

„VICTOR BABEȘ” UNIVERSITY OF MEDICINE
AND PHARMACY OF TIMIȘOARA
THE FACULTY OF PHARMACY
Department of Pharmacognosy

GHIȚU A. ALEXANDRA-MĂLINA



ABSTRACT

Scientific Coordinator
Prof. Dr. Farm. DANCIU CORINA

Timișoara
2021

**„VICTOR BABEȘ” UNIVERSITY OF MEDICINE
AND PHARMACY OF TIMIȘOARA
THE FACULTY OF PHARMACY
Department of Pharmacognosy**

GHIȚU A. ALEXANDRA-MĂLINA



ABSTRACT

***IN VITRO* EVALUATION OF THE NATURAL BIOACTIVE
COMPOUND APIGENIN ON EXPERIMENTAL MODELS OF
MALIGNANT MELANOMA: ANTIPROLIFERATIVE,
PRO-APOPTOTIC, ANTIANGIOGENETIC AND
IMMUNOMODULATORY POTENTIAL**

Scientific Coordinator
Prof. Dr. Farm. DANCIU CORINA

**Timișoara
2021**

IN VITRO EVALUATION OF THE NATURAL BIOACTIVE COMPOUND APIGENIN ON EXPERIMENTAL MODELS OF MALIGNANT MELANOMA: ANTIPROLIFERATIVE, PRO-APOPTOTIC, ANTIANGIOGENETIC AND IMMUNOMODULATORY POTENTIAL

I. INTRODUCTION. AIMS OF THE STUDY.

Phytotherapy is one of the current research topics for the 21st century, numerous studies in the literature attesting to the therapeutic properties of compounds from vegetal origin (1). The latest statistics show that over 60% of current medication is sourced from natural compounds, the effectiveness of these substances being proven both for the treatment and prevention of various diseases. 83% of the new active molecules admitted in the therapeutic management of cancer are derived from bioactive compounds (2), (3).

According to the latest WHO statistics, cancer is one of the leading causes of death worldwide (10 million deaths) and cardiovascular disease (17.9 million deaths), chronic respiratory disease (3.8 million deaths) and diabetes (1.6 million deaths). deaths) fall into the category of the most common causes of death, along with cancer (4).

The purpose of anticancer treatments is to ensure the healing, or to obtain new palliative treatments against this pathology. Recent research aims to develop a strategy to eradicate this disease, which acts targeted on cancer cells, with the aim of stimulating antiproliferative and / or pro-apoptotic and / or antiangiogenetic processes. Developing treatment methods target the tumor microenvironment, with the activation / inhibition of molecular targets that are key factors in the evolution, progression and resistance of malignant cells (5).

The most common types of skin cancer are: basal cell carcinoma (the most common, but also the least dangerous); squamous cell carcinoma (has a high ability to spread in the body and high risk of superinfection) actinic keratoses (represent precancerous lesions) and melanoma. Less common may be: Merkel cell carcinoma, Kaposi's sarcoma and sebaceous gland carcinoma. (6) Melanoma is the most aggressive form of cancer, due to its high metastatic capacity (7).

One of the most common inconveniences in cancer therapy is the appearance of numerous side effects, chemotherapy not being sufficiently selective in eradicating the malignant process. At the same time, the rapidity of the installation of drug resistance contributes to the failure of treatment and to the increase in the number of deaths due to this disease. (8), (9).

Among the natural compounds with anticancer properties (preclinical/ clinical experimental studies), respectively their derivatives can be mentioned: resveratrol, curcumin, quercetin, kaempferol, apigenin, daidzein, miricetin, genistein, lignin, hydroxycinnamic and hydroxybenzoic acids, epigallocatechins, sulforaphane, β -sitosterol, campesterol, stigmasterol, oleanolic acid, ursolic acid, artemisinin, amentoflavone, carnosol, vinca-alkaloids, taxanes, epipodophyllotoxins, irinotecan, topotecan, anthracyclines, berberine, amentoflavone, eupatilin, lycopene, lutein, fucoxanthin, canthanxanthin, hypericin, gingerol (3), (10).

Some examples of phytochemicals with *in vitro* activity on different cell lines include: artemisinin (*Artemisia annua* L.) inhibits the multiplication of breast cancer cell lines [MDA-MB-231 and MCF-7], pancreatic cancer [Mia PaCa-2], prostate cancer [PC-3], lung cancer [A459] (11); quercetin (*Allium cepa* L.) has antimelanoma effect on murine melanoma cell line [B16 – BL6]; amentoflavone (*Biophytum sensitivum* L.) inhibits the metastatic process on murine melanoma cell line [B16F10]; fucoxanthin (*Fucus luminaria* L.) inhibits the development of melanoma cell lines [SK-MEL-

28 and B16F10]; daidzein and genistein (*Glycine max* L.) have a synergistic effect on skin line carcinoma [A431] (2); Epigallocatechin gallate (EGCG) (*Camellia sinensis* L.) exerts a cytotoxic effect on breast cancer cell lines [MDA-MB-231 and MCF-7] (12). Polysaccharides (PGL) from *Ganoderma lucidum* L. inhibits the development of murine melanoma [B16F10] and colon cancer [HCT-116, SW 480 and AH-130], (13), berberine (*Tinospora cordifolia* L.) is active on human cervical carcinoma cell line [SiHa] and oral cavity carcinoma [Kb] (14); boldines (*Peumus boldus* L.) inhibit the development of breast cancer cell lines [MCF-7 and MDA-MB-231]; thymokinone (*Nigella sativa* L.) suppresses tumor development of human lung cancer cell line [A549] (2); (*Solanum lycopersicum* L.) has anticancer action on various prostate cancer cell lines [LNCaP, CaP and PC3] (15), gingerol (*Zingiber officinale* L.) acts antiproliferative on lung cancer cell lines [A549], gastric [HGC], breast [MDA-MB-231] (16); ursolic acid (*Rosmarinus officinalis* L.) decreases tumor angiogenesis on melanoma cell line [SK-MEL-2] (17); curcumin (*Curcuma longa* L.) inhibits the development of human melanoma cell line [A375 and C8161] (18), resveratrol (*Vitis vinifera* L.) suppresses the development of colorectal cancer cell line [LoVo], breast cancer cell line [MCF-7], ovarian cancer cell line [CAOV-3 and OVCAR-3], cervical cancer cell line [HeLa and SiHa], gastric cancer cell line [SGC -7901] (19).

Antitumor properties of natural compounds have been attested by numerous *in vivo* studies. Among the natural molecules with therapeutic effect on animal experimental model, there can be listed: xenograft berberine (*Coptis chinensis* L.) [human gastric cancer cell line BGC-823 and SGC7901 inoculated into albino mouse strain] (20), sulforaphane (*Brassica oleracea* L.) on experimental animal model [murine melanoma cell line B16F-10 inoculated in mouse strain C57BL / 6], apigenin (*Matricaria chamomilla* L.) on experimental animal model [LNCaP prostate cancer cell line inoculated in the strain of TRAMP C57BL / TGN mice] (21), gingerol (*Zingiber officinalis* L.) on experimental animal model [HGC gastric cancer cell line inoculated on albino athymic mice] (16), ursolic acid (*Rosmarinus officinalis* L.) on CAM model [SK-MEL-2 human melanoma cell line inoculated on CAM model] (17), curcumin (*Curcuma longa* L.) on experimental animal model [A375 human melanoma cell line inoculated in BALB / c albino mouse strain] (18).

