# Effects of ethnomedicinally used plant extracts on wound healing studied by an *in vitro* cell culture model

**Doctoral (PhD) Thesis**

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# I. INTRODUCTION

Application of medicinal plants has primary importance in several regions of Transylvania, part of Romania. In these areas many diseases are still treated with agents and methods based on the experiences of generations, e.g. with drugs of plant origin. These drugs are used mainly by the older generations living in isolated villages, where no permanent medical, veterinary and pharmacy services are available. Due to their hard worker lifestyle, people often suffer from physical (skin) injuries, therefore herbs positively affecting the wound healing process have a crucial role in these indications.

The special secondary metabolites found in herbs especially polyphenols, can stimulate the process of wound healing in several ways (antimicrobial, antioxidant, anti-inflammatory effects) and also by increasing the cellular proliferation and migration activities. Flavonoids belonging to the group of polyphenols can be divided into 6 subgroups: flavones, flavanones, flavonols, isoflavones, flavan-3-ols (catechin monomers and condensed tannins - proanthocyanidins), and anthocyanidins. Phenolic acids can also be further subdivided into benzoic acid derivatives and cinnamic acid derivatives. Among the anti-inflammatory effects of polyphenols the inhibition of inflammatory enzymes such as cyclooxygenase-2 (COX2), lipoxygenase, phospholipase A2 and inducible nitric oxide synthase maybe mentioned which, in turn reduces the concentration of prostaglandins and leukotrienes being responsible for the inflammatory response.

Another key step in the wound healing process is the migration of epithelial cells to the injured area followed by their cell proliferation. The most common cell types of skin tissues involve fibroblasts and keratinocytes, which play an essential role in the wound healing procedure.

Polyphenols can prevent the non-homeostatic, oxidative injuries caused by elevated free radical levels through a variety of antioxidant mechanisms; the most common among them is the direct neutralization of reactive oxygen species (ROS) by the transfer of one hydrogen atom. Additional defense mechanisms include the activation of antioxidant enzymes, inhibition of oxidase enzymes, chelation, or increasing elevated uric acid levels.

The mechanisms of antibacterial activity of phenolic compounds have not yet been fully elucidated, as they may act in several ways. It is a general observation that Gram-positive bacteria are more susceptible to the inhibitory effect of polyphenols and other antibacterial agents than Gram-negative ones, which can be explained by their different cell wall structure.

The studied medicinal herbs in the thesis are the followings: *Anthyllis vulneraria* L. (common kidney-vetch, *A. vulneraria,* Leguminosae) is an annual, biennial or perennial plant that occurs in fields and meadows throughout Europe. Flavonoids, saponins and tannins have been detected mainly in the aerial part of the plant. In the Transylvanian folk medicine, the plant is mainly used for the treatment of inflammation and wounds as a foment.

*Fuchsia magellanica* Lam. and *Fuchsia triphylla* L. (hardy fuchsia, *F. magellanica* and lady's eardrops, *F. triphylla*, Onagraceae) are perennial cultivated plants all over in Europe. High levels of anthocyanins have been detected in both the flowers and berries of fuchsia species, while leaves contain significant amounts of flavonoids, primarily flavonols and flavonoids. In the Transylvanian folk medicine, the fresh leaves of fuchsia species are

mainly used for wounds, and they are also used in raw form for the treatment of boils and dermatitis.

*Lysimachia nummularia* L. (creeping Jenny, *L. nummularia*, Myrsinaceae) is an evergreen plant that lives mostly in ditches and wet grasslands and in some places as a cultivated species throughout Europe. Based on earlier phytochemical studies, the plant contains mainly flavonoids, phenolic acids, saponins, and tannins. In the Transylvanian folk medicine, the aerial part of the species is used both externally and internally: externally mainly for the treatment of various skin diseases, wounds and abscesses.

## II. MAJOR AIMS OF THE STUDY

The compounds and the mode of action of medicinal plants at the cellular level are sometimes scarce in the literature therefore, in the dissertation we conducted our research based on the following two main objectives.

### II.1. Development of a new methodology for measuring cytotoxicity and testing the reliability of available cellular viability methods

At the beginning of my PhD research, we searched for answers to the following central questions:

- Can a multiparametric cytotoxicity test be developed that allows the determination of several different intracellular components from the same cell sample by a one-step extraction?
- Since protein synthesis is an ATP-dependent process, can the quantitative measurement of green fluorescent protein (GFP) fluorescence be used in cell lines expressing GFP to assess viability?
- What limiting factors may arise when a method is set up for measuring cytotoxicity? Are there any compounds that can interfere with methods for measuring viability or cytotoxicity?

### II.2. Chemical and biological study of herbs involved in wound healing

In the further part of the dissertation, we focused on the following goals and on answering the related questions:

- One major goal was to collect folk medicinal data on the knowledge of the inhabitants of the Homoród Valley in Transylvania, especially for the local treatments for wounds, ulcers and other skin diseases, in addition to the previous records.
- We aimed to identify plant species which, despite their wide application in folk medicine to skin diseases and wounds possess a limited literature data on their phytochemical properties and biological mechanisms of action.
- The main question of our chemical studies was whether we can obtain quantitative and qualitative data on the active substances in the tested plants?
- In the course of our biological research, we searched for answers to the following questions:
    - At which dose the tested plant extracts can be used in cellular assays that do not cause a decrease in viability?
    - Do the extracts have antimicrobial and antioxidant effects, and at what extent?

- Do the extracts stimulate the ability of cells to migrate, and at what extent?
- Can it be assessed by the changes in signaling protein expression which pathways are affected by the plant extracts to augment their biological effects?

## III. MATERIALS AND METHODS

### III.1. Cell cultures

Our studies were performed on adherent **A549-GFP** lung carcinoma cell line, on **HepG2** human hepatoma cell line, **HaCaT** normal human keratinocyte cell line and on **3T3** normal mouse embryonic fibroblast cell line. The cells were grown in high glucose (4500 mg/l) DMEM medium (completed with 10% fetal bovine serum (FBS), penicillin-streptomycin antibiotic mixture at 37 ºC in a 5% $CO_2$ thermostat)).

