„BABEȘ-BOLYAI" UNIVERSITY CLUJ-NAPOCA

FACULTY OF BIOLOGY AND GEOLOGY

DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY

DOCTORAL THESIS

THEAPEUTIC POTENTIAL OF *ALLIUM SATIVUM* L. AND *ALLIUM FISTULOSUM* L. PLANTS

- Summary -

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Introduction

The aim of this PhD Thesis was to investigate the biological properties of *Allium* extracts and to highlight the therapeutical potential of allicin, the active compound from *Allium* extracts, by using *in vitro* models – human cell cultures and fungi cultures, and *in vivo* models – Wistar rats. *Allium* extracts and allicin were analyzed from phytochemical point of view and their antioxidant, antifungal and antitumoral properties were analyzed by conventional golden standard methods.

For performing the presented studied we used cutting edge techniques and state of the art protocols which allowed us to obtain solid data in accordance with the literature. The results are opening new perspectives for further studies in phytomedicine.

The investigation of antifungal effect of *Allium sativum* extract and allicin was highlighted on an onychomycosis infection with *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa*; for the first time was identified a nail infection caused by a co-infection of the above-mentioned fungi. The antifungal effect of garlic extract and allicin was analyzed simultaneously with the investigation of the antioxidant effect, seeking for a potential method to reduce oxidative stress and local inflammation generated by the fungi co-infection. Thus, we wanted to obtain a new therapy which can inhibit both fungal infection and local inflammation.

Worldwide, access to efficient therapy with less costs are a must, especially for populations with low income and poor communities. There is a continuous need for alternative and accessible therapies for fighting against many diseases, like fungal infections, which have higher incidence in populations with poor financial level. Natural extracts, like *Allium sativum* extract, could represent a novel therapy against fungal infections.

Starting from our previous idea, the study was extended in order to investigate the effect of synthesized allicin against cancer cells.

World health organization (WHO) estimated around 9.6 million new deaths in 2018 due to cancer. The trend is still unfavorable, new cancer cases are reported and the incidence is growing from year-to-year due to pollution, genetic imbalance and other factors to which people are constantly exposed.

Lung and colorectal cancer are the most common types of cancer within men while breast cancer leads the statistic in women (Siegel et al., 2019).

In the last decades, many antitumoral therapies started from natural compounds or synthetic derivates, nature serving as model for nowadays therapies, one example is Paclitaxel (Waver, 2014) which has its origin in *Taxus brevifolia*, being very effective in combination with conventional cytostatic drugs like carboplatin or cisplatin in ovarian cancer.

Allicin, the active compound in garlic, was studied by many research groups, especially regarding its antitumoral potential.

In this thesis we investigated the antitumoral effect of allicin from many points-of-view: (i) regarding tumor cell proliferation; (ii) regarding morphological changes induced by treatment; (iii) regarding its potential to inhibit colony formation and cell migration, two cancer hallmarks; (iv) regarding the induced cell death, in the case of colorectal cancer and lung cancer.

Allicin was teste both individually and in combination with 5-fluorouracil.

All these aspects are presented in detail in the following pages, structured as three studies, with the common element being allicin and *Allium* extracts. These results are obtained with an interdisciplinary collaboration and involving research teams from Faculty of Biology and Geology from ”Babeș-Bolyai” University , Cluj-Napoca, Faculty of Chemistry and Chemical Engineering from ”Babeș-Bolyai” University , Cluj-Napoca, Biological research institute from Cluj-Napoca, “Ion Chiricuta” Oncology Institute, Cluj-Napoca and ”Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca.

Objectives and scope

Study objectives were (a) to obtain and investigate the phytochemical composition of *Allium* extracts, (b) to investigate the antifungal and antioxidant effect of *Allium sativum* extract and allicin by comparing it with a conventional and commercial anti-inflammatory compound, (c) to evaluate the antitumoral potential of allicin as individual treatment and in combination with conventional cytostatic drugs, by investigating the mechanism of induced cell death, (d) to investigate the inhibitory effect against cell migration and colony formation, two cancer features.

The aim of the doctoral thesis is to study two very common species of *Allium*, cultivated and used by humans since ancient times. Using different research methods, we contributed to the enrichment of knowledge about the therapeutic potential of the plants *Allium sativum* and *Allium fistulosum*, reflected in publications. Of particular importance is research of allicin effects. The research results provide important perspectives for use in phytomedicine.

I. Theoretical part

1. Biological characterization of Allium species

1.5. *Allium sativum*

A. sativum (figure 1) is used for its therapeutic properties, and literature studies suggest that it prevents many chronic diseases due to the large amounts of flavonoid quercitrin. This flavonoid contributes to the prevention of cardiovascular diseases and has antitumor properties (D'Andrea, 2015). Garlic extract reduces oxidative stress and has hypoglycemic properties, balancing postprandial blood glucose (O'Keefe and Bell, 2007).

A. sativum is one of the most widely cultivated vegetable, with crops of about 10 million tons per year, according to the American Agriculture Organization. Garlic is a rich source of bioactive compounds with therapeutic potential (Cerella et al., 2011; Corzo-Martínez et al., 2007).