Certain natural compounds have also been included in clinical trials. There can be mentioned some examples: homoharingtonin (*Cephalotrox fortune* L.) is approved for the treatment of chronic myeloid leukemia. The first combination antigen-anticancer treatment is IgG4 kappa and calicheamicin 101 (*Micromonospora echinospora* L.) studied in non-Hodgkin's lymphoma. The compound IMG-901 (HuN901-DM1) 235, a conjugate of maytansine (*Actinosynnema pretiosum* L.) and the antibody huN901, has also been evaluated for the treatment of multiple myeloma and lung cancer (22). In the review made by Chinembiri *et al.* there are mentioned clinical studies showing the effect of curcumin in the colorectal cancer, prostate cancer, breast, pancreatic and biliary cancer. It is known that brentuximab vedotin has been approved for the treatment of anaplastic lymphoma and Hodgkin's lymphoma since 2011 (10), and the compounds of marine origin (aplidine (plitidepsin) - non-Hodgkin's lymphoma; bryostatin-1- ovarian cancer, salinosporamide A-multiple myeloma, solid tumors, lymphomas and zalypsis-Ewing sarcoma) are used in treatment regimens for various cancers (23).

Many compounds are currently included in various clinical trials. Among which: genistein for prostate cancer (24); desmodium (a mixture of triterpenes, saponins, alkaloids, polyphenols, flavonoids and tryptamine derivatives for breast cancer (25); quercetin for prostate cancer (26); isoquercetin for kidney cancer (27); resveratrol for colon cancer and gastrointestinal tumors (28), (29); phenols (hydroxytyrosol, procyanidins, hesperidin, eriocitrin, curcumin, resveratrol, punicalagin, ellagic acid), methylxanthines (theobromine and caffeine) for breast cancer (30); epirubicin and vinorelbine for breast cancer (31); vincristine for chronic lymphocytic leukemia (32); irinotecan (semisynthetic derivative of camptothecin) for colorectal and metastatic cancer (33).

The process of angiogenesis is closely related to the evolution of cancer processes, and among the species with anti-angiogenic qualities can be specified the following: *Vitis vinifera* L. (resveratrol), *Camellia sinensis* L. (epigallocatechin-3-gallate), *Silybum marianum* L. (silymarin), *Genista tinctoria* L. (genistein) (34), *Taxus brevifolia* L. (taxol) (35), *Curcuma longa* L. (curcumin) (36), *Panax ginseng* L.,

(ginsenosid-Rg3) (37), *Magnolia officinalis* L (magnosaline) (38), *Rabdosia rubescens* L. (oridonine) (39), *Scutellaria baicalensis* L. (baicalin), *Allium hirtifolium* L., (quercitin), *Artemisia annua* L. (artemisinin) (40), *Zingiber officinalis* L. (gingerol) (41), *Glycyrrhiza glabra* L. (glabridin, isoliquiritigenin) (42), *Glycine max* L., (genistein, daidzein) (43).

The main objective of this PhD thesis is to elucidate the complex mechanism of anti-melanoma action of flavone apigenin on the A375 human melanoma cell line, by various *in vitro* tests, while determining the effect of apigenin on dendritic cells and implicitly on the immune system. Secondary, tests were performed on other human melanoma (SK-MEL-24) and murine (B16A5) cell lines, respectively on the human cervical cancer cell line (HeLa).

Flavonoids are among the most significant classes of pharmacologically active compounds. They are present in the chemical composition of many medicinal plants, fruits and vegetables and are subclassified according to: the number / place of grafting of the phenyl radical, the presence / absence of the double bond at position 2-3, the nature of the substituents, the number nuclei in the molecule in: flavones, flavonols, flavanones, flavanonols, isoflavones, biflavonoids, neoflavonoids, chalcones, aurones, catechins and anthocyanins (44).

Apigenin belongs to the subclass of flavones, and from a pharmacological point of view it is characterized by a variety of properties, among which we can mention: vasoprotective, anti-inflammatory, hypotensive, antiviral, antibacterial effects, and in the last few years, specialized studies have evaluated and confirmed various *in vitro* and *in vivo* studies, its antitumor effects on different cell lines (45),(1).

Chamomile - *Matricaria chamomilla* L. (synonym *Matricaria recutita* L.) is highlighted as one of the most important sources of apigenin. It belongs to the *Asteraceae* family and has an apigenin content in the range of 5010-5320 mg / 100 g dried product. (46), (47). Remarkable quantities can also be found in: *Petroselinum crispum* L. (1200-1350 mg/100 g dried product) (47), *Apium graveolens* L (108 mg/100 g dried product) (48), *Rosmarinus officinalis* L (43.8 mg/100 g dried product) (47), *Camelia sinensis* L (41.4- 175.7 mg/100 g dried product), *Origanum vulgare* L. (15.6- 19.4 mg/100 g dried product) (47), *Piper nigrum* L (4.98 mg/100 g dried product) (48), *Capsicum annum* L (3.5 mg/100 g dried product) (49).

II. MATERIALS AND METHODS

II.1. Study I: Evaluation of the antiproliferative, proapoptotic, antiangiogenetic and immunomodulatory potential of apigenin on A375 human melanoma cell line.

II.1.1. The evaluation of the antiproliferative potential of apigenin (Api) on A375 human melanoma cell line was performed using the MTT method. This type of assay it is widely used for the determination of cell viability and consists of the reduction of the yellow tetrazolium salt [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide or MTT] to purple formazan crystals. The process is performed by viable cells. (50).

II.1.2. Cell cycle analysis by flow cytometry following *in vitro* Api incubation for A375 human melanoma cell line. The principle of this method is based on the simultaneous determination of the physical and chemical parameters of each analyzed cell in a system, at the same time performing the sorting of the cells for their use in subsequent tests. Thus, in order to determine the cell distribution following incubation with Api on A375 human melanoma cell line, the cellular DNA content and the phases of the cell cycle were evaluated, by means of flow cytometry analysis. (51).

II.1.3. The evaluation of the anti-migratory effect, by the "Scratch Assay" method after incubation with Api, *in vitro*, for A375 human melanoma cell line. By this method, the antimigration potential of Api was determined *in vitro* on A375 human melanoma cell line. The method is based on the evaluation

of the ability to inhibit cell migration and metastasis, the growth and development of A375 melanoma, cells being evaluated compared to the control line. (52).

II.1.4. The determination of the caspase 3 activity by colorimetric method, after incubation with Api, *in vitro*, for A375 human melanoma cell line. To highlight the presence / absence of caspase-3, a colorimetric method was used to determine the activity of this enzyme. Determination of caspase-3 expression was an important factor in the present study because caspases are key factors in the apoptosis process. (53).