### III.2. Multiparametric cytotoxicity test

To establish the new method, A549 cells expressing GFP were treated with metabolic inhibitors: sodium fluoride (NaF 2.5; 5; 10; 15 and 20 mM), cycloheximide (CHX 0.02; 0.04; 0, 08; 0.18 and 0.35 nM) and ochratoxin A (OTA 5; 12.5 and 20 μM). Following the treatments, ATP, total nucleic acid (propidium iodide staining), and intracellular total protein contents were determined from the same cell samples.

### III.3. Effect of β-cyclodextrins (CD) on the resazurin fluorescence viability test

In our experiments, we investigated the interaction of three types of beta-CD with resazurin (a viability test molecule which is transformed to resorufin by the action of intracellular reductase enzymes) and resorufin. The CDs were native beta-cyclodextrin (BCD), hydroxypropyl-beta-cyclodextrin (HPBCD), and heptakis-2,6-di-O-methyl-beta-cyclodextrin (DIMEB).

#### III.3.1. Measurement of the molecular interactions of CDs by fluorescence spectroscopy

Resorufin-CD interactions were examined by fluorescence spectroscopy where emission spectra were recorded. CDs (BCD, HPBCD, DIMEB) were added in increasing concentrations (0-1200 μM) to 0.4 μM resorufin.

#### III.3.2. Effect of CDs on cellular cytotoxicity data

The potential interfering effect of CDs was examined on HepG2 cell line by the resazurin assay. Additionally, we also determined total intracellular ATP and protein contents in 96-well cell culture plates. Cells were treated with 0; 0.25; 0.5 and 1 mM CD concentrations and 2 μM resazurin together and separately for 30 and 120 min, respectively. After the treatments, the emission signal of the samples was measured on a plate reader.

### III.4. Ethnobotanical research in Transylvania

Ethnobotanical surveys were conducted in Aldea, Comănești, Ghipeș, Călugăreni, Rareș, Chinușu, Mărtiniș, Petreni, Sânpaul, Orășeni, Bădeni, Locodeni, Lueta, and Crăciunel. We conducted semi-structured interviews with the informants. During the interviews the

drugs of plant, animal and other origin were mentioned by their use in the treatment of various disease groups.

### III. 5. Collection and preparation of extracts of the studied plant species

Based on the performed ethnobotanical surveys, we selected those 4 plant species for which none or only a few phytochemical and pharmacological data could be found in scientific databases. Based on these literature reviews, *A. vulneraria, F. magellanica, F. triphylla* and *L. nummularia* were selected for further studies. For sampling, the ethnomedicinally mentioned plant parts were collected. 50% ethanolic and aqueous extracts were prepared from the dried and ground plant samples, based on their ethnomedicinal use.

### III. 6. Polyphenol analysis with high performance liquid chromatography (HPLC), diode array detector and MS analysis using electrospray ionization

Measurements were performed on an Agilent 1100 HPLC system equipped with a diode array detector (DAD). For the mobile phase we used 0.3% acetic acid solution (A) and methanol (B). The gradient program for the optimized system was as follows: between 0 and 30 min, solution B increased from 5% to 100%, and then it was maintained at 100% for 5 min, with decreasing from 100% to 5% in 1 min. The detection wavelength was set to 280 nm. Each fraction was identified by ESI-MS.

### III.7. Cytotoxicity testing of the of plant extracts with a plate reader

The cytotoxic effect of the extracts on 3T3 and HaCaT cell lines was tested according to the description in subsection III.2. based on our multiparametric method, except for GFP measurement. The concentrations of the extracts used were as follows: *A. vulneraria*: 500-2500 μg/mL (ethanolic extract) and 4000-8000 μg/mL (aqueous extract); *F. magellanica* and *F. triphylla*: 50-800 μg/mL (ethanolic extract) and 120-1000 μg/mL (aqueous extract); *L. nummularia*: 250-1500 μg/mL (ethanolic extract) and 3000-7000 μg/mL (aqueous extract).

### III.8. Cytotoxicity measurement of plant extracts with a flow cytometer

Due to their more strong biological effect, only ethanolic extracts were tested: *A. vulneraria* 50; 100 and 200 μg/mL, *F. magellanica* and *F. triphylla* 2.5; 5 and 10 μg/mL, while L. nummularia 10; 25 and 50 μg/mL concentrations. The experiments were performed on a BD FACS Canto II flow cytometer. Discrimination between early, late apoptotic and necrotic cells, was performed using Annexin V and 7AAD (7-aminoactinomycin D) dyes.

### III.9. Investigation of the antimicrobial effect of plant extracts

The bacterial strains used were *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes)* and *Pseudomonas aeruginosa* (*P. aeruginosa*). The $MIC_{80}$ values of the aqueous and the ethanolic extracts of the plants, i.e. the concentration that inhibits the growth of the given microorganism by 80%, were determined by microdilution technique. The optical density of the treated bacterial suspensions at 580 nm was used to calculate the $MIC_{80.}$

### III.10. Non-enzymatic total antioxidant capacity (TAC) studies on a plate reader

#### III.10.1. Oxygen Radical Absorption Capacity – ORAC

The method is based on the kinetic observation of the decrease in the light emission of 400 nM fluorescein solution in a 96-well plate oxidized by 400 mM AAPH (2,2'-azobis (2-amidinopropane). The measurement was monitored on a plate reader at 37 °C and the antioxidant capacity calculation was based on the area under the emission curves (AUC).

#### III.10.2. Enhanced chemiluminescence method - ECL

The assay was developed in white 96-well plates using the luminescence mode of the plate reader. Prior to measurement, detection reagent (0.15 M borate buffer supplemented with 0.45 mM luminol and 1.8 mM para-iodophenol) and 15 μU/mL peroxidase enzyme were mixed. The kinetic measurement was started by automatic injection of $H_2O_2$ (hydrogen-peroxide). The calculation was also done by the luminescence AUC values.

#### III.10.3. Measurement of antioxidant capacity based on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging

The assay procedure in 96-well plates was as follows: 200 μM DPPH solution and 100 mM acetate buffer were added to 50 μL of sample/standard. After 60 min incubation, the absorbance change was measured on a plate reader at 517 nm.