Garlic inhibits carcinogenic activity and induces cell cycle arrest in malignant cells (especially in the G2 / M phase) (Xiao et al., 2005). Apoptosis and histone methylation are also stimulated (Iciek et al., 2009). The antitumor activity of garlic is due to the antimutagenic and antigenotoxic activity of active compounds, such as polyphenols, dialyl sulfates and dialyl disulfates (Guyonnet et al., 2002).

The polyphenols in garlic are bioactive compounds with multiple biological properties: antioxidant, antitumor, antibacterial and anti-inflammatory; plants rich in polyphenols have the ability to inhibit oxidative degradation of lipids and reduce oxidative stress (Capasso, 2013; Nencini et al., 2007; Thomson and Ali, 2003; Duthie et al., 2000).

Figure 1. *Allium sativum* (stem and leaves - left; bulbs - right) (Original: Marcel Pârvu).

Polyphenols are secondary metabolic compounds; they are generally involved in protecting plants from UV radiation or pathogens. But the antioxidant nature of these compounds makes them particularly important for modern medicine.

Studies on polyphenols suggest that they provide protection against various types of cancer, reduce the risk of diabetes and degenerative diseases (Arts and Hollman, 2005; Bianchini and Vainio, 2001).

Starting from alignment, numerous organosulfur compounds can be obtained from the composition of garlic, through biotransformation processes (Figure 2). Aliin is converted by cutting, crushing or grinding by the enzyme aliinase into molecules of sulfenic acid, which after condensation of two molecules will give rise to allicin. This is practically the common point of the various organosulfur compounds in garlic (and other Allium species). Following decomposition, either vinyl compounds or dialyl sulfides (DAS), dialyl disulfides (DADS) or dialyl trisulfides (DATS) are formed. DAS, DADS and DATS, by reduction, will form allyl mercaptans and allyl persulfides, but DADS and DATS by nucleophilic substitution and addition of glutathione will form S-allyl-glutathione which through a series of substitutions will form allyl-glutathione-disulfide (GSS) (Trio et al., 2014).

Condensation of allicin forms ajoena, addition of a glutathione will result in s-allyl mercaptogutathione (SAMG), and addition of an L-cysteine will result in s-allyl mercaptocysteine (SAMC) (Mitrová et al., 2018; Trio et al., 2014) .

Organosulfur compounds in garlic are not all present in the fresh bulb. Basically, the presence or absence of these compounds is strictly related to the state of the garlic. Fresh bulb contains an impressive amount of allicin, the essential oil is rich in DADS and DATS, while crushed and dried garlic powder contains allicin, DADS and DATS. Whole garlic cloves contain large amounts of DADS, DATS, ajoena and allicin-derived vinyl compounds. But adult garlic extract contains SAMC (Trio et al., 2014).

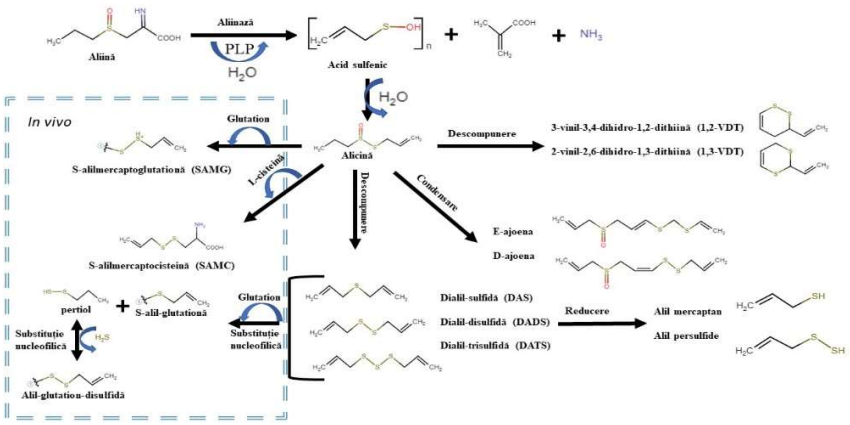


Figure 2. Alliin biotransformation in organosulfur compounds (adapted from Trio et al., 2014).

Antibacterial properties

Allicin, along with other sulfur compounds, is responsible for the antimicrobial properties of garlic. A. sativum is effective against many bacteria, be they Gram-negative or Gram-positive (Oosthuizen et al., 2017; Reiter et al., 2017; Harris et al., 2001; Abdullah et al., 1988).

Aqueous, alcoholic and ether extract have been tested against many pathogenic bacteria, and Gram-positive bacteria such as *Staphylococcus aureus* have been much more sensitive to these extracts than Gram-negative bacteria. Aqueous garlic extract, administered together with common antibiotics, can be used to combat nosocomial infections (common in hospitals) (EL-mahmood, 2009).

Antifungal properties

The antifungal effect of *A. sativum* is determined by the active compounds in its composition: aliina, alicina and ajoena. Allicin has broad-spectrum antifungal activity and shown a strong antifungal effect against *Candida albicans* (Ankri and Mirelman, 1999). The allicin obtained from *A. sativum* was also tested in combination with Amphotericin B (a strong and toxic antifungal, used mainly in patients with progressive and possibly fatal fungal infections) against *C. albicans*, and the results showed that the allicin improves the effect of amphotericin B against *C. albicans* (An et al., 2009).