II.1.5. The evaluation of the apoptosis phenomenon (early / late and necrosis) by the Annexin V-PI technique, following incubation with Api *in vitro* for A375 human melanoma cell line. The evaluation of this phenomenon was performed using the Annexin V-PI combination, which provides information necessary for the evaluation and differentiation of cells in the early apoptosis phase (Annexin-V-positive, PI-negative), or late (Annexin-V-negative, PI - positive), viable cells (Annexin-V-negative, PI-negative), or necrotic cells (Annexin-V-positive, PI-positive). The Annexin V-PI association does not perform the coloring of viable cells, either in early apoptosis, but only in necrotic cells, or in late apoptosis, because they have a compromised membrane, allowing PI to penetrate inside the cells and thus react with nucleic acids. forming a red fluorescence (54).

II.1.6. The evaluation of cytotoxicity by determining the amount of LDH, after incubation with Api, *in vitro*, for A375 human melanoma cell line. The principle of this method is based on the release of the cytosolic enzyme LDH into the medium, which will later be quantified as an enzymatic reaction that produces formazan. The amount of formazan produced is directly proportional to the LDH secreted into the environment and is an indicator of the cytotoxic effect. (55).

II.1.7. The evaluation of the bioenergetic profile, using the extracellular flow analyzer (Seahorse XFe24), of A375 human melanoma cell line, after incubation with Api. The changes of the bioenergetic profile of the cell line of interest provides information on the antiproliferative and cytotoxic action of the flavone in this study. Thus, by measuring the specific parameters during the experiment [(a) basal respiration, b) Leak stage, c) maximum respiration, d) adenosine triphosphate (ATP) turnover, e) reserve capacity - an image can be made regarding modification of the cellular energy profile following stimulation with Api (56), (57).

II.1.8. The evaluation of the antiangiogenic profile of Api using the technique of normal and tumor chorioallantoic membrane with A375 human melanoma cells. Evaluation is performed on both normal CAM and tumor CAM (A375 human melanoma cell line). The method used requires the use of fertilized chicken eggs (*Gallus gallus domesticus*). In the first stage, the effect of Api on CAM in normal development was followed to determine the tolerability of Api on normal tissues and the antiangiogenic potential, and in the second stage A375 human melanoma cell line was inoculated on normal CAM to develop tumors. On these areas with tumor development, treatment was applied with: a) Api 30 μ M, b) Api 60 μ M, c) DMSO 1% - used as a control solution and the evolution was followed for a period of several days (58) (59).

II.1.9. The evaluation of the immunomodulatory potential on human dendritic cells following incubation with Api, by analyzing the effect on cell differentiation, morphology and proliferation using XTT technique and fluorescence microscopy. The immunomodulatory potential of Api in dendritic cells was assessed by XTT analysis and fluorescence microscopy. The XTT method is based on the reduction of the yellow tetrazolium (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-nitro) benzene hydrate from sulfonic acid or XTT) to formazane of orange color by viable cells through NADH produced by mitochondria; thus a water-soluble orange product called formazan is formed (60).

Regarding the highlighting of the CD morphology, fluorescence microscopy was used in which the DAPI solution (4', 6-Diamidine-2-phenylindole dihydrochloride) (Roche diagnostics, Mannheim, Germany) and the phalloidin solution Alexa Flour 488 were used as reagents. The DAPI method involves the blue fluorescence labeling of the exposed nuclear material, and a purple-fluorescent coloration is visible by the use of the phalloidin-Alexa Flour reagent. These types of reagents are also

used to highlight the phenomena of apoptosis, staining cells that have alterations in the cell membrane. (61).

II.1.10. Evaluation of the immunomodulatory potential on human dendritic cells, following incubation with Api, by analyzing the effect on some cytokines (IL6, IL10, tumor necrosis factor α , (TNF α)). This analysis was performed by fluorescence microscopy, a technique described above (61).

II.2. Study II: Evaluation of the antiproliferative, cytotoxic and anti-angiogenic potential of apigenin on human melanoma cell line SK-MEL-24

II.2.1. In the first stage, the antiproliferative activity of Api on the human melanoma cell line SK-MEL-24 was evaluated using the MTT technique, a method used in the previous study. This analysis was performed following the same protocol (62), and SK-MEL-24 human melanoma cells were stimulated with different concentrations of Api [0.3, 1, 3, 10, 30 and 60 μ M] (63).

II.2.2. The evaluation of the cytotoxic effect of Api on the human melanoma cell line SK-MEL-24 was performed using the LDH method, also used in previous tests. (63).

II.2.3. Evaluation of the antimigratory potential of Api on the human melanoma cell line SK-MEL-24 was performed using the Scratch assay method. Stimulation of melanoma cells was performed with Api [30 and 60 μ M] (63).

II.2.4. Evaluation of the anti-angiogenic potential of Api by the CAM method with tumor development. Two Api concentrations [30 and 60 μ M] were tested for this analysis, and 0.5% DMSO was used for the control line. The method used was represented by the method developed by Ribatii *et al.*, but with slight modifications (58), (64).

II.3. Study III: Evaluation of the cytotoxic and anti-angiogenic potential of apigenin on murine melanoma cell line B164A5.

II.3.1. Evaluation of the antiproliferative activity of Api on the murine melanoma cell line B164A5, by means of MTT analysis. The same protocol was followed as in previous studies. Different concentrations of Api [1, 3, 10, 30 and 60 μ M] were tested on the murine melanoma cell line B164A5 for a period of 72 h (65).

II.3.2. The cytotoxic potential of Api on the murine melanoma cell line B164A5 was evaluated using the LDH technique, following the protocol related to the LDH method. (65).

II.3.3. Using the CAM assay method, the anti-angiogenic potential of Api was evaluated on the murine melanoma cell line B164A5. The method is described above (58) (59).

II.4. Study IV: *In vitro* evaluation of apigenin and apigenin-7-O-glycoside on human cervical cancer cell line HeLa.

II.4.1. Evaluation of the antiproliferative activity of Api and Api-7-O-glycoside on the human cervical cancer cell line HeLa. The method was performed by MTT analysis, the protocol being described above (66).

II.4.2. Evaluation of the pro-apoptotic potential by the DAPI method and the Annexin V-PI technique. The DAPI colorimetric method was used to highlight the nuclei, being tested two concentrations of Api (aglycone), respectively Api-7 (heteroside) [10 μ M and 30 μ M]. The same concentrations of Api and Api-7 were tested by the Annexin V-PI method (66).

III. RESULTS

III.1. Study I

III.1.1. The results obtained after the evaluation of Api [0.3-60 μ M] on A375 human melanoma cell line proves the presence of the antiproliferative effect of the highlighted phytochemical, the effect being detected starting with the concentration of 30 μ M, and the IC₅₀ value being reached at the concentration of 33.02 μ M Api (62).