#### III.10.4. Trolox Equivalent Antioxidant Capacity – TEAC

First the $ABTS^{\bullet+}$ (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) radical was formed as follows: $K_2S_2O_8$ - potassium persulfate (125 mM) and ABTS (7 mM) were mixed in a ratio of 49:1. The solution was allowed to stand in the dark at room temperature for 12 hours. 20 μL of diluted plant sample and 80 μL of $ABTS^{\bullet+}$ solution were added to 96-well plates. After 20 min of incubation the absorbance change was measured on a plate reader at 734 nm.

#### III.10.5. Analysis of TAC values

In case of all 4 methods for measuring total antioxidant capacity, calibration lines obtained from a dilution series of Trolox standards (water soluble vitamin E derivative) were used. For the ORAC and ECL tests, results were expressed in Trolox equivalent (TE)/g of dry matter. For the DPPH and TEAC methods, the 50% radical neutralizing effect ($IC_{50}$) of the plant extracts was expressed, i.e. that concentration in μg/mL which reduces the absorbance of the DDPH or ABTS radical by 50%. Thus the obtained values for the TAC methods were not directly comparable with each other.

### III.11. Measurement of intracellular antioxidant capacity

The applied dichlorofluorescein diacetate (DCFH-DA) and dihydrorhodamine123 (DHR123) tests were performed in 96-well plates. HaCaT and 3T3 cells were incubated with 50 μM DCFH-DA or 10 μM DHR123 and with plant extracts. Kinetic measurement began after the addition of 1 mM AAPH. Fluorescence intensities were monitored on a plate reader. The radical neutralizing effect of the extracts was expressed as the percentage of inhibition ($IC_{50}$).

### III.12. *In vitro* study on cell migration ("wound healing test")

The migration ability of HaCaT and 3T3 cells treated with plant extracts was examined in 24-well plates where 500 μm wide cell-free areas were established in the wells. The closure rate of these "wounds" were monitored and quantified. Cells were treated with subtoxic concentrations of plant extracts: for *A. vulneraria* 50; 100 and 200 μg/mL, for *F. magellanica* and *F. triphylla* 2.5, 5 and 10 μg/mL, for *L. nummularia* 10; 25 and 50 μg/mL and the positive control was PDGF-BB growth factor at a concentration of 15 ng/mL. Cell migration was examined by taking a photograph in every 4 hour for 24 h using a phase-contrast thermostat microscope (JuLi Stage, NanoEnTek) with time-laps imaging technique. When evaluating the results, the closure rate (in %) of the cell-free area was determined compared to that of the untreated control. A sum of the closure curve replicates was generated for each extract and the areas under the curve (AUC) were calculated.

### III.13. Protein expression studies

Based on the results of our previous measurements, only the 50% ethanolic extracts of *F. magellanica* and *F. triphylla* were tested on HaCaT cell line at concentrations of 2.5; 5 and 10 μg/mL. The treated cells were lysed with a solution containing protease and phosphatase inhibitors followed by total protein assay, then 15 μg of protein from the lysates was denatured with SDS and beta-mercaptoethanol and analyzed by western blot. For investigation of the antioxidant cell signaling pathways superoxide dismutase-2 (SOD2) and catalase (CAT) antibodies were used. For the anti-inflammatory processes COX2 antibody, while for studying the signaling routes in migration, proliferation and in overall cellular survival, antibodies against protein kinase B (Akt1), p38, extracellularly mediated kinase 1, 2 (Erk1 / 2) were applied. We also tested their phosphorylated forms. The enhanced chemiluminescence (ECL) signal obtained during visualization of the immune complexes was compared to the signal of β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading controls. The ECL was detected with a G: Box Chemi XX6 System and densitometry was performed with its built in software.

### III.14. Statistical analysis

Our data were evaluated using the SPSS statistical analysis software version 22 (IBM Corporation). Statistical evaluation was performed by an independent sample T-test to compare aqueous and ethanolic extracts, while one-way ANOVA was used to compare treated and control groups. To examine the differences between the TAC methods, Principal Component Analysis (PCA) was performed. $P<0.05$ values were considered to be statistically significant.

## IV. RESULTS AND DISCUSSION

### IV.1. Development of a novel multiparametric cytotoxicity plate reader method

The new method is based on a lysis reagent (boric acid/NaOH buffer at alkaline pH of 9.2) supplemented with Triton-X-100 and EDTA. Using the buffer, all adherent cells are lysed at identical speed and the reagent stabilizes the intracellular protein and ATP contents for at least 30 minutes.

After 4 h of NaF incubation, the measured intracellular parameters were significantly reduced in a dose-dependent manner. The most striking difference was observed in ATP levels, as the highest NaF concentration (20 mM) almost completely depleted the ATP content (2%, compared to untreated control cells). This is most probably due to the fact that NaF is a common enzyme inhibitor compound. This also includes the inhibition of some glycolytic enzymes, which largely contribute to ATP production, especially in tumor cells where the energy production route depends on glycolysis at a high extent.

After 24 hours of OTA treatment, a dose-dependent decrease was observed for each of the intracellular factors tested. The highest OTA concentration (20 μM) reduced GFP levels to 82%, while total intracellular protein decreased to 75% compared to the control. OTA mediates its complex effects partially through the induction of oxidative stress and energy depletion.

The effect of CHX was examined at two different incubation times (4 hours and 24 hours, respectively). 24-hour treatment reduced GFP levels to 40%, total protein content to 50%, and PI signal to 60%. CHX is a widely used compound to inhibit protein synthesis in eukaryotes. Treatment for 4 hours increased ATP levels, while incubation for 24 hours caused a significant decrease. The reason for the opposite findings is presumably due to the apoptosis-inducing effect of CHX through inhibition of protein synthesis. The apoptotic steps require energy (ATP) to perform the downstream processes smoothly. Furthermore, protein synthesis is a highly energy-dependent intracellular procedure therefore, if CHX inhibits the pathways of protein synthesis, this can result in the accumulation of unused ATP in the cells.

As a general observation in our studies, we found that regardless of the treatments the GFP signal intensity changed parallel with the cell number and the cellular protein content.