Alcoholic garlic extract has a strong antifungal effect against *M. guilliermondii* and *R. mucilaginosa* (Pârvu et al., 2019).

Antiparasitic and antiviral properties

Allicin is antiparasitic against many parasites in the human gut, such as *Giardia lamblia* and *Entamoeba histolytica* (Ankri and Mirelman, 1999).

A. sativum has antiviral properties. The antiviral activity of the active compounds in garlic was studied, and the largest share, as antiviral activity, has ajoene, followed by allicin, allyl-methyl and thiosulfinate (Weber et al., 1992).

An investigation of the effect of alitridine (diallyl-trisulfide extracted from *Allium sativum*) on HCMV (Cytomegalovirus) replication and viral gene expression suggested that alitridine has anti-HCMV effects (Zhen et al., 2006).

Antihypertensive and antitrombotic properties

A. sativum has antihypertensive properties, demonstrated in numerous studies in the literature. Aqueous garlic extract lowers blood pressure and bradycardia by direct mechanism, without involving the cholinergic pathway, thus suggesting a peripheral mechanism for hypotension (Nwokocha et al., 2011; Ginter and Simko, 2010; Hiyasat et al., 2009; Brace, 2002).

It has been shown that the administration of aqueous garlic extract inhibits lipid peroxidation and prevents the depletion of glutathione, allowing its functional recovery, thus reducing oxidative stress and lowering blood pressure and inflammation (Sener et al., 2007) . It is also assumed that the decrease in blood pressure in rats, after administration of *A. sativum*, is partially mediated by nitric oxide (NO), because its synthesis is intensified (Al-Qattan et al., 2006).

Allicin, the major active substance in garlic, has immunomodulatory effects and stimulates B lymphocytes to secrete IgM and IgG antibodies. This ability is potentiated when allicin is converted together with glutathione into S-allyl-mercaptoglutation; in addition, antibody secretion is also influenced by the level of oxidative stress in the cell (Toma et al., 2019).

Anti-inflammatory properties

The active compounds of garlic are involved in the modulation of intracellular signals, especially of the Nrf2-ARE pathway (ARE is the antioxidant response element). Nrf2 is a transcription factor, which complexes with MafK to bind the antioxidant response element (ARE) and to initiate transcription. Nrf2 mediates the expression of phase 2 metabolizing enzymes, such as HO-1, GST and Glel. In the case of colon cancer, garlic induces the translocation of Nrf2, activating antioxidant enzymes and aiming to promote apoptosis. DAS, DADS and DATS also stimulate the expression of antioxidant enzymes, inducing the activation of Nrf2-ARE in liver cells, and their modulation capacity increases with increasing numbers of sulfur atoms. DAS and DATS inhibit reactive oxygen species-induced NFkB, allowing cross-talk between anti-inflammatory signaling pathways (Figure 3). DATS and DADS have the ability to inhibit inflammatory processes, and organosulfur molecules have anti-inflammatory potential; allicin attenuates tumor necrosis factor α (TNFα), DADS inhibits the secretion of proinflammatory molecules, and DATS inhibits cellular production of interleukins (IL-6, IL-10, IL-12) and TNFα (Trio et al., 2014).

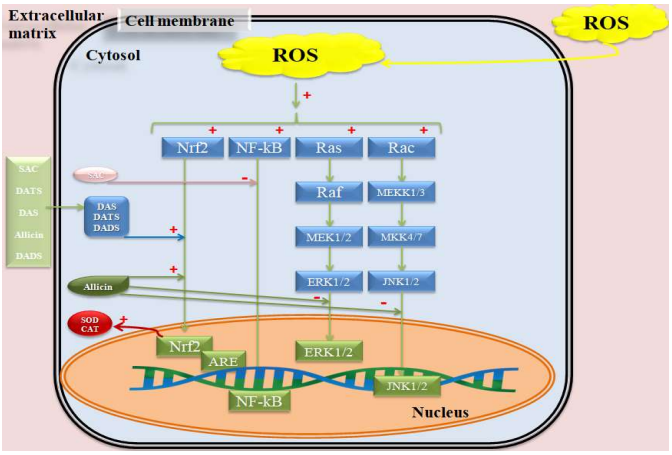


Figure 3. Scheme of the antioxidant mechanism of action of garlic organosulfur compounds (adapted from Trio et al., 2014). SAC (S-Allyl-Cysteine), DAS (Dialyl-sulfides), DADS (Dialyl-disulfides), DATS (Dialyl-trisulfides), ROS (Reactive oxygen species). The sulfur compounds in the garlic composition modulate the ROS signaling pathway and influence the expression of Nrf-2, NF-kB, JNK 1/2 and ERK1 / 2, resulting in a modulation of gene expression and activation of the Nrf-2-ARE complex which initiates the synthesis of enzymes of the antioxidant system such as SOD (Superoxide dismutase) and CAT (Catalase).

A. sativum and allicin toxicity

Studies on the toxicity of garlic extract indicate that although it is of plant origin, the dose administered must be taken into account, but also the fact that in combination with certain drugs it can create discomfort or side effects.

A study by Tattelman shows that patients receiving anticoagulants should take doses of garlic extract with caution; thus, high doses of *A. sativum* together with the administration of anticoagulants can lead to prolonged bleeding time to the detriment of patients (Tattelman, 2005).