III.1.2. Following the analysis of the cell cycle by flow cytometry, it was shown that at both 30M and 60 μ M, Api induces cycle arrest in G2 / M phase and leads to an increase in the percentage of A375 cells in this phase, from 18,946. \pm 1.91% (control line) at 33,423 \pm 0.15% at a concentration of 30 μ M, respectively at 33,653 \pm 0.96% at a concentration of 60 μ M (62).

III.1.3. The recorded results demonstrated that both 30 μ M and 60 μ M Api led to a significant inhibitory effect on cell migration of A375 human melanoma cells compared to the control line. The changes recorded were as follows: Api 30 μ M-24 h has produced a change in the migration path of melanoma cells [from 550.84 μ m to 418.90 μ m], and in the case of Api 60 μ M-24 h [from 575.05 μ m to 537.59]. The values of the induced cell recovery rate were 23% for Api 30 μ M and 6.84% for Api 60 μ M. The apigenin-induced cell migration inhibition rate was 77% for Api 30 μ M and 93.16% for Api 60 μ M. Another change produced was on the cell shape and morphology after applying Api 60 μ M (62).

III.1.4. At 72 h post stimulation with Api 30 μ M an increased activity of the enzyme caspase-3 was detected, but the phenomenon was different in the case of incubation with Api 60 μ M, in which case the activity of caspase-3 did not increase, possible effect due to the presence of cytotoxic effect at this concentration and incubation time. At the same time, the values of caspase-2 and p53 protein were monitored, but their expression was not recorded. The phenomenon installed in the case of this analysis is called Hormesis phenomena, by increasing the biphasic response (low doses - obtaining therapeutic effect, high doses - obtaining cytotoxic effect (62).

III.1.5. Following the use of the Annexin V-PI technique, it was highlighted that after the incubation with Api 30 μ M, as well as with Api 60 μ M, the phenomena of early and late apoptosis and late necrosis were installed, respectively. In the case of Api 30 μ M, the phenomenon of early apoptosis (8.5 \pm 1.8% of cells) was mainly observed, while in the case of Api 60 μ M, it mainly induced the phenomenon of late apoptosis (12.25 \pm 2.9% of cells). (62).

III.1.6. Following the quantification of the amount of LDH to evaluate the cytotoxic effect induced by Api on A375 human melanoma cell line, the following results were recorded: at 72 h after Api stimulation, the amount of LDH released was different depending on the concentration tested. The cytotoxicity rate for Api 30 μ M was 20.75%, and for DMSO, the cytotoxicity rate was 1.12%. With the increase of Api concentration to 60 μ M, the cytotoxicity rate on the A375 melanoma line did not increase, but a similar value has been reached, namely, 19% (62).

III.1.7. The results obtained after recording the parameters observed in the case of determining the bioenergetic profile, showed that in terms of basal respiration, the cells remained unchanged, and on basal rates a dose-dependent increase was observed (basal respiration for Api 30 μ M is 137.9 \pm 31.4 pmol / min vs. Control 266.07 \pm 20.8 pmol / min, and in the case of Api 60 μ M the basal respiration is 22.2 \pm 5.5 pmol / min vs. Control). In the Leak stage, there was a decrease in OCR due to the blockade of ATP production after the injection of oligomycin. At the highest applied Api dose [60 μ M], a decrease in Leak stage (34.9 \pm 7.1 pmol / min vs. Control 135.01 \pm 14.6 pmol / min), and maximum respiratory rate (12.4 \pm 5.3 pmol / min) was observed. vs Control 46.5 \pm 5.9 pmol / min. In this experiment the maximum respiration was lower than the basal respiration due to the tumor cells moving to a glycolytic state. The maximum respiration for Api 30 μ M was 53.3 \pm 6.9 pmol / min, in exchange for Api 60 μ M values of 12.4 \pm 5.3 pmol / min were obtained. Turnover of ATP decreased after stimulation with Api, an effect observed in both tested concentrations, but also in DMSO. (ATP turnover for Api 60 μ M, was 22.1 \pm 8.3 pmols / min vs. Control, and in the case of Api 30 μ M a value of 12.65 \pm 6.5 pmol / min was

recorded vs. Control 131.05 ± 7.1 pmol / min). Thus, following the stimulation with Api, there was a change in the bioenergetic profile in the case of A375 human melanoma cell line, a more pronounced phenomenon in the case of Api 60 μ M. From what is known from the literature, these would be the first data regarding the effect of Api on mitochondrial respiration, as well as on glycolysis in A375 human melanoma line. Regarding the changes on the reserve capacity, Api considerably reduces the reserve capacity of A375 tumor cells (Api reserve capacity for 30 μ M is -9.8 ± 5.8 pmol / min vs. Control -219 ± 24.5 pmol / min, and in the case of Api 60 μ M -84.6 ± 31.4 pmol / min vs Control). Regarding ECAR values, Api treatment produced a significant dose-dependent decrease in ECAR, indicating that Api also induces a deficiency in cellular glycolytic activity (Api 30 μ M -11.4 ± 2.8 mpH / min vs. Control 65.04 ± 1.5 mpH / min, and for Api 60 μ M -45.1 ± 7 mpH / min vs Control) (62).

III.1.8. In normal CAM, stimulation with Api 30 μ M produced a significant inhibition of the angiogenesis process, compared to the control line, and Api 60 μ M caused an irritative and fibrotic process involving a significant number of capillaries around the injured area (62).

In the case of Api testing on CAM with tumor development, it was found that both Api concentrations produced an impairment of tumor angiogenesis, affecting the migration of cancer cells and reducing the number of capillaries. (62).

III.1.9. Evaluation of the effect on dendritic cells (CD) was performed in the presence, or absence of lipopolysaccharides (LPS), after incubation with different concentrations of Api and DMSO. Post-activation with LPS, vehicle-incubated CDs expanded, but this cell expansion was significantly diminished by incubation with 30 μ M Api and 60 μ M Api, respectively. At very low doses of Api [1 μ M] there was no change compared to the control sample. Another aspect was the change in the metabolic activity of CD, a phenomenon observed in the case of high doses of Api, both after 24 h from the application of Api, but even more pronounced after 48 h from the stimulation with Api. The appearance of CD was also altered, with LPS stimulation being observed, a typical clustered aggregation in the control line, but this phenomenon was not present in the case of stimulation with high dose Api [60 μ M]. (62).

The morphology of CD was also modified, following the stimulation with Api 60 μ M, CD adopting a round shape, and in the case of stimulation with LPS, no standard development phenomena on CD were highlighted. In the case of Api 1 μ M and 30 μ M, no changes in CD morphology was visible compared to the control line. In contrast, in the case of 60 μ M Api incubation, CD morphology was affected. By adding LPS, under normal conditions CDs were activated. In this case, following the application of Api 30 and 60 μ M, CDs did not develop following activation by LPS (62).