## IV.2. Investigation of resorufin-CD interactions

### IV.2.1. Fluorescence spectroscopy

CDs increased the fluorescence intensity of resorufin in a concentration-dependent manner, and a shift in the maximum of the emission wavelength was observed. The strongest increase in intensity was observed for DIMEB. Since the CDs alone did not show fluorescence under the conditions used, our observations confirm the formation of resorufin-CD complexes. Considering the strong fluorescence increase of resorufin in the presence of CDs, the resorufin-CD interaction may interfere with the results of the resazurin-based cellular viability studies. This phenomenon may be most significant in the case of DIMEB, as methylated CDs enter the intracellular space by endocytosis (whereas BCD and HPBCD are unable to do so).

### IV.2.2. Resazurin-based cellular viability measurements on a plate reader

The intracellular effect of CDs was examined after two incubation periods (30 min and 120 min). HepG2 cells were treated simultaneously with CD and resazurin (co-treatment). Furthermore, since CDs can affect the viability of HepG2 cells, we also examined how the separate treatment of the cells (first with CD and then with resazurin after removal of the CD) can affect their viability.

Intracellular ATP levels and total protein contents from separate CD and consecutive resazurin treatments were compared. Based on both ATP and protein data, a CD-induced

viability decrease was observed. In the presence of higher DIMEB concentrations, a stronger decrease in viability was found. This may explain why the temporary signal increase observed for 0.25 mM DIMEB after 120 min of incubation decreases at 0.5 and 1 mM DIMEB concentrations.

Our results indicate that various CDs are able to influence the results of the resazurin viability test in a complex way. Thus, in experiments using CDs, the results of the resazurin-based cell viability assay do not appear to be reliable.

**IV.3. Results of ethnobotanical collections**

Within the frame of our work in Transylvanian ethnomedicine, among the selected plants the highest number of local names has been described in the case of *L. nummularia* (*fillérfű, piculavirág, íneresztőfű, pillérfű, fillérlapi, folyóka*). *A. vulneraria* was known as *medvetalpa* or *szipókavirág.* Because *F. magellanica* and *F. triphylla* are not named separately by the local people, the name fuchsia was commonly used for each species in the genus. Among the used plant parts, the aerial parts and the leaves were most frequently mentioned, that were applied externally to treat skin diseases, mainly as a compress. In the case of fuchsia species, the leaves are used for "*szökés*" which means blue-black bleeding, deep abscess, or virulent acne.

**IV.4. Semi-quantitative analysis of phenolic compounds in plant extracts by LC-DAD-ESI-MS/MS method**

A total of 82 components were separated in the samples, including gallic acid derivatives, hydroxycinnamic acid derivatives, and flavonoid glycosides, moreover 8 anthocyanin compounds were detected in *F. magellanica* and *F. triphylla* extracts.

Flavonol glycosides are predominant in the *A. vulneraria* extracts. The highly typical aglycones were kaempferol and quercetin, as well as methylated forms of isorhamnetin and rhamnocitrine aglycones. Also, the aqueous extract contained a wide variety of caffeic, coumarin and ferulic acid derivatives.

In addition to the common flavonol derivatives, fuchsia extracts showed unusual quercetin galloyl hexosides and kaempferol galloyl hexoside in large amounts in the *F. magellanica* sample. Anthocyanin derivatives were also dominant in the leaf extracts: peonidin dihexoside isomer was present in both fuchsia samples, while cyanidin dihexoside was found only in the *F. magellanica* extracts.

*L. nummularia* also contained large amounts of flavonol glycosides, however, myricetin glycosides were predominant in the sample. Myricetin 3-O-deoxyhexoside was the major component in both the aqueous and the ethanolic extracts. In the aqueous extract coumarin and ferula glucarate isomers were characteristic.

**IV.5. Results of the plate reader cytotoxicity assays**

A general observation for the examined plant samples was that the ethanolic extracts were more effective in reducing ATP levels, cell numbers, and protein contents in both cell lines in a dose-dependent manner compared to the aqueous extracts of the same plant. However, no significant differences depending on the solvent type were observed in fuchsia species, as both the ethanolic and aqueous extracts showed almost similar toxic effects in the studied cell lines. The explanation of the differences between the solvents could not be

attributed to the ethanol content of the samples, since it was maximized at 1.5%, which does not affect the viability of the cells significantly. Therefore, the toxic effect was due to the higher amounts of active ingredients in the ethanolic extracts. The 3T3 fibroblast cell culture was more sensitive to the exposure of the leaf extracts than HaCaT keratinocytes. A possible explanation for this might be that fibroblasts are located in the dermis, while keratinocytes can be found closer to the epidermis, so they need to be more resistant to the external and potentially damaging factors.

**IV.6. Flow cytometric cytotoxicity results**

Based on the plate reader tests, the presumed subtoxic concentrations of ethanolic extracts of plants were subjected to apoptosis-necrosis assays by Annexin V-7AAD flow cytometry on 3T3 and HaCaT cell lines. The results obtained for the two different cell lines were similar: *A. vulneraria* at a concentration of 200 µg/mL, *F. magellanica* and *F. triphylla* at a concentration of 10 µg/mL and *L. nummularia* at a concentration of 50 µg/mL did not cause any significant apoptotic/necrotic effect, as the majority of the cells remained intact (the proportion of necrotic cell death rate was below 4% in both cell lines).

**IV.7. Determination of MIC values for antimicrobial activity**

Ethanolic extracts of *F. magellanica* and *F. triphylla* can be considered to have "strong" antimicrobial properties against *S. aureus, B. subtilis, S. pyogenes,* and *P. aeruginosa*, as their $MIC_{80}$ values ranged from 5 to 60 µg/mL compared to erythromycin positive control with $MIC_{80}$ values ranging from 0.1 to 42 µg/mL. Ethanolic extracts of *A. vulneraria* and *L. nummularia* showed "moderate" antimicrobial activity against *B. subtilis* and *S. pyogenes*, and *A. vulneraria* had a "weak" activity against *S. aureus*. It was an interesting observation that only the aqueous extracts of fuchsia species were effective against the tested bacterial strains, including *S. aureus, B. subtilis,* and *S. pyogenes* ($MIC_{80}$ values: between 17-65 µg/mL). None of the tested plant extracts showed inhibitory effect against *E. coli.*

We did not find any previous data on the antimicrobial effects of the studied plant species, therefore we compared our results with literature sources based on their active ingredients. In the fuchsia species their anthocyanin content might be responsible for the outstanding antimicrobial activity. Furthermore, fuchsia species also contained hydroxybenzoic acids, which are known as potent antimicrobial compounds. The main flavonoids in the leaf extracts of *L. nummularia* were myricetin derivatives knowing to have a strong antibacterial effect however, in our study we found only a weaker activity.

