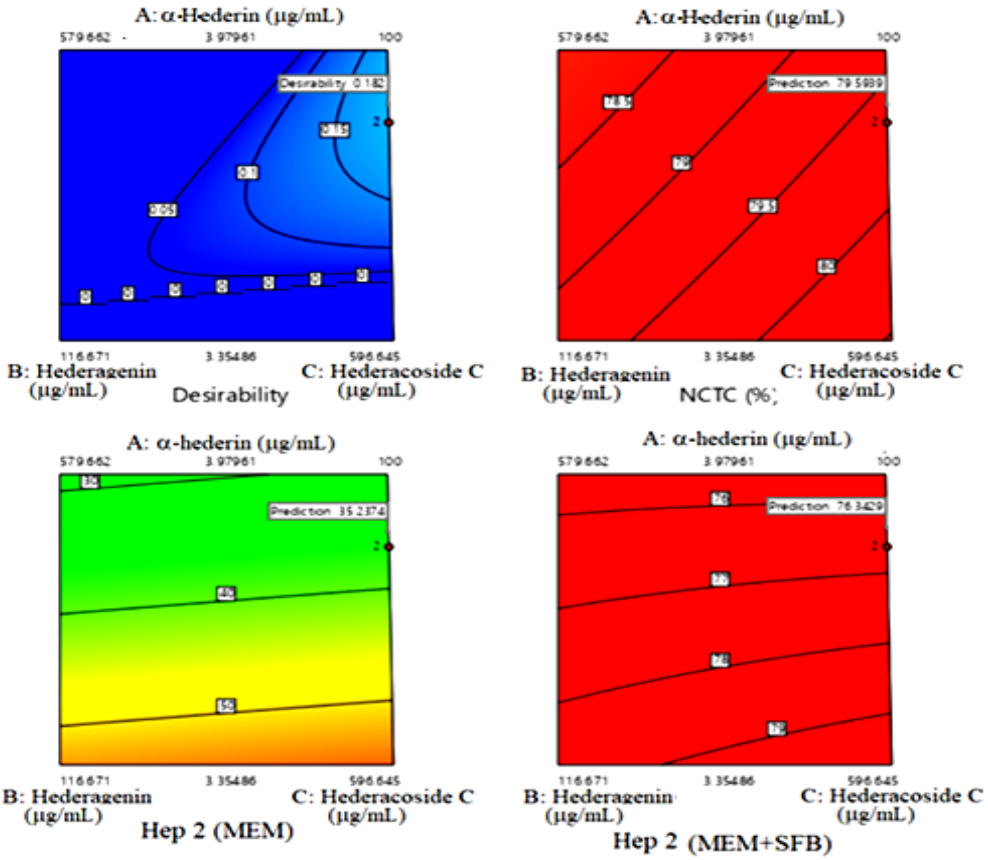


Figure 9.4. Desirability of the response area and viability of NCTC and Hep-2 treated cells.

Table 9.9. Optimal variant.

Solution no.	Saponin concentration (µ g / mL)			Cell viability (%)			Desirability
	α-Hederin	Hederagenin	Hederacoside C	NCTC	Hep-2 (MEM)	Hep-2 (MEM + SFB)	
1.	3.863	100.000	596.137	79.594	34.833	76.368	0.182

ORIGINAL CONTRIBUTIONS

The scientific research of this thesis was focused on studies that aimed to highlight and optimize the antiproliferative effects of saponins of the type hederagenin, α-hederin and hederacoside C from *Hedera helix* L. leaves extracts and theirs fractions, respectively of the standard commercialized compounds.

The performed studies have generated the following original contributions:

❖ Crude saponins mixture was isolated by precipitation with diethyl ether of the ethanolic extract obtained by maceration of the two types of plant material: unselected ivy leaves and selected palm form leaves, respectively (Method I). The isolation yields were 4.78 % and 3.89 %, respectively. CSS and LC-MS analysis of saponin fractions isolated from unselected ivy leaves indicated the predominant presence of hederagenin in A2 fraction in the amount of 8.5 μg / mL and hederacoside C in fractions A8 and A9 with 12.76 and 14.47 μg / mL, respectively.

❖ Crude hederagenin was isolated by two methods:

- from the ethanolic extract resulted by repeated maceration of ivy leaves, followed by acid hydrolysis and purification with acetonitrile (Method II) with hederagenin isolation yield (RTI.8.1.) of 9.22 % - the best efficiency among the three applied saponin isolation methods.

- from ivy leaves ethanolic extract obtained by refluxing, subjected to acid hydrolysis and purification with lead acetate, (Method III), with an isolation yield of hederagenin (RTI.9.2.) of 6.42%.