1.6. *Allium fistulosum*

Allium fistulosum L. (Welsh onion) is a species cultivated in Romania (Figure 4). Its leaves have a great nutritional value, and can be eaten both fresh and cooked, in addition they are green all year round.

Due to its rich content of sulfur compounds, flavonoids and fatty acids, *A. fistulosum* has antifungal, antioxidant and antihypertensive properties (Lee et al., 2018; Vlase et al., 2013).

A. fistulosum has been used to treat colds, headaches, abdominal pain and cardiovascular disease by adherents of traditional medicine. There are studies that show that *A. fistulosum* prevents the formation of clots, has antioxidant, antihypertensive properties and is a very good antihypolipidemic product (Sung et al., 2015). Welsh onions are of great interest to researchers, due to their composition rich in propylene sulfides, compounds with strong antibacterial and anti-inflammatory effect (Liu et al., 2016).

Figure 4. *Allium fistulosum* (original: Marcel Pârvu).

Welsh onions, along with other members of the *Alliaceae* family, have special medicinal properties, reduce blood pressure and inhibit platelet aggregation. *A. fistulosum* modulates cardiovascular functions, restores the tone of arteries (especially the aorta), but the vasoconstrictor or vasodilator effect is dependent on the way the extract was prepared (Chen et al., 2000).

2. Allicin

Allicin is an organosulfur compound present in garlic, onions and other Allium species. The biosynthesis of allicin is carried out according to Figure 5, starting from alliin, under the action of the enzyme alliinase. The basic substrate of alline is cysteine. Following this reaction, allysulfenic acid, pyruvic acid and an NH3 group are formed. Two molecules of allysulfenic acid condense and form allicin by releasing a molecule of water (Salehi et al., 2019; Si et al., 2019; Chen et al., 2018; Huang et al., 2017; Borlinghaus et al., 2014).

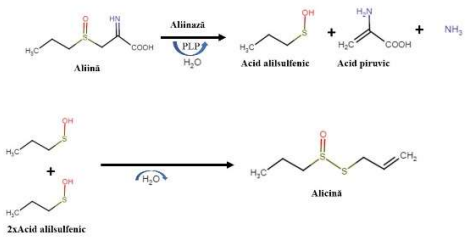


Figure 5. Allicin biosynthesis (adapted from Salehi et al., 2019).

Allicin has antibacterial, antiviral, antitumor, anticoagulant, antihypertensive, antiparasitic and hepatoprotective effects. Also, allicin is effective against several species of fungi (Chen et al., 2019; Huang et al., 2017; Salama et al., 2014; Ilić et al., 2011; Pârvu et al., 2011; Pârvu et al., 2010; Josling, 2003).

Allicin reduces lipid deposits and has anti-inflammatory properties (Marón et al., 2020; Eilat et al., 1995;). Excess lipids promote the development of adipose tissue which is an important source of estrogen. There is a close link between the risk of developing breast cancer and high levels of circulating estrogen, so many women who are in the postmenopausal period have an increased risk of developing breast cancer (Yue et al., 2003).

Allicin has beneficial effects in inhibiting neuroblastoma proliferation in mice, and administration in combination with cyclophosphamide translates into increased efficacy of conventional treatment; an improvement in T cell subset and an enhanced immunity have also been observed (Gao et al., 2015). Allicin has an antitumor effect against various types of tumor cells, such as those of the colon and lung (Țigu et al., 2020).

3. Types of cancer

Cancer is an uncontrolled growth of cells, a process that involves the activation of oncogenes or the inhibition of tumor suppressor genes. In the advanced stages, tumor cells can metastasize due to loss of cell adhesion to the substrate or adhesion between cells (Vogelstein and Kinzler, 2004).

The effect of epigenetic factors is decisive in the development of tumor cells. Epigenetic changes can induce pro-cancerous characteristics of non-tumor cells, by disrupting the function of certain specific genes and by modulating the processes that determine the cell cycle or initiating apoptosis (Sarkar et al., 2013; Sarkar et al., 2013; Gal-Yam et al.,, 2008).

The most common types of cancer are breast, colorectal and lung cancer, which have a very high incidence worldwide and are responsible for many deaths, both in men and among women (the case of breast cancer) (Siegel et al., 2019).

3.1. Breast cancer

According to statistics, in 2018, 268,670 new cases of breast cancer were registered in the United States of America (Siegel et al., 2018).

A possible cause of developing breast cancer is considered exposure to significant amounts of estrogen. Exposure to high concentrations of estrogen significantly increases the risk of developing liver, cervical and vaginal cancer (Henderson et al., 1988).

Three mechanisms have been described as possibly involved in the manifestation of estrogen carcinogenicity: receptor-mediated hormonal activity (closely related to stimulating cell proliferation, which increases the chances of accumulation of errors in DNA) (Adlercreutz et al., 1994); activation of cytochrome P450 (metabolically mediated activation) which intensifies the rate of mutations (Liehr et al., 1986); the inability of the DNA repair mechanism to remedy the mistakes made after division and thus certain errors can induce carcinogenicity of estrogen (Yan and Roy, 1995).