**IV.8. Results of total antioxidant capacity studies**

In general, the ethanolic extracts of the selected plants showed higher antioxidant capacities than their corresponding aqueous extracts. In case of all the 4 methods used, fuchsia species showed the highest TAC values systematically *(F. triphylla > F. magellanica > L. nummularia > A. vulneraria)* (Fig. 1*)*.

The high TAC values of fuchsia species may be primarily explained by their large anthocyanin contents because they are known as strong antioxidant compounds. Based on literature survey regarding TAC measurements (TEAC, ORAC), the antioxidant capacity of anthocyanins seems to be 3-6 times higher than that of the Trolox standard at the same

concentrations. Considering these findings anthocyanins appear to be more effective than the classical antioxidants.

In addition to earlier reports of other researchers, we also found correlations between TEAC and DPPH, as well as between ORAC and ECL methods. The data of ethanolic extracts of *F. magellanica* and *F. triphylla* correlated much more with each other than the corresponding aqueous extracts. The differences between antioxidant methods can be explained by their chemical background. Chemically, the ECL and ORAC tests are based on a hydrogen atom transfer (HAT) mechanism, while the TEAC and DPPH methods are single electron transfer (SET) reactions.

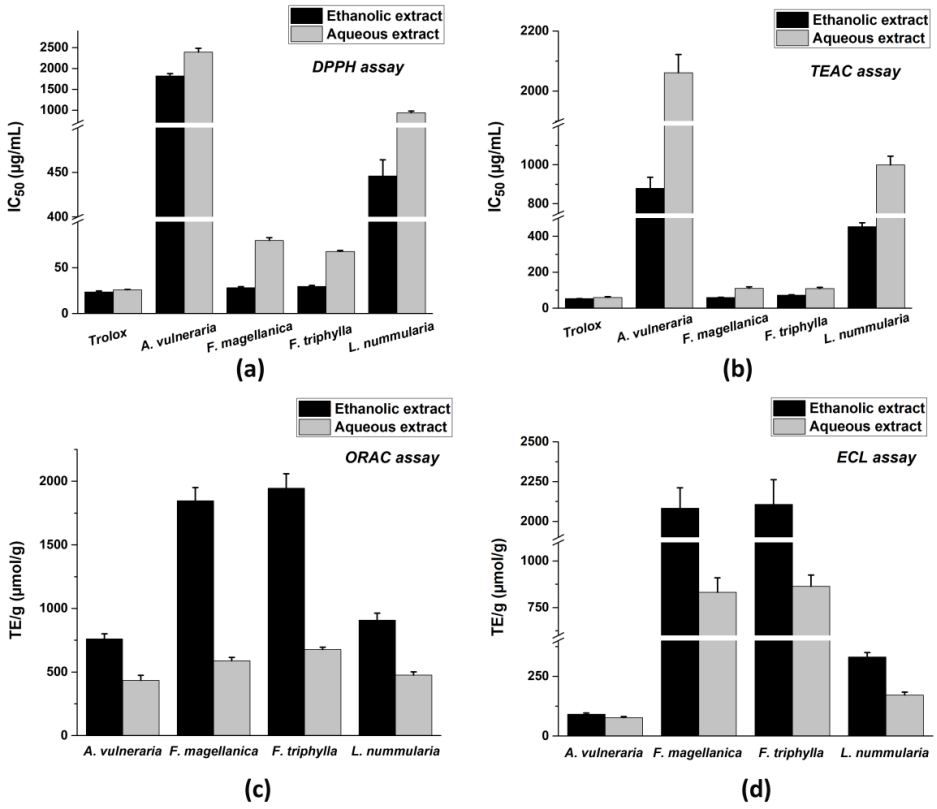


***Figure 1.*** *Total antioxidant capacity (TAC) of 50% (v/v) ethanolic and aqueous extracts of four selected medicinal plants (A. vulneraria, F. magellanica, F. triphylla and L. nummularia) measured by different spectroscopic methods: (**a**) DPPH assay; (**b**) TEAC assay; (**c**) ORAC assay and (**d**) ECL assay. Mean ± SD of 5 independent experiments, each in 3 replicates. The aqueous and ethanolic extracts were compared with t-probe (**p < 0.01, ***p < 0.001)*

## IV.9. Results of intracellular ROS measurements

In the cell-based antioxidant activity tests we got similar results compared to the previous TAC assays.

Ethanolic and aqueous extracts of the majority of the studied species strongly inhibited the fluorescent signal of oxidized DCF and rhodamine. The calculated 50% inhibitory concentrations are seen in Fig. 2 and 3. The strongest inhibitory effect of DCF and rhodamine fluorescence was seen in the case of ethanolic and aqueous extracts of fuchsia species in both cell lines compared to that of the other plant extracts (Fig. 2 and 3). In the case of DCF, neither the aqueous nor the ethanolic extract of *A. vulneraria* showed antioxidant activity in

the 3T3 cell line, and only the ethanolic extract had measurable antioxidant properties in HaCaT cell cultures (Fig. 2).

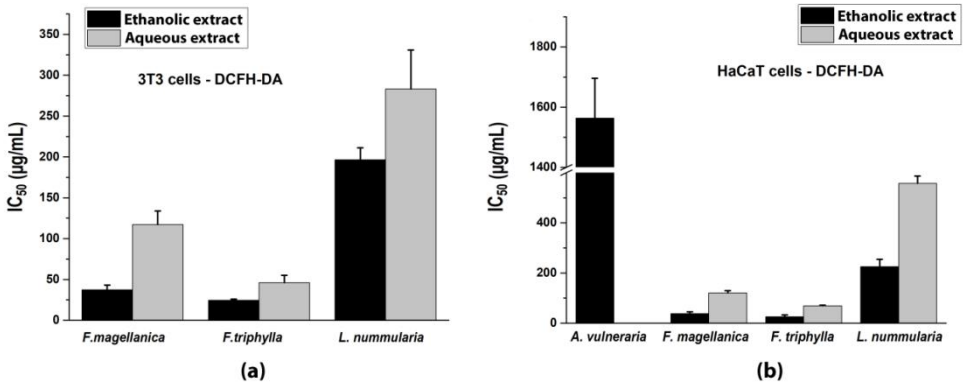


***Figure 2.*** *Intracellular antioxidant capacities of studied plant extracts with DCFH-DA staining in 3T3 fibroblast cells* ***(a)*** *and in HaCaT keratinocyte cells* ***(b)****. Mean ± SD of five independent experiments, n = 5×4 replicates for each concentration. The aqueous and ethanolic extracts were compared with t-probe (**p < 0.01, ***p < 0.001)*