The quantity of hederagenin in the sample of crude isolated hederagenin RTI.8.1. estimated based on the chromatograms LC-MS was 20.50 mg / mL, approx. 5x more than in the source extract. Tested *in vitro*, crude hederagenin RTI.8.1. in concentration range 2-200 mg / mL manifested biocompatibility with normal mouse fibroblast NCTC cells, and had antitumor activity on human cervix epithelial tumor cells Hep-2 at concentration of 100 - 400 mg / mL, with an IC_{50} of 320 mg / mL close to the standard IC_{50} of 250 mg / mL. These results indicate the antiproliferative capacity of the crude hederagenin isolated from *Hedera helix* extract and its potential to be used as a therapeutic agent in antitumor therapy.

❖ The Flash-chromatographic method with UV-Vis detection was used to obtain the refluxed hydroethanolic extract fractions from *Hedera helix* leaves, which were biologically and biochemically characterized. The antiproliferative activity of the studied samples was dependent with the saponins content, and their antioxidant activity was correlated with theirs polyphenols and flavonoids content. *In vitro* testing of fractions 7 and 8 rich in saponins, on human cervix epithelial tumor cells, manifested strong anti-proliferative effects, with IC_{50} values of 181 mg / mL for Fr.7 tested in MEM, respectively 115.7 μg / mL in MEM with FBS, while Fr.8 tested in MEM with FBS registered an IC_{50} of 368.9 μg / mL. Both fractions tested on normal mouse fibroblast NCTC cells, were non-cytotoxic in the concentration range of 2-200 μg / mL.

❖ The optimization of a saponins mixture consisting of: α-hederin, hederagenin and hederacoside C with maximum anti-proliferative effect was performed by mathematical design using Response Surfaces Method (RSM) generated by Design Expert 11. software. Software processing of individual *in vitro* saponin tests on normal mouse fibroblast NCTC cells and human cervical epithelial Hep-2 tumors in MEM and MEM with FBS at 24 hours of treatment,

generated 14 possible mixtures of the three saponins, these mixture variants also being tested *in vitro.* Mathematical modeling with RSM of the obtained data indicated as solution the variant in which α-hederin: hederagenin: hederacoside C have the mass ratio 3,863: 100,000: 596,137 (μg), this variant of saponins mixture generated maximum antiproliferative effect on Hep-2 tumor cells cultured in MEM without FBS (viability 34.83 %), and at the same time present biocompatibility on normal NCTC cells (viability 79.59 %).

PERSPECTIVES FOR DEVELOPMENT

- Our studies indicate that the method of hederagenin isolation from *Hedera helix* leaves ethanolic extract resulted by repeated maceration of ivy leaves, followed by acid hydrolysis and purification with acetonitrile (Method II) proved to be effective with a yield of 9.22 %. In perspective this method completed with the performing of efficient purification stages of the isolated compound, may become an innovative technology for the hederagenin obtaining.

- Ivy extract fractions rich in saponins, resulted by Flash-chromatography with UV-Vis detection, with strong antiproliferative properties, have the potential to become useful agents in anti-tumor therapies, but for the confirmation of these specific properties, but additional studies are required specific to cellular and molecular biology, as well as their testing on a wider range of tumor cell lines.

- The saponin mixture optimized by mathematical modeling, consisting of α-hederin: hederagenin: hederacoside C in the mass ratio 3,863: 100,000: 596,137 (μg) established using RSM and Design Expert 11 software, exhibit antitumor effect on human cervix epithelial tumor cells Hep-2. Our study shows the potential of this saponins mixture with the mentioned mass ratio to be used in pharmacology applications.

Selective Bibliography:

1. P. Ovadje, A. Roma, M.Steckle, L.Nicoletti, J.T. Arnason, S. Pandey, Advances in the research and development of natural health products as pain stream cancer therapeutics evidence-based complementary and alternative medicine, Review Article, Hindawi Publishing Corporation, (2015), pp.1-12.

2. Burlando, L. Verotta, L. Cornara, E. Bottini-Massa, Herbal Principles in Cosmetics: Properties and Mechanisms of Action, CRC Press, 2010, Print *ISBN: 978-1-4398-1213-6*, eBook.

3. W.C. Evans, Trease and Evans Pharmacognosy (16th ed.). Elsevier Science limited, UK, 48 (2009).

4. J. Ackerfield , J. Wen, A morphometric analysis of *Hedera* L. (the ivy genus, Araliaceae) and its taxonomic implications, ADANSONIA, ser. 3, 24 (2002), pp.197-212.

5. P.A. Gleeson, M.A. Jermyn, Alteration in the composition of β-lectins caused by chemical and enzymic attack, Austral J Plant Physiol, 6 (1979), pp. 25-38.