3.2. Colorectal cancer

Colorectal cancer is located in the region of the colon and rectum, with symptoms such as the presence of blood in the stool, reduced stool diameter, weight loss and often a feeling of constant fatigue (Whiffin et al., 2014).

In 2018, 97,220 new cases of colon cancer, 43,030 new cases of rectal cancer and 8,580 new cases of anal cancer were registered in the United States (Siegel et al., 2018).

Colorectal cancer is one of the most common types of cancer, being the 3rd most common type of cancer in men and the 2nd in women (Muller et al., 2016). Most colorectal cancers occur sporadically, only 20-30% have hereditary components (Whiffin et al., 2014).

In 2018, 97,220 new cases of colon cancer, 43,030 new cases of rectal cancer and 8,580 new cases of anal cancer were registered in the United States (Siegel et al., 2018).

3.3. Lung cancer

The lungs are the primary organ of the human respiratory system and are located on either side of the heart. Located in the thoracic cavity, the lungs are uneven (the right one being larger), are part of the lower respiratory tract that begins with the trachea, and in the lung, branches into the bronchi and bronchioles, and the bronchioles continue with an alveolar duct that ends with pulmonary alveoli (Figure 10) (Arakawa et al., 2000).

Lung cancer is differentiated into two types of cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC - non-small cell carcinoma). Histologically, lung carcinoma can be classified into SCLC, squamous cell cancer, adenocarcinoma, and large cell lung cancer (Lemjabbar-Alaoui et al., 2015; Riihimaki et al., 2014).

Most lung cancers indicate paracrine and autocrine stimulation. NSCLC and SCLC overexpress tyrosine kinase receptors, especially EGFR (Epidermal Growth Factor Receptors),

sometimes even producing ligands for EGFR. Thus, in the signaling path, the transcription is activated; In most cases, transcription is overactivated, resulting in numerous cells that will further divide and lead to invasion and metastasis. In many cases, pro-angiogenic factors are activated (Imyanitov et al., 2005).

Lung cancer is caused by DNA degradation, but also by epigenetic changes (Hammerschmidt and Wirtz, 2009). Smoking is one of the main causes of the development of lung cancer, containing over 70 carcinogens (Hecht, 2012). Passive smoking is also a cause of lung cancer in non-smokers. Asbestos is another compound that potentiates the development of lung cancer and many other lung diseases (O'Reilly et al., 2007). Air pollution and exposure to other physical and chemical factors, such as radiation or toxic gases, fuel the risk of developing lung tumors, affecting the development of respiratory and lung tract cells (Cogliano et al., 2011).

The repair mechanisms no longer cope with the changes in the cells, so that a large number of activated oncogenes accumulate. The most affected signaling pathway, in the case of lung cancers, is G proteins coupled with autocrine receptors, the p53 tumor suppression system and the RAS / RAF / MEK / MAPK pathway (Imyanitov et al., 2005).

4. Onychomycosis

Onychomycosis is a nail infection characterized by discoloration, thinning and detachment of the nail from the conjunctival bed (Gupta et al., 2000). This is an infection with a high frequency in people over 60 years. In general, onychomycosis is caused by dermatophytes (Westerberg and Voyack, 2013). In the most common cases, onychomycosis is caused by infection with dermatophytes of the genus *Trichophyton*. Microorganisms, such as *Candida*, cause infections especially in the fingernails (Thomas et al., 2010). Infections caused by molds that do not fall into the group of dermatophytes are less common, but there have been studies showing that these infections are closely related to immunodeficiency caused by viral infections (Surjushe et al., 2007).

Treatment for onychomycosis requires a therapeutic combination that completely inhibits the development of the infectious agent. Treatment varies depending on the degree of infection, but the patient's history must also be taken into account, so as to avoid interaction with other existing medications (Scher et al., 2007).

Rhodotorula mucilaginosa is a yeast species that generally infects immunocompromised hosts (Ioannou et al., 2019). In some situations, it may be responsible for producing onychomycosis, even in patients without immunodeficiencies (Ge et al., 2019; Simon et al., 2014; Loss et al., 2011). *R. mucilaginosa* is resistant to most azoles, sometimes it can be controlled with ketoconazole (Ge et al., 2019).

Meyerozyma guilliermondii generally causes skin infections in humans, being associated with onychomycosis (Guler et al., 2017; Ghannoum et al., 2000; Dick et al., 1985). A co-infection with *R. mucilaginosa* and *M. guilliermondii* has been reported in an elderly person with a medical history indicating hepatitis B virus infection (Pârvu et al., 2019).

II. Personal contribution

1. Materials and methods

1.1. Allicin synthesis and characterization

Allicin was synthesized and analyzed according to the method described by Jansen et al., 1987 (Jansen et al., 1987; Pârvu et al., 2019). These determinations were made by Lecturer Dr. Augustin Cătălin Moț, in the laboratory of Chemistry - Faculty of Chemistry and Chemical Engineering of Babeș-Bolyai University, Cluj-Napoca**.**

1.2. Isolation, cultivation and molecular identification of *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* fungi producing onychomycosis

M. guilliermondii (Wick.) Kurtzman & M. Suzuki and *R. mucilaginosa* (A. Jörg.) F.C. Harrison were isolated from the onychomycosis-affected nails of an 88-year-old patient with chronic HBV hepatitis. The fungal samples were identified by morphology and characteristics of the culture colonies after incubation at 22 ° C for three days. The experiments were performed in triplicate (Pârvu et al., 2019). These determinations were made by Prof. Univ. Dr. Marcel Pârvu, in the Botany laboratory of the Faculty of Biology and Geology, UBB Cluj-Napoca.