Similar to the DCF signal, extracts of fuchsia species (both ethanolic and aqueous) inhibited the fluorescence intensity of rhodamine to the greatest extent. In the case of DHR123 treatment, although the ethanolic and aqueous extracts of *A. vulneraria* showed quantifiable results for both cell lines however, the aqueous fraction had only a weak effect (Fig. 3).

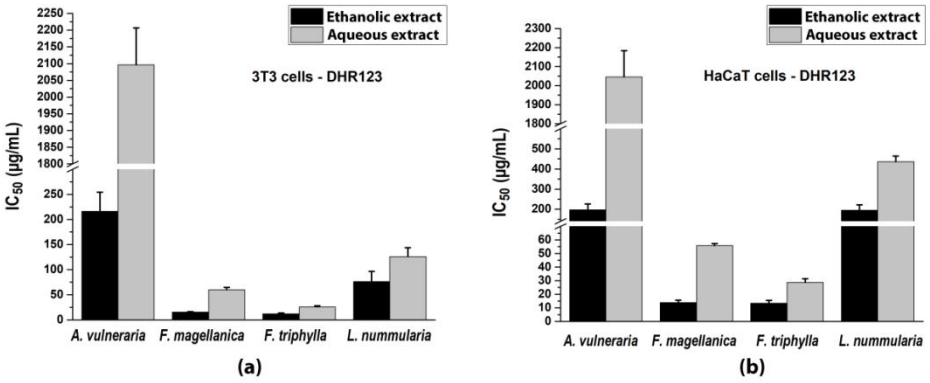


***Figure 3.*** *Intracellular antioxidant capacities of studied plant extracts with DHR123 (a) on 3T3 fibroblast cells* ***(a)*** *and on HaCaT cell culture* ***(b)****. Mean ± SD of 5 independent experiments, n = 5 × 4 replicates for each concentration. The aqueous and ethanolic extracts were compared with t-probe (**p < 0.01, ***p < 0.001)*

### IV.10. Results of the *in vitro* migration test

The positive control PDGF-BB at 15 ng/mL had a stimulating effect on the migration of both cell lines (117.05 ± 6.72% ($p<0.01$)) for 3T3 cells and 115.16 ± 8.26% ($p<0.01$) for HaCaT cells when compared to the untreated samples (Fig. 4). For all studied plants, it was a remarkable observation that the less concentrated extracts caused the more effective stimulation in cell migration compared to the higher extract concentrations.

Of the four plants examined, *A. vulneraria* exerted the mildest stimulating effect, as it caused an increase of 102.08 ± 11.18% in 3T3 cells and 108.57 ± 10.92% in HaCaT cells,

respectively, at a concentration of 50 µg/mL. *L. nummularia* showed no significant migration effect on the tested cell lines. An increased migration and closure rates were observed in *F. magellanica* and *F. triphylla* treated cells compared to the untreated cells. Among the 2 fuchsia species, *F. magellanica* had the strongest migration-stimulating effect on both cell lines, with a closure rate of 120.2 ± 10.17% ($p<0.01$) on 3T3 cells and 114.61 ± 3.72% ($p<0.01$) on HaCaT cells at 2.5 µg/mL concentrations.

For our plants, we have not found yet any previous scientific publications studying their biological effects, especially on cell migration. At the same time, literature data demonstrate that high levels of anthocyanins of fuchsia species stimulate the wound healing process by enhancing fibroblast and keratinocyte migration. Furthermore, hydroxybenzoic acid derivatives measured in fuchsia species (e.g. gallic acid in *F. magellanica* extract, or ellagic acid in both fuchsia extracts) increase the migration of keratinocytes and fibroblasts.