6. S. Fazio, J. Pouso, D. Dolinsky, A. Fernandez, M. Hernandez, G. Clavier, M. Hecker, Tolerance safety and efficacy of *Hedera helix* extract in inflammatory bronchial diseases under clinical practice conditions: A prospective, open, multicentre post marketing study in 9657 patients, Phytomedicine, (2006), doi 10.1016/j.phymed.2006.05.003.

7. D. Hofmann, M. Hecker, A. Vopl, Efficacy of dry extract of ivy leaves in children with bronchial asthma—A review of randomized controlled trials, Phytomedicine, 10 (2003), pp. 213-220.

8. H. Suleyman, V. Mshvildaze, A. Gepdiremen, R. Elias, Acute and chronic anti-inflammatory profile of the ivy plant, *Hedera helix*, in rats, Phytomedicine, 10 (2003), pp. 370-374.

9. R.M. Facino, M. Carini, R. Stefani, G. Aldini, L. Saibene, Anti-elastase and anti-hyaluronidase activities of saponins and sapogenins from *Hedera helix*, *Aesculus hippocastanum*, and *Rusucus aculeatus*: Factors contributing to their efficacity in the treatment of venous insufficiency, Arch. Pharm. (Weinheim), 328 (1995), pp. 720-724.

10. S. Danloy, J. Quetin-Leclercq, P. Coucke, M.C. De Pauw-Gillet, R. Elias, G. Balansard, L. Angenot, R. Bassleer, Effects of α-hederin, a saponin extracted from *Hedera helix*, on cells cultured *in vitro*, Planta Medica, 60 (1994), pp. 45-49.

11. J. Liu, Y. Liu, P. Bullock, C.D. Klaassen, Suppresion of liver cytochrome P450 by α-hederin: Relevance to hepatoprotection, Toxicol. Appl. Pharmacol., 134 (1995), pp. 124-131.

12. J. Liu, S. Choudhuri, Y. Liu, H. Kreppel, GK.Andrews, C.D. Klaassen, Induction of metallothioninen by α-hederin, Toxicol. Appl. Pharmacol., 121 (1993), pp. 144-151.

13. C. Ciocă, C. Mărgineanu, V. Cucu, The saponins from *Hedera helix* with antibacterial activity, Pharmazie, 33 (1978), pp. 609:610.

14. J. Julien, M. Gasquet, C. Maillard, P. Timon-David, Extracts of the ivy plant, *Hedera helix*, and their antihelminthic activity on liver flukes, Planta Med, (1985), pp. 203:205.

15. B. Majester-Savornin, R. Elias, A.M. Diaz-Lanza, G. Balansard, M. Gasquet, F. Delmas, Saponins of the ivy plant, *Hedera helix*, and their leishmanicidic activity, Planta Med, (1991), pp. 57:261.

16. A. Gepdiremen, V. Mshvildadze, H. Suleyman, R. Elias, Acute anti-inflamatory activity of four saponins isolated from ivy: α-hederin, hederasaponin-C, hederacolchiside-E and hederacholchiside-F in carrageenan-induced rat paw edema, Phytomedicine, 12 (2005), pp. 440-444.

17. J. Quetin-Leclercq, R. Elias, G. Balansard, Cytotoxic activity of some triterpenoid saponins, Planta Med, 58(1992), pp. 279-280.

18. L. Cheng, T.S. Xia, Y.F. Wang, W. Zhou, X.Q. Liang, J.Q. Xue, L. Shi, Y. Wang, Q. Ding, M. Wang, The anticancer effect and mechanism of α-hederin on breast cancer cells, International Journal Of Oncology, 45 (2014), pp.757-763.

19. N. Dias, C. Bailly, Drugs targeting mitochondrial functions to control tumor cell growth, Biochem Pharmacol, 70 (2005), pp. 1-12.

20. J.J., Liu Lin M., J.Y. Yu, B. Liu , J.K. Bao, Targeting apoptotic and autophagic pathways for cancer therapeutics, Cancer Lett, 300 (2011), pp. 105-114.

21. Q.F. Wang, J.C. Chen, S.J. Hsieh, C.C. Cheng, S.L. Hsu, Regulation of Bcl-2 family molecules and activation of caspase cascade involved in gypenosides-induced apoptosis in human hepatoma cells, Cancer Lett, 183 (2002), pp. 169-178.

22. J. Wang, XZ. Zhao, Q. Qi, et al., Macranthoside B, α-hederagenin, saponin extracted from *Lonicera macranthoides* and its anti-tumor activities *in vitro* and *in vivo*, Food Chem Toxicol, 47 (2009), pp. 1716-1721.