This study was approved by the ethics commission of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, and the patient was informed and filled in the consent and enrollment form in the study.

M. guilliermondii and *R. mucilaginosa* species were molecularly confirmed targeting Internal Transcribed Spacer using ITS1 and ITS2 primers (White et al., 1990). Molecular analysis was performed by Dr. Cristina Mircea, in the Molecular Biology laboratory of the Institute for Interdisciplinary Research in Bio-Nano-Sciences.

1.3. *Allium* extract preparation

Allium plant extracts were prepared by cold, at room temperature, for three days (Anonymous, 1993; Ionescu-Stoian, 1977; Pârvu et al., 2015), using small fragments of plants that were immersed and extracted in 70% alcohol (Merck, Bucharest, Romania). The plants were collected from the "Alexandru Borza" Botanical Garden in Cluj-Napoca.

Leaves were used to prepare the *Allium fistulosum* extract, and bulbs were used to prepare the *Allium sativum* extract. For each *Allium* species used, in this study, a specimen-voucher was kept at the Herbarium of the "Babeș-Bolyai" University, Cluj-Napoca, Romania (*Allium sativum* L. - CL666161; *Allium fistulosum* L. - CL659761). The fluid extract obtained by filtration was prepared in a ratio of 1: 1.2 with 30% ethanol for *A. fistulosum* and 1: 1 with 20% ethanol for *A. sativum*, the ratios being expressed in mass: volume (g: mL). These determinations were made by Prof. Univ. Dr. Marcel Pârvu, in the Natural Extracts Laboratory of the Faculty of Biology and Geology, UBB Cluj-Napoca.

1.3.1. Phytochemical characterization

Separation of the compounds was performed using the Agilent 1200 HPLC system (Waldbronn, Germany) (Pârvu et al., 2019).

These determinations were made by Lecturer Dr. Augustin Cătălin Moț, in the laboratory of Chemistry - Faculty of Chemistry and Chemical Engineering of Babeș-Bolyai University, Cluj-Napoca.

1.3.2. Antifungal effect

In order to determine the antifungal effect of *Allium sativum* extract, by the agar dilution method, the fungal cells were inoculated with different concentrations of garlic extract: 2%, 4%, 6%, 8%, 10% and 12% for *M. guilliermondii* and a concentration of 14% was added in the case of *R. mucilaginosa*. The negative control was without extract. A control with an antifungal compound based on naphthifine hydrochloride (Exoderil Sandoz, 10mg / mL) was used, at various concentrations: 0.5%, 1%, 3%, 4% and 5% for *M. guilliermondii* and 0.1%, 0.5 %, 1%, 1.5%, 2%, 2.5% and 3% for *R. mucilaginosa*.

The antifungal effect was expressed in minimum inhibitory concentration (MIC / CMI), where the percentage of mycelial growth inhibition (P) for each concentration was calculated using the formula P = (CT) x 100 / C, where C is the diameter of the control colony, and T the diameter of the treated colony (Pârvu et al., 2019).

These determinations were made by Prof. Dr. Marcel Pârvu, in the Laboratory of Natural Extracts of the Faculty of Biology and Geology, UBB Cluj-Napoca.

1.3.3. Antioxidant effect

In vitro antioxidant activity was evaluated using DPPH and ABTS radical dissociation tests according to the protocol published in the literature (Perez et al., 1975). The results are expressed in quercitin equivalents, obtained by the calibration curve, using quercitin standard (Pârvu et al., 2019).

For the evaluation of the antioxidant activity in vivo, Albino Wistar male rats, weighing between 200 and 250 g, from the biobase of the "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania (Andreicuţ et al., 2018) were used.

The determination of the biomarkers of systemic oxidative stress was performed by determining the total oxidative status (TOS) in the serum, by colorimetric reaction, and the results were expressed in μmol H_2O_2 equivalent / L. The total antioxidant response in serum (ART) was determined by colorimetric method, and the results expressed in Trolox equivalent / L. The oxidative stress index (OSI) is an indicator of the degree of oxidative stress and was represented as TOS / TAR (Pârvu et al., 2014).

The Griess reaction was used to determine the synthesis of nitric oxide and the results were expressed in μmol / L (Pârvu et al., 2014). Malondialdehyde (MDA) was measured using thiobarbituric acid. Total thiols were estimated using Ellman reagent. All tests for serum determinations were performed by spectrophotometric methods using the Jasco V-530 UV-Vis Spectrophotometer (Jasco International Co. Ltd, Tokyo, Japan).

These determinations were made by Prof. Univ. Dr. Alina Elena Pârvu, in the pathophysiology laboratory - UMF Cluj-Napoca.