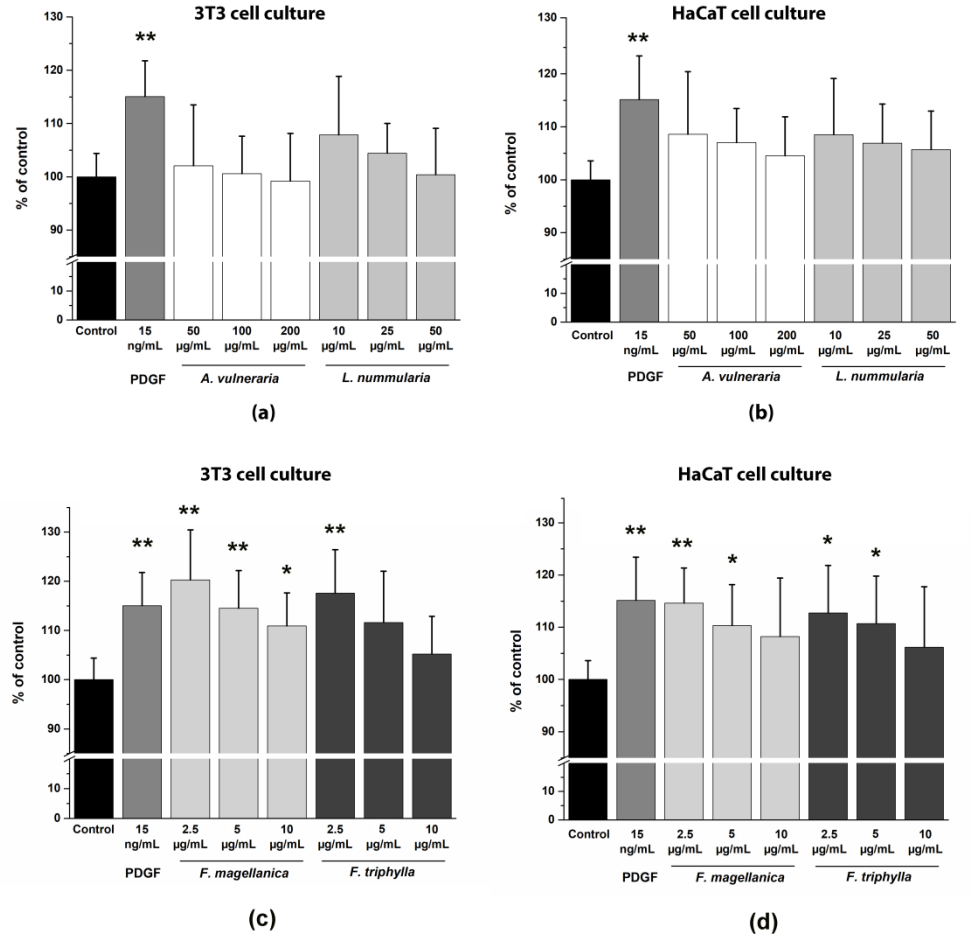


***Figure 4.*** *Comparative analysis by time-lapse imaging of wound closure ability of 50% (v/v) ethanolic extracts of A. vulneraria (**a, b**), L. nummularia (**a, b**), F. magellanica (**c, d**) and F. triphylla (**c, d**) on 3T3 (**a, c**) and HaCaT (**b, d**) monolayer cultures. (Mean ± standard deviation, 3 independent experiments, n = 3 * 3 parallel measurements at all concentrations). Significant change to untreated control, one-way Anova test (*p < 0.05, **p < 0.01)*

## IV.11. Results of signal transduction processes by western blot technique

### IV.11.1. Anti-inflammatory signaling pathway assay at the protein level

Inhibition of COX2 is a key point to investigation of the anti-inflammatory effect. LPS positive control alone caused an increase in expression of COX2 at 173.26 ± 9.92% ($p < 0.05$), whereas LPS and *F. triphylla* co-treatment dose-dependently inhibited COX2 expression (2.5 μg/mL: 162.28 ± 11.11% ($p < 0.05$); 5 μg/mL: 100.80 ± 9.61%; 10 μg/mL: 94.40 ± 12.32%). However, compared to LPS treatment, *F. magellanica* did not reduce but unexpectedly increased the expression of COX2 in a concentration-dependent manner. At the highest concentration of 10 μg/mL, an increase in the expression of 205.95 ± 23.79% ($p < 0.05$) was observed.

As we did not find any previous data of the plant extracts' effects on the studied signal transduction processes we compared our obtained western blot results with the literature data based on the drug composition and contents. It has been shown that besides cyanidin, delphinidin and petunidin can also inhibit COX2 expression, whereas pelargonidin, peonidin, and malvidin cannot inhibit it. Our results are in contradiction with the literature data, as the extract of *F. magellanica* increased the level of COX2 protein in spite of the fact that we could not detect any cyanidin derivative from this plant. Interestingly, a peonidin derivative was isolated from both fuchsia species, which does not inhibit COX2 and cannot be responsible for the different COX2 expression trends obtained for fuchsias. Several anthocyanins could not be accurately identified in the ethanolic extract of *F. triphylla* in the absence of adequate literature sources, which may have decreased COX2 expression.

Another difference between the two Fuchsia species, which may explain the different COX2 expression levels, is their ellagic acid and quercetin galloyl hexoside contents, which occur in four times higher amounts in the ethanolic extract of *F. triphylla* than in the ethanolic extract of *F. magellanica*. Ellagic acid has been shown to inhibit LPS-induced COX2 protein expression in monocytes while it did not affect the COX1 protein. Furthermore, a glycosidic component similar to quercetin galloyl hexoside, quercetin galloyl glucopyranoside, also inhibits COX2 protein levels in macrophages.

### IV.11.2. Signaling study of antioxidant effects at the protein level

Both *F. magellanica* and *F. triphylla* increased the levels of antioxidant CAT and SOD2 proteins compared to the control cells. In the case of CAT the highest concentration of plant extracts (10 μg/mL) increased the protein expression at the largest extent, causing even higher signal levels than $H_2O_2$. While $H_2O_2$ treatment increased the protein levels by 143.03 ± 20.80%, the treatment by *F. magellanica* caused an increase of 165.44 ± 13.10%, and *F. triphylla* 194.24 ± 25.13% ($p<0.05$) in the cells. Fuchsia extracts also had a stronger effect on SOD2 levels than $H_2O_2$ by itself (146.28 ± 15.60%). In the case of *F. magellanica*, the dilute extract with a concentration of 2.5 μg/mL caused an increase in the expression level of 165.29 ± 21.02%, and *F. triphylla* at a concentration of 5 μg/mL an increase of 189.08 ± 13.19% ($p<0.05$) in HaCaT cells compared to the untreated controls.

The change in the level of antioxidant proteins may have been caused by anthocyanins of the plant extracts, which have been shown to increase the expression of antioxidant enzymes (CAT, SOD2, SOD1) at the protein level.

### IV.11.3. Protein expression of signaling pathways related to cellular migration, proliferation and overall cell survival

Both *F. magellanica* and *F. triphylla* ethanolic extracts dose-dependently increased the amount of phosphorylated p38 protein compared to untreated cells. The largest increase was found by the highest concentrations (10 μg/mL) in both plant samples *(F. magellanica* caused an increase of 134.39 ± 22.68%, while *F. triphylla* of 146.61 ± 25.86% compared to the control). Although the p38/MAPK signaling pathway in wound healing is not yet fully elucidated, recent studies suggest that it is involved in directing the migration of keratinocytes to the lesion area. Because Fuchsia extracts increased p38 protein phosphorylation, we hypothesize that keratinocyte migration and wound closure are associated with p38/MAPK pathway activities.

In the study of the Erk/MAPK pathway, *F. magellanica* decreased the phosphorylation of Erk1/2 in a concentration-dependent manner (56.75 ± 2.84% at the highest concentration of 10 μg/mL), whereas *F. triphylla*, although not dose-dependently, increased phosphorylated protein expression (174.80 ± 8.34% ($p<0.05$) in cells treated with the dilute concentration of 2.5 μg/mL. Our results are inconsistent with the literature data, because gallic acid found in *F. magellanica* extract activates the Erk1/2 signaling pathway in both human keratinocytes and fibroblasts however, we observed a decreased expression.