III.1.10. Cytokine secretion was analyzed to see if the reduced activity of LPS-stimulated cells produced certain functional consequences. Following the application of high doses of Api, the secretion of cytokines was strongly blocked, and the application of DMSO caused a significant increase in IL-6 and TNF- α secretion, possibly due to the increase in membrane permeability. IL-6 and IL-10 secretion was almost completely blocked with 30 μ M and 60 μ M Api stimulation, and TNF- α secretion was reduced by approximately 60%. Low doses of Api did not cause any effect compared to the control group (62).

III.2. Study II

III.2.1. Following the MTT analysis, the cell viability on the human melanoma cell line SK-MEL-24 was diminished, the highest doses of Api being responsible for the most significant antiproliferative effect on melanoma cells (at 30 μ M - cell viability was $75.08 \pm 5.5\%$ vs. Control and for 60 μ M - cell viability was $62.9 \pm 5.4\%$ vs. Control) (63).

III.2.2. In the case of LDH analysis, the tested Api concentrations were 30 μ M and 60 μ M, as they recorded a significant antiproliferative effect on the SK-MEL-24 cell line. Following stimulation with Api, a significant cytotoxic effect was observed, depending on the dose of Api tested. The cytotoxicity rate for the 30 μ M concentration was $9 \pm 1.1\%$ vs. Control ($1.6 \pm 0.7\%$), respectively for the concentration of 60 μ M was $11.1 \pm 2.4\%$ vs. Control (63).

III.2.3. During the dose range, Api led to inhibition of SK-MEL-24 human melanoma cell migration, depending on the dose tested. In the case of the 30 μM concentration, the migration inhibition rate was 6.2%, and in the case of the 60 μM concentration, the recorded value was lower, of 3.4%. (63).

III.2.4. The CAM method showed that both concentrations of Api produced changes in tumor growth and development (human melanoma cell line SK-MEL-24), the tumor area becoming less compact and the appearance of blood vessels underwent changes, forming a dense vascular network, of the wheel spokes type converging towards the tumor cells, these aspects being installed 24 h after stimulation. After 48 hours, the area of the tumor cells was limited, the number of capillaries was also reduced and the percentage of blood vessels in the peritumoral areas, which acquired an irregular appearance. No major differences were observed between the two Api concentrations tested, but in the case of Api 60 μM a higher decrease in neoangiogenesis and tumor cell development was observed. (63).

III.3. Study III

III.3.1. Following MTT analysis, Api was shown to have antiproliferative effects on murine melanoma cell line B164A5, depending on the dose of compound used. Cell viability after Api application was $76 \pm 1.7\%$ vs. Control in case of 30 μM concentration, and in case of 60 μM concentration a cell viability of $57.8 \pm 1.8\%$ vs. Control (65).

III.3.2. The values recorded from LDH analysis demonstrated the cytotoxic effect of Api on murine melanoma cell line B164A5. The cytotoxicity rate at the 30 μM concentration was $13.3 \pm 1.7\%$, and at the 60 μM concentration the cytotoxicity rate was $15.1 \pm 1.8\%$, the values being reported to the control group. (65).

III.3.3. Following CAM analysis with tumor development with B164A5 murine melanoma cells, after application of Api [30 and 60 μM - 48 h], the following aspects were observed: tumor areas were reduced, cell agglomerations were decreased, and a lower cell density was observed around the application ring. Following stimulation with a concentration of 30 μM , the capillaries became thinner and uneven, and in the case of a concentration of 60 μM , the blood vessels showed an irregular appearance. In both tested concentrations, cell migration was decreased, but the number of cells was reduced especially at the highest dose [60 μM] (65).

III.4. Study IV

III.4.1. Through the MTT method, it has been shown that both Api and Api-7-O-glycoside affect cell viability on human cervical cancer cell line HeLa. The dose range used was [0.3; 1; 3; 10; 30 μM]. The IC_{50} value was reached at the concentration of 12.08 μM for Api, respectively at the concentration of 18.28 μM for Api-7-heteroside. It can be seen that aglycone was more active compared to the conjugated form, but glucose (elemental conjugate) did not produce significant changes in the proliferation of human cervical cancer cells HeLa (66).

III.4.2. Through the DAPI method, the pro-apoptotic potential of the studied natural compounds was highlighted. Condensation of the nuclei and their decreased number are the result of impaired cell membrane integrity, events visible after incubation with Api and Api-7-O-glycoside, the human cervical cancer cell line HeLa (66).

Regarding the pro-apoptotic potential on the human cervical cancer cell line HeLa, following the analysis of Annexin V-PI, it was shown that both Api and Api-7-O-glycoside induced early apoptosis, late apoptosis and necrosis, but most often the phenomena of early apoptosis can be observed. After incubation with Api [10 μM] the percentage of viable cells was $79 \pm 2\%$, and for Api -7- O-glycoside [10 μM] a value of $84 \pm 3\%$ was obtained. In the case of Api stimulation [30 μM], the percentage of viable cells recorded was $67.5 \pm 2.5\%$, and for Api-7-O-glycoside [30 μM] a percentage of $72.5 \pm 2.5\%$ viable cells was determined. (66).

IV. CONCLUSIONS

As a result of the studies performed, the following aspects can be concluded:

1. Api exerts antiproliferative effects (dose and time dependent) on A375 human melanoma cell line, the IC₅₀ value being recorded at a concentration of 33.02 μ M.
2. Following the determination of the effect of Api on the cell cycle, both tested concentrations [30 μ M and 60 μ M] led to the cycle arrest in the G2 / M phase, to the inhibition of DNA and enzyme synthesis and affected the cell division process of A375 human melanoma cell line. The following percentages recorded were: $18,946 \pm 1.91\%$ (control line), $33,423 \pm 0.15\%$ [Api 30 μ M] and $33,653 \pm 0.96\%$ [Api 60 μ M].
3. The antimigratory effect of Api was detected both at the concentration of 30 μ M and at the concentration of 60 μ M, through the Scratch assay analysis. In order to extrapolate this effect *in vitro*, further *in vivo* tests are needed to confirm a potential antimetastatic effect.
4. Api [30 μ M-72h] produced the activation of the enzyme caspase-3, but in the case of the concentration of [60 μ M-72h], this mechanism was not visible, obtaining a cytotoxic effect.
5. Following the Annexin V-PI analysis, the following phenomena were recorded: following the incubation of the A375 cell line with phytocompound in a concentration of 30 μ M, early apoptosis phenomena were mainly detected, and in case of higher concentration [60 μ M], a higher number of events associated with late apoptosis occurred. Necrotic cells represent a low percentage in this case.
6. The cytotoxic effect induced by Api [30 μ M and 60 μ M] was present, but manifested itself in a reduced manner and without significant differences between the two tested concentrations.
7. After incubation with the studied phytocompound, only in the case of the concentration of 60 μ M, changes were detected on the energy profile of A375 human melanoma cell line. The recorded changes can be used for further studies of Api as an active antitumor agent on mitochondrial respiration.
8. Regarding the effects of Api on the process of normal angiogenesis, the lower concentration produced a stronger inhibitory effect, while Api 60 μ M, produced irritation, inflammation and fibrosis in CAM. On tumor angiogenesis (A375 human melanoma cell line), Api 30 μ M led to a decrease in the number of blood vessels inside the application ring and aspect of capillaries, as well as a decrease in tumor areas. In the case of Api 60 μ M the limitation of tumor growth was more pronounced, as well as the number of interconnected capillaries, but the vascularization did not change as in the case of Api 30 μ M, a process possible due to the irritative effect on normal CAM.
9. Following the incubation of dendritic cells with phytocompound [30 μ M and 60 μ M] different events occurred: the expansion of dendritic cells was significantly diminished and the morphological changes were visible only in the case of high dose of Api. Also, the specific activity of lipopolysaccharides that activate the function of dendritic cells and initiate the inflammatory process has been diminished. The concentration of 60 μ M produced the change of the morphology of the dendritic cells, these adopting a round shape, and in the case of low doses of Api [1 μ M and 30 μ M] these changes did not occurred.
10. Following *in vitro* tests to evaluate the effect of Api on the secretion of IL-10, IL-6 and TNF- α , cytokine secretion was strongly blocked by high concentrations of Api, but low doses of Api did not produce any effect. Considering the doses and the incubation time, Api does not lead to the stimulation of the immune system, although this event would have been a desideratum of this study.
11. Regarding the studies performed on the human melanoma cell line SK-MEL-24, Api [30 and 60 μ M] affected the cell viability, registering cell viability values of $75.08 \pm 5.5\%$ vs. Control [Api 30 μ M] and $62.9 \pm 5.4\%$ [Api 60 μ M], vs. Control. The cytotoxic effect generated by the same doses of natural compound led to dose-dependent values, so that at the concentration of 30 μ M, the values were $9 \pm 1.1\%$ vs. Control ($1.6 \pm 0.7\%$), and in the case of the concentration of 60 μ M they were $11.1 \pm 2.4\%$ vs. Control. Following the CAM analysis, it was shown that Api led to the inhibition