1.4. Scanning electron microscopy

The morphology of *R. mucilaginosa* and *M. guilliermondii* fungi was analyzed for control cells using the JSM 5510 LV electron scanning microscope (SEM) (JOEL Co, Tokyo, Japan). The samples were prepared according to the literature (Moț et al., 2017). These determinations were performed in the Electron Microscopy laboratory "Constantin Crăciun" - UBB Cluj-Napoca.

1.5. Transmision electron microscopy

The ultrastructure of the control fungi and those treated for one hour with MIC extract was analyzed using the transmission electron microscope (TEM) JEOL JEM 1010.

The samples were prepared according to the literature (Moț et al., 2017). These determinations were performed in the Electron Microscopy laboratory "Constantin Crăciun" - UBB Cluj-Napoca.

1.6. Cell culture

Cells (Table 2) BJ, DLD-1, MDA-MB-231, MCF-7 and SK-MES-1 were purchased from ATCC (American Type Culture Collection) through LGC Standards GmbH, Wesel, Germany. Normal human HaCaT keratinocytes were provided by the cell line service of the German Cancer Research Center - Heidelberg, Germany. These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.7. Cell viability assay

After 24 h of treatment, the cell viability rate was measured using the MTT technique (3-(4.5 dimethyl thiazol-2-yl) -2.5 diphenyl tetrazolium bromide) at 570 nm (Sigma-Aldrich, St. Louis, US). The plates were read using the TECAN SPARK 10M spectrophotometer (TECAN, Austria GmbH, Grodig, Austria).

Using the same protocol, the antiproliferative effect of allicin, 5-FU (5-Fluorouracil) and therapeutic combinations was evaluated by the MTT method (Țigu et al., 2020). These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.8. Fluorescence confocal microscopy

After 24 hours of exposure to half of the IC_{50} and the combination of these, morphological changes were followed using the triple staining method developed in the MedFuture cell therapy laboratory, UMF Cluj-Napoca and published in a previous paper (Budisan et al., 2019). The images were processed with ImageJ software (Țigu et al., 2020). These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.9. Biochemical determinations: LDH, CAT and Caspase 3

The cells were incubated in 12-well plates at a concentration of 100,000 cells per well at a final volume of 2000 μL. After 24 h of incubation, the cells were treated with three different concentrations of extract: 10%, dose of IC_{50} and dose below IC_{50}. The supernatant was collected after 24 hours of incubation with extracts, and by freezing / thawing the cellular homogenate was obtained. Lactate dehydrogenase (LDH) and Catalase (CAT) were determined from the cell supernatant to check their level in the culture medium. LDH activity was measured at 340 nm using the LDH Activity Kit for in-vitro studies (Biomaxima, Lublin, Poland), and samples were analyzed on the 2000 Evolution spectrophotometer (Biochemical Systems International, Italy). Catalase activity was determined at 240 nm using the VWR UV-1600PC UV-Vis spectrophotometer (Vives - Bauza et al., 2007). Caspase 3 (Casp3) was determined using the supernatant, using the kit for activated Caspase 3 (Invitrogen), and the plate was read using the TECAN SPARK10M spectrophotometer (TECAN, Austria GmbH, Grodig), following the protocol in the analysis kit. These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca and within the Cluj-Napoca Institute for Biological Research.

1.10. Flow cytometry analysis

To investigate cell death, such as apoptosis or necrosis, after 24 hours of treatment, the technique of detecting cell death by flow cytometry was applied (Țigu et al., 2020). These determinations were made by Dr. Ancuța Jurj, at the Genomics Center - UMF Cluj-Napoca.

1.11. Wound healing assay

Colorectal cancer and lung cancer cells were cultured in flat-bottomed 96-well plates. In each well, 50,000 cells were seeded and incubated overnight. After 24 h, the treatment was added for one day, and then the culture medium was changed and the cells deprived of fetal bovine serum (Liang et al., 2007). The wound was created using a pipette tip and monitored until the wound in the control sample was completely closed. Images were processed using ImageJ software (Țigu et al., 2020). These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.12. Colony assay

For each treatment and control, at both cell lines used, 500 cells were seeded per well, in 6-well plates. After 24 hours of treatment, the culture medium was changed and the cells were analyzed for a period of two weeks, in order to obtain colonies. After two weeks, the cells were fixed with fresh methanol fresh from the freezer and stained with purple crystals dissolved in methanol. The reagents were purchased from Sigma-Aldrich, St. Louis, USA) (Țigu et al., 2020; Rafehi et al., 2011). These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.13. Western blot

The cells were resuspended in Trizol, and the protein fraction was used for subsequent determinations. (Țigu et al., 2020). These activities were carried out within the Research Center for Advanced Medicine - MedFuture - UMF Cluj-Napoca.

1.14. Ethics and animals

The animal model experiments were carried out at the Faculty of Medicine at the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca and at the Faculty of Veterinary Medicine at the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. All experiments were organized in accordance with Law 43/2014 on the protection of animals used for scientific purposes and European Directive 63/2010. The experiments obtained the approval of the Sanitary Veterinary and Food Safety Directorate of Cluj-Napoca (no. 22 / 13.12.2016).