Fuchsia extracts do not contribute to the enhancement of the phosphoinositide 3-kinases (PI3K/Akt) signaling pathway, as they did not increase the level of phosphorylated Akt however, the most diluted extract of *F. magellanica* (2.5 μg/mL, 76.66 ± 3.54% ($p <0.05$)) and the most concentrated extract of *F. triphylla* (10 μg/mL, 81.93 ± 6.37%) decreased it. Anthocyanins can inhibit the PI3K/Akt pathway as it was seen in a cell type typically found in the skin (HUVEC endothelial cells). Furthermore, ellagic acid has been shown to inhibit the migration of human endothelial cells.

## V. SUMMARY, NEW SCIENTIFIC RESULTS

### V.1. Development of a novel method for measuring cytotoxicity and testing the reliability of already existing procedures

1. In order to determine several parameters from the same adherent cell sample, we developed a borate buffered-detergent containing lysis buffer suitable for plate reader experiments, during which 4 parameters could be examined sequentially from one sample (intracellular ATP, GFP, nucleic acid and total protein content). The addition of 20 mM $Na_2EDTA$ to the lysis buffer stabilizes ATP levels for up to at least 30 min after the lysis.
2. We have demonstrated by our experiments that we can quantify and compare the expressed GFP fluorescence intensity with other viability markers using other well established viability parameters performed by plate reader methods. This has been proven by using several metabolic inhibitor treatments where the signal of GFP correlated well with the nucleic acid content of cells (the signal of propidium iodide) and with the total protein content as well.
3. In our experiments, we demonstrated that estimating cell viability by determining a single viability factor (e.g., measuring enzyme activity by the resazurin method) can

be misleading. This is because some β-cyclodextrins (BCD, HPBCD, DIMEB) form stable complexes with resazurin and resorufin, influencing the fluorescent signal of resorufin and presumably the rate of cellular uptake of resazurin. These results indicate that the different CDs can influence the results of the resazurin viability test in a complex way. Thus, in experiments that also use CDs, the reliability of resazurin-based studies appears to be highly questionable.

**V.2. Investigation of plant species selected based on ethnobotanical collections**

4. Based on the results of ethnopharmacobotanical collections, it can be highlighted that the traditional knowledge related to the local use of herbs plays an important role in Transylvanian ethnomedicine, mainly among the elderly generations. For the selected 4 plant species (*Anthyllis vulneraria, Fuchsia magellanica, Fuchsia triphylla, Lysimachia nummularia*), we did not find any preliminary studies on their biological effects, especially in wound healing.
5. Flavonol glycosides are predominant in the *A. vulneraria* sample. In contrast, fuchsia species contain, in addition to common flavonol compounds, various cinnamic acid and benzoic acid derivatives, such as caffeic acid, ellagic acid, and gallic acid derivatives, kaempferol and quercetin galloyl glycosides. Among them, ellagic acid and quercetin galloyl hexoside are present in four times greater amounts in the ethanolic extract of *F. triphylla* than in the ethanolic extract of *F. magellanica*. In the leaves of *F. magellanica* and *F. triphylla*, several anthocyanin components are also present, such as cyanidin and peonidin derivatives. In the leaf extracts of *L. nummularia*, the major flavonoids were myricetin derivatives.
6. The ethanolic extracts of *F. magellanica* and *F. triphylla* did not decrease cellular viability below 50 μg/mL, while their aqueous extracts' limits were 120 μg/mL. The ethanolic extract of *L. nummularia* could be used at a maximum concentration of 250 μg/mL, while the aqueous extract at a concentration of 3000 μg/mL did not cause significant cell-damaging effects. The least cytotoxic plant was *A. vulneraria*, as its ethanolic extract at a concentration of 500 μg/mL and its aqueous extract at a dose of 4000 μg/mL did not decrease the viability of 3T3 and HaCaT cells.
   Biological effects of ethanolic extracts of the plants were also examined on a flow cytometer. Plant extracts did not induce cytotoxic effects up to: *F. magellanica* and *F. triphylla* 10 μg/mL, *L. nummularia* 50 μg/mL and *A. vulneraria* 200 μg/mL.
7. Among the tested 4 plants, the extracts of fuchsia species showed a significant antimicrobial activity against *P. aeruginosa, S. aureus, B. subtilis*, and *S. pyogenes* strains. The weakest antimicrobial effect was measured in the extracts of *A. vulneraria*. None of the tested plant extracts showed inhibitory activity in *E. coli*.
8. Both fuchsia species showed the highest TAC values, and intracellular ROS neutralizing effect among the studied species (*F. triphylla* > *F. magellanica* > *L. nummularia* > *A. vulneraria*), in this order.
9. Among our analyzed plants, *F. magellanica* and *F. triphylla* exerted a stimulatory effect on cell migration similar to or even at a higher extent than PDGF-BB did in 3T3 and HaCaT cell lines.

10. The inhibition of the COX2 enzyme was observed only in the case of *F. triphylla* extract, which reduced its expression level close to the control value, while *F. magellanica* dose-dependently increased COX2 expression, exceeding the effect of LPS positive control.

    In the study of the two antioxidant enzymes (CAT and SOD2), the ethanolic extracts of both fuchsia species stimulated their expression. A dose-dependent increase was observed for CAT protein.

    Extracts of fuchsias also regulate two pathways of the MAPK cascade, on the one hand through phosphorylation of p38 protein and on the other, through phosphorylation of Erk1/2 proteins. Interestingly, in the case of p38 both extracts increased the level of the phosphorylated protein. However, *F. magellanica* decreased the Erk1/2 level, while *F. triphylla* increased it. The extracts of the tested fuchsia species are not involved in enhancing the PI3K/Akt signaling pathway.

In general, among the selected 4 plant species, *F. magellanica* and *F. triphylla* show outstanding biological effects, mainly through their anthocyanin and phenolic acid contents, which may be responsible for their effective role in our wound healing model.

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## VII. LIST OF PUBLICATIONS

### *Articles related to this thesis*

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**Cumulative impact factor related to my thesis: 12.432**

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