of cell migration (human melanoma cell line SK-MEL-24), the values recorded for the rate of inhibition of cell migration being 6.2% [Api 30 μ M] and 3.4 % [Api 60 μ M]. There were no significant differences between the two tested concentrations, only a pronounced effect in the case of Api 60 μ M. Both concentrations of phytocompound led to limited growth and migration of SK-MEL-24 tumor cells, also inhibited tumor angiogenesis and contributed to the reduction of blood vessels inside the ring.

12. The experimental study of Api on the murine melanoma line B164A5 showed the antiproliferative and cytotoxic properties of this flavone at both tested concentrations [30 and 60 μ M], but the effects were stronger at the higher dose of Api. Tumor angiogenesis was also affected in both concentrations used, depending on the dose tested. These results may represent a new direction of research for further studies on murine models.

Given the results presented above, it can be concluded that the antiproliferative effect of Api was more pronounced in A375 human melanoma cell line, compared to human melanoma cell line SK-MEL-24 and murine melanoma cell line B164A5. Also, after incubation with this phytocompound, the cytotoxic effect was more visible in the case of the A375 human melanoma cell line.

13. Regarding the *in vitro* anticancer activity of Api and Api-7-O-glycoside, the results showed that in both cases the proliferation of human cervical cancer cells HeLa was affected, but a more intense activity was observed in the case of aglycone (Api) compared to heteroside (Api-7). IC₅₀ values were obtained at 12.08 μ M for Api and 18.28 μ M for Api-7-O-glycoside, respectively. Regarding the pro-apoptotic effect, it also manifested itself in both cases, and the phenomena was dependent on the dose of Api, respectively, Api-7-O-glycoside.

Along with the previously presented studies, other groups of researchers have highlighted the antitumor activity of Api on other human melanoma [518A2, A2058, C8161, RPMI-7951] and murine cell lines [B164A5, B16F10, B16-BL6]. The present results complement the data on the antiproliferative, pro-apoptotic and antiangiogenic activity of flavone on A375 human melanoma cell lines and SK-MEL-24. Following the tests performed, Api does not contribute to the stimulation of dendritic cells, so it is not a research direction that deserves to be exploited.

In short, the mechanisms of action by which Api inhibits the tumor process include inhibiting cell proliferation, cell cycle arrest, decreasing cell migration, manifesting the cytotoxic effect, stimulating apoptotic processes, inhibiting tumor angiogenesis and affecting the bioenergy profile.

There is also currently an increased number of studies on the mechanism of action of this phytocompound on various experimental animal models of melanoma. Regarding clinical trials, there are currently no ongoing studies with Api for the management of melanoma cancer.

The results obtained during this PhD thesis provides a complete and complex picture of the action of the phytocompound *in vitro* on selected cell lines, respectively *in vivo* on experimental models of normal and tumor angiogenesis. All this information is new and completes the existing data in the literature on the topic of interest, contributing to the elucidation and understanding of the complex mechanism of action of Api on experimental models.