23. H.M. Park, S.J. Kim, J.S. Kim, H.S. Kang, Reactive oxygen species mediated ginsenoside Rg3- and Rh2-induced apoptosis in hepatoma cells through mitochondrial signaling pathways, Food Chem Toxicol, 50 (2012), pp. 2736-2741.

24. J. Chun, I.J. Ha, Y.S. Kim, Antiproliferative and apoptotic activities of triterpenoid saponins from the roots of *Platycodon grandiflorum* and their structure-activity relationships, Planta Med., 79 (2013), pp. 639-645.

25. S. Mo, H. Xiong, G. Shu, et al., Phaseoloideside E, a novel natural triterpenoid saponin identified from *Entada phaseoloides*, induces apoptosis in Ec-109 esophageal cancer cells through reactive oxygen species generation, J. Pharmacol. Sci., 122 (2013), pp. 163-175.

26. C.Z. Wang, X.L. Li, Q.F. Wang, S.R. Mehendale, AB. Fishbein, A.H. Han, S. Sun, C.S. Yuan The mitochondrial pathway is involved in American ginseng-induced apoptosis of SW-480 colon cancer cells, Oncol. Rep., 21 (2009), pp. 577-584.

27. S.M. Swamy, B.T. Huat, Intracellular glutathione depletion and reactive oxygen species generation are important in α-hederin-induced apoptosis of P388 cells, Mol. Cell. Biochem,. 245 (2003), pp. 127-139.

28. S. Hiai, H. Oura, T. Hakajima, Color reaction of some sapogenins and saponins with vanillin and sulfuric acid, Planta Medica, 29 (2), (1976), pp. 116-122.

29. A. Suhaib, A. Hussien, J. Zainab, J. Awad, Isolation and characterization of triterpenoid saponin hederacoside C. present in the leaves of *Hedera helix* L. cultivated in Iraq, Iraqi J Pharm Sci, 23(2), (2014), pp. 33-41.

30. J.B. Harborne, Phytochemical methods. John Wiley & Sons, Inc. New York, 1973.

31. R. Tatia, C. Zălaru, I. Ţârcomnicu, L. Moldovan, O. Crăciunescu, I. Călinescu, Isolation and characterization of hederagenin from *Hedera Helix* L. extract with antitumor activity, Rev.Chim.(Bucharest), 70 (4), 2019, pp.1157-1161.

32. R. Tatia, A. Toma, L. Moldovan, C. Zălaru, I. Călinescu, Phytochemical and antiproliferative potential of *Hedera helix* extract fractions, Buletin Ştiinţific U.P.B., Seria B: Chimie şi Ştiinţa Materialelor, 2020, articol în curs de publicare.

33. R. Tatia, C. Zălaru, O. Crăciunescu, L. Moldovan, A. Oancea, I. Călinescu, Optimization of triterpene saponins mixture with antiproliferative activity, Appl. Sci., 9, 5160, 2019; doi:10.3390/app, 9235160.

RESULTS DISSEMINATION

Scientific articles:

1. Rodica Tatia, Agnes Toma, Lucia Moldovan, Christina Zălaru, Ioan Călinescu, Phytochemical and antiproliferative potential of *Hedera helix* extract fractions, Buletin Ştiinţific U.P.B., Seria B: Chimie şi Ştiinţa Materialelor, 2020, articol în curs de publicare (F.I. = 0,41).

2. Rodica Tatia, Christina Zălaru, Oana Crăciunescu, Lucia Moldovan, Anca Oancea, Ioan Călinescu, Optimization of triterpene saponins mixture with antiproliferative activity, Appl. Sci., 9, 5160, 2019; doi:10.3390/app, 9235160, (F.I. = 2,474).

3. Rodica Tatia, Christina Zălaru, Isabela Ţârcomnicu, Lucia Moldovan, Oana Crăciunescu, Ioan Călinescu, Isolation and characterization of hederagenin from *Hedera Helix* L. extract with antitumor activity, Rev.Chim.(Bucharest), 70 (4), 2019, pp.1157-1161, (F.I. = 1,755).

International Conferences:

1. Rodica Tatia, Lucia Moldovan, Christina Zălaru, Isabela Ţârcomnicu, Mihaela Enache, Ioan Călinescu, Antiproliferative activity evaluation of some saponin extracts from H*edera helix*, ICGEB Workshop Modern Biotechnological Advances for Human Health – BAHH2, May 28 – 31 2019, Bucharest, Romania.

2. Rodica Tatia, Ioan Calinescu, Christina Zălaru, Isabela Ţârcomnicu, Lucia Moldovan, *In vitro* cytotoxicity evaluation of isolated saponins from *Hedera helix* L. extract, RICCE 20th Romanian International Conference on Chemistry and Chemical Engineering, September 6 – 9 2017, Poiana Braşov, Romania.