REFERENCES

1. Mateescu I, Paun L, Popescu S, Roata G, Sidorof M. Medicinal and aromatic plants - A statistical study on the role of phytotherapy in human health. *Bull UASVM Anim Sci Biotechnol*. 2014;71(1), p. 14–9.
2. Majolo F, de Oliveira Becker Delwing LK, Marmitt DJ, Bustamante-Filho IC, Goettert ML. Medicinal plants and bioactive natural compounds for cancer treatment: Important advances for drug discovery. *Phytochem Lett.* 2019;31, p. 196–207.
3. Sauter ER. Cancer prevention and treatment using combination therapy with natural compounds. *Expert Rev Clin Pharmacol*. 2020;13(3), p. 265–85.
4. WHO statistics [Internet]. World health statistics 2020: monitoring health for the SDGs, sustainable development goals. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO. Disponibil la: <https://apps.who.int/iris/bitstream/handle/10665/332070/9789240005105-eng.pdf>.
5. Wang JJ, Lei, KF, Han F. Tumor microenvironment: recent advances in various cancer treatments. *Eur Rev Med Pharmacol Sci*. 2018; 22(12), p. 3855–64..
6. Euromelanoma Disponibil la: <https://www.euromelanoma.org/romania/informa%C8%9Bii-generale/tipuri-de-cancer-de-piele>. 2020.
7. Redondo-Blanco S, Fernández J, Gutiérrez-Del-Río I, Villar CJ, Lombó F. New insights toward colorectal cancer chemotherapy using natural bioactive compounds. *Front Pharmacol*. 2017; 8, p. 109. Disponibil la: doi:10.3389/fphar.2017.00109
8. Efferth T, Saeed MEM, Mirghani E, Alim A, Yassin Z, Saeed E, et al. Integration of phytochemicals and phytotherapy into cancer precision medicine. *Oncotarget*. 2017 25;8(30), p. 50284–50304. Disponibil la: doi: 10.18632/oncotarget.17466.
9. Aghajan ZM, Schuette P, Fields TA, Tran ME, Siddiqui SM, Hasulak NR et al. Theta oscillations in the human medial temporal lobe during real-world ambulatory movement. *Curr Biol*. 2017, 27(24), p. 3743–3751.e3. Disponibil la: doi: 10.1016/j.cub.2017.10.062. Epub 2017 Nov 30.
10. Chinembiri TN, du Plessis L H, Gerber M, Hamman, JH, du Plessis J. Review of natural compounds for potential skin cancer treatment. *Mol Basel Switz*. 2014;19(8), p. 11679–721.
11. Lang SJ, Schmiech M, Hafner S, Paetz C, Steinborn C, Huber R, et al. Antitumor activity of an *Artemisia annua* herbal preparation and identification of active ingredients. *Phytomedicine*. 2019;62, p. 152962. Disponibil la: <https://doi.org/10.1016/j.phymed.2019.152962>.
12. Costa D, Santos R, Andrade EDS, Themistocles BL, da Silva BG, Fialho E, et al. Green tea extract (*Camellia sinensis*) as a potential antitumoral agent on breast cancer cells (FS13-04-19). *Curr Dev Nutr*. 2019; 3(Suppl 1):nzz030.FS13-04-19.
13. Sohretoglu D, Huang S. Ganoderma lucidum polysaccharides as an anti-cancer agent. *Anticancer Agents Med Chem*. 2018;18(5), p. 667–74.
14. Bala M, Pratap K, Verma PK, Singh B, Padwad Y. Validation of ethnomedicinal potential of *Tinospora cordifolia* for anticancer and immunomodulatory activities and quantification of bioactive molecules by HPTLC. *J Ethnopharmacol*. 2015;175, p. 131–7.
15. Mirahmadi M, Azimi-Hashemi S, Saburi E, Kamali H, Pishbin M, Hadizadeh F. Potential inhibitory effect of lycopene on prostate cancer. *Biomed Pharmacother*. 2020;129, p. 110459. Disponibil la: doi: 10.1016/j.biopha.2020.110459. Epub 2020 Jun 30.
16. Dissanayake KGC, Waliwita WALC, Liyanage RP. A review on medicinal uses of *Zingiber officinale* (ginger). *Int J Health Sci Res*. 2020;10(6), p. 142–8.
17. Caunii A, Oprean C, Cristea M, Ivan A, Danciu C, Tatu C et al. Effects of ursolic and oleanolic on SK MEL 2 melanoma cells: *In vitro* and *in vivo* assays. *Int J Oncol*. 2017;51, p. 1651–60.
18. Zhao G, Han X, Zheng S, Li Z, Sha Y, Ni J et al. Curcumin induces autophagy inhibits proliferation and invasion by downregulating AKT/mTOR signaling pathway in human melanoma cells. *Oncol Rep*. 2016;35, p.1065–74.
19. Rauf A, Imran M, Butt MS, Nadeem M, Peters DG, Mubarak MS. Resveratrol as an anti-cancer agent: a review. *Crit Rev Food Sci Nutr*. 2018;58(9), p. 1428–47.

20. Wang J, Wang L, Lou GH, Zeng HR, Hu J, Huang QW, et al. Coptidis rhizoma: a comprehensive review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *Pharm Biol.* 2019;57(1), p. 193–225.
21. Shukla S, Bhaskaran N, Babcook MA, Fu P, MacLennan GT, Gupta S. Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoxO pathway. *Carcinogenesis.* 2014;35(2), p. 452–60.
22. Butler MS. Natural products to drugs: Natural product-derived compounds in clinical trials. *Nat Prod Rep.* 2008, 25(3), p. 475-516. Disponibil la: doi: 10.1039/b514294f.
23. Giddings LA, Newman DJ. Microbial natural products: molecular blueprints for antitumor drugs. *J Ind Microbiol Biotechnol.* 2013;40, p. 1181–210.
24. Cancer trials- Genistein [Internet]. Disponibil la: <https://clinicaltrials.gov/ct2/show/NCT00584532?term=genistein&cond=Cancer+Prostate&draw=2&rank=2>
25. Clinical trials- Desmodium-Breast cancer. Disponibil la: <https://clinicaltrials.gov/ct2/show/study/NCT03959618?term=desmodium&cond=cancer&draw=2&rank=1>
26. Clinical trials-Quercetin. Disponibil la: <https://clinicaltrials.gov/ct2/show/NCT01912820?term=quercetin&cond=cancer+prostate&draw=2&rank=2>
27. Clinical trials-Isoquercetin. Disponibil la: <https://clinicaltrials.gov/ct2/show/NCT02446795?term=isoquercetin&cond=cancer+kidney&draw=2&rank=1>
28. Clinical trials-Resveratrol-Colon cancer. Disponibil la: <https://clinicaltrials.gov/ct2/show/NCT00256334?term=resveratrol&cond=cancer+colon&draw=2&rank=1>
29. Clinical trials-Resveratrol-Gastrointestinal tumors. Disponibil la: <https://www.clinicaltrials.gov/ct2/show/NCT01476592?term=resveratrol&cond=cancer&draw=2&rank=4>
30. Clinical trials-Polyphenols. Disponibil la: <https://www.clinicaltrials.gov/ct2/show/NCT03482401?term=phenolics+polyphenols&cond=breast+cancer&draw=2&rank=1>
31. Clinical trials-Epirubicin and Vinorelbine. Disponibil la: <https://www.clinicaltrials.gov/ct2/show/NCT00176488?term=epirubicin+vinorelbine&cond=breast+cancer&draw=2&rank=1>
32. Clinical trials-Vincristine. Disponibil la: <https://www.clinicaltrials.gov/ct2/show/NCT01463852?term=vincristine&cond=chronic+lymphocytic+leukemia&draw=2&rank=1>
33. Clinical trials-Irinotecan. Disponibil la: <https://www.clinicaltrials.gov/ct2/show/NCT01220063?term=irinotecan+hydrochloride&cond=Colorectal+Cancer&draw=2&rank=3>
34. Lu K., Bhat M. and Basu S. Plants and their active compounds: natural molecules to target angiogenesis. *Angiogenesis.* 2016;19(3):287–95.
35. Bocci G., Di Paolo A. and Danesi R. The pharmacological bases of the antiangiogenic activity of paclitaxel. *Angiogenesis.* 2013/02/07 ed iulie 2013;16(3), p. 481–92.
36. Nagaraju G. P., Zhu S., Ko J. E., et al. Antiangiogenic effects of a novel synthetic curcumin analogue in pancreatic cancer. *Cancer Lett.* 2015;357(2), p. 557–65.
37. Yu Y., Zhang C, Liu L, Li X. Hepatic arterial administration of ginsenoside Rg3 and transcatheter arterial embolization for the treatment of VX2 liver carcinomas. *Exp Ther Med.* 2013;5(3), p. 761–6.
38. Al-Abd AM, Alamoudi AJ, Abdel-Naim AB, Neamatallah TA, Ashour OM. Anti-angiogenic agents for the treatment of solid tumors: Potential pathways, therapy and current strategies - A review. *J Adv Res.* 2017;8(6), p. 591–605.
39. Tian L, Xie K., Sheng D, Wan X, Zhu G. Antiangiogenic effects of oridonin. *BMC Complement Altern Med.* 2017;17(1):192. Disponibil la: <https://doi.org/10.1186/s12906-017-1706-3>.
40. Bahmani M, Shirzad H, Shahinfard N, Sheivandi L, Rafieian-Kopaei M. Cancer phytotherapy: Recent views on the role of antioxidant and angiogenesis activities. *J Evid-Based Complement Altern Med.* 2017;22(2), p. 299–309
41. Samad NA, Abdul AB, Rahman HS, Abdullah R, Tengku Ibrahim TA, Othman HH. Antiproliferative and antiangiogenic effects of zerumbone from *Zingiber zerumbet* L. Smith in sprague dawley rat model of hepatocellular carcinoma. *Phcog Mag.* 2019;15 , p .277–82.

42. Shah SL, Wahid F, Khan N, Farooq U, Shah AJ, Tareen S, et al. Inhibitory effects of *Glycyrrhiza glabra* and its major constituent glycyrrhizin on inflammation- associated corneal neovascularization. *Evid-Based Complement Altern Med ECAM*. 2018; 2018, ID 8438101, 8 pages. Disponibil la: <https://doi.org/10.1155/2018/8438101>.
43. Mirossay L, Varinská L, Mojžiš J. Antiangiogenic effect of flavonoids and chalcones: An update. *Int J Mol Sci*. 2017;19 (1), p. 27. Disponibil la: doi: 10.3390/ijms19010027.
44. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. *J Nutr Sci*. 2016;5, p. e47. Disponibil la: doi:10.1017/jns.2016.41.
45. Yan X., Qi M., Li P, Zhan Y, Shao H. Apigenin in cancer therapy: anti-cancer effects and mechanisms of action. *Cell Biosci*. 2017; 7,p. 50. Disponibil la: <https://doi.org/10.1186/s13578-017-0179-x>.
46. Fajemiroye JO, Ferreira NL, de Oliveira LP, Elusiyan CA, Pedrino GR, da Cunha LC et al. *Matricaria recutita* and its isolate-apigenin: economic value, ethnopharmacology and chemico-biological profiles in retrospect. *Res Rev J Pharmacogn Phytochem*. 2016; 4(4), p. 2347–2332.
47. Hostetler GL, Ralston RA, Schwartz SJ. Flavones: food sources, bioavailability, metabolism and bioactivity. *Adv Nutr. Mai* 2017; 8(3), p. 423–35.
48. Ali F, Rahul, Naz F, Jyoti S, Siddique YH. Health functionality of apigenin: A review. *Int J Food Prop*. 2017; 20: 6, p. 1197–238.
49. Carvalho Lemos V., Reimer JJ, and Wormit A. Color for Life: Biosynthesis and distribution of phenolic compounds in Pepper (*Capsicum annuum*). *Agriculture*. 2019; 9(4), p. 81. Disponibil la: doi:10.3390/agriculture9040081.
50. MTT Assay Disponibil la: <https://www.sigmaaldrich.com/technical-documents/protocols/biology/roche/cell-proliferation-kit-i-mtt.html>.
51. McKinnon K.M. Flow Cytometry: An Overview. *Curr Protoc Immunol*. 2018;120:5.:1.1-5.1.11. Disponibil la: doi: 10.1002/cpim.40.
52. Liang C.C., Park A.Y., Guan J.L. *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc*. 2007;2(2)p. 329–33.
53. McIlwain DR, Berger T and Mak TW. Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol*. 2013;5(4), p. a008656. Disponibil la: doi: 10.1101/cshperspect.a008656.
54. Demchenko AP. Beyond annexin V: fluorescence response of cellular membranes to apoptosis. *Cytotechnology*. 2013;65(2), p. 157–72.
55. Chan F, Moriwaki K and De Rosa, M. Detection of necrosis by release of lactate dehydrogenase activity. In *Immune Homeostasis: Methods in Molecular Biology (Methods and Protocols)*; Snow, A., Lenardo, M. (Eds) Humana Press. 2013;979, p. 65–70.
56. Duicu O.M.; Scurtu, I., Popescu, R. Sturza A, Coricovac D, Danila MD, et al. Assessment of the effects of methylene blue on cellular bioenergetics in H9c2 cells. *Rev Chim*. 2015; 66, p. E519-522.
57. Zhang J, and Zhang Q. Using Seahorse machine to measure OCR and ECAR in Cancer Cells. *Cancer Metab*. 2019, p. 353-363.
58. Ribatti D, Vacca A, Roncali L, Dammacco F. The chick embryo chorioallantoic membrane as a model for *in vivo* research on anti-angiogenesis. *Curr Pharm Biotechnol*. 2000;1, p. 73–82.
59. Avram S, Coricovac DE, Pavel IZ, Pinzaru I, Ghiulai R, Baderca F, et al. Standardization of A375 human melanoma models on chicken embryo chorioallantoic membrane and Balb/c nude mice. *Oncol Rep*. 2017;38, p. 89–99.
60. XTT Assay. Disponibil la: <http://home.sandiego.edu/~josephprovost/XTT%20Protocol.pdf>.
61. Sreelatha S, Jeyachitra A, Padma PR. Antiproliferation and induction of apoptosis by *Moringa oleifera* leaf extract on human cancer cells. *Food Chem Toxicol*. 2011; 49(6), p. 1270–5.
62. **Ghiu A**, Schwiebs A, Radeke HH, Avram S, Zupko I, Bor A, et al. A comprehensive assessment of apigenin as an antiproliferative, proapoptotic, antiangiogenic and immunomodulatory phytochemical. *Nutrients*. 2019;11(4), p. 858. Disponibil la: doi: 10.3390/nu11040858.

63. **Ghiu A**, Pavel IZ, Avram S, Kis B, Minda D, Dehelean CA, et al. An *in vitro in vivo* evaluation of the antiproliferative and anti-angiogenic effect of flavone apigenin against SK MEL-24 human melanoma cell line. *Analytical Cellular Pathology*, 2021; vol. 2021, Article ID 5552664, 11 pages. Disponibil la: <https://doi.org/10.1155/2021/5552664>.
64. Avram S., Ghiulai R., Pavel I.Z., et al. Phytocompounds Targeting cancer angiogenesis using the chorioallantoic membrane assay. În: *Natural Products and Cancer Drug Discovery*. Intechopen. 2017. p. 45–66. Disponibil la <https://www.intechopen.com/chapters/55790>.
65. **Ghiu A**, Avram Ș, Pavel IZ, Scurtu AD, Minda D, Danciu C, et al. Evaluation of the cytotoxic and antiangiogenic potential of flavone apigenin using the B16A5 mouse melanoma cell line. *Med Evol.* 2020; 25, p. 288–95.
66. Minda D, Avram Feflea S, Pavel I, Kis B, **Ghiu A**, Zupko I, et al. An *in vitro* evaluation of apigenin and apigenin-7-O-glucoside against HeLa human cervical cancer cell line. *Rev Chim.* 2020; 71, p. 140–4.