The animals were bought from the biobase of the University of Medicine and Pharmacy "Iuliu Haţieganu" Cluj-Napoca, white rats of the Wistar breed, adult females weighing between 150-180 g, F1 generation. They were housed in zoo-hygienic conditions, light-dark cycle 12/12 h, at 20ºC, protected from noise. The animals were handled carefully and quietly. Access to standard water and food was ensured, ad libitum, the standard diet consisting of special granules for rodents, obtained from the Cantacuzino Institute, Bucharest (Farcaș et al., 2019; Toma et al., 2017; Pârvu et al., 2014 ; Roman and Puică, 2013).

2. Results and discussions

1.15. Allicin: antitumoral potential

1.15.1. Antiproliferative effect of Allicin and 5-FU against colorectal and lung cancer cell lines

In this study, allicin was synthesized as an adjuvant, a sulfur compound synthesized in house, in combination with 5-FU. Natural compounds interact with different molecular mechanisms; Depending on the nature of the pathology, some natural compounds reduce DNA degradation, oxidative stress, inhibit proliferation or reduce genetic alterations, being used as antitumor agents (Moosavi et al., 2018; Nabavi et al., 2018; Braicu et al., 2017; Budisan et al., 2017; Rajput and Mandal, 2012). Allicin is a natural compound, with adjuvant potential, with numerous biological effects demonstrated and intensively studied in recent decades; the antioxidant, anti-inflammatory, immunostimulatory or antifungal effects have been of interest to numerous research groups (Pârvu et al., 2019; Toma et al., 2019). Allicin consumption, by adding garlic to the diet, significantly reduces the risk of developing chronic inflammation, increased oxidative stress and DNA destruction (Padiya et al., 2014; Schafer and Kaschula, 2014; Dhawan and Jain, 2005).

The aim of this study is to highlight the synergistic effect of the therapeutic combination of 5-FU and allicin against lung and colorectal cancer. By reducing the dose of 5-FU and replacing it with allicin, similar or better results than conventional therapy (with 5-FU) are targeted.

1.15.1.1. Viability assay

The inhibitory effect was evaluated for different concentrations of 5-FU and allicin, by analyzing the viability of normal fibroblast (BJ) cells, colorectal adenocarcinoma (DLD-1) and squamous cell adenocarcinoma (SK-MES-1), after 24 h, by the MTT method (Figure 11). After 24 h of 5-FU treatment, all three lines showed a similar IC_{50}, 195.5 µM for BJ, 214.3 µM for DLD-1 and 202.2 µM for SK-MES-1, indicating a nonspecific action of 5-FU. This compound is unable to distinguish tumor cells from normal cells; this is a general aspect for cytostatic compounds. Following allicin exposure for 24 h, the SK-MES-1 line was most sensitive with an IC_{50} of 8,625 µM, then the BJ line with an IC_{50} of 33.17 µM, and the colorectal cancer line was the most resistant with an IC_{50} of 53.53 µM, highlighting a different effect compared to 5-FU (Figure 12).

After obtaining IC_{50} values, the effects of the therapeutic combination between 5-FU and allicin were investigated. The antiproliferative effect of the combination of 5-FU and allicin in half of the IC_{50} was significantly better compared to individual treatments, as shown in Figure 13. The antiproliferative effect on colon and lung cells was more pronounced in the group treated with the combination. of 5-FU and allicin, compared with 5-FU and allicin as single treatments at the IC_{50} dose.

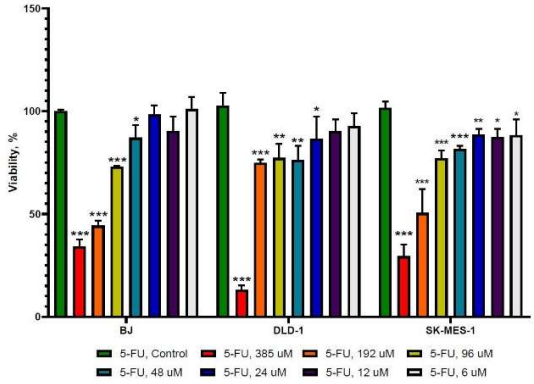


Figure 11. Cell viability analysis, after 5-FU treatment. It inhibited cell growth in a dose-dependent manner, with a similar effect for the three incubated cell lines, for 24 h with 6, 12, 24, 48, 96, 192 and 385 μM 5-FU. The results with P <0.05 were considered statistically significant (* P <0.05, ** P <0.01 and *** P <0.001) (Țigu et al., 2020).

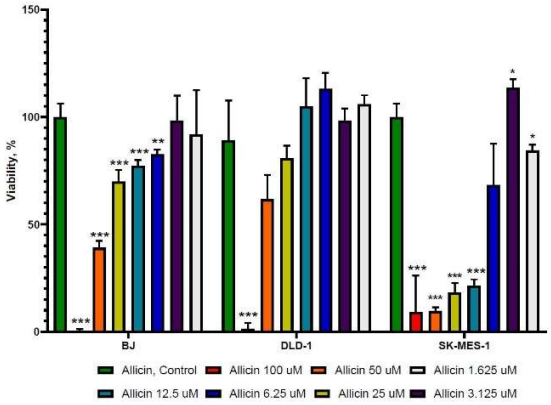


Figure 12. Cell viability analysis, after exposure to 24 hours of allicin treatment. Allicin had a different inhibitory effect for the three cell lines treated with 1,625, 3,125, 6.25, 12.5, 25, 50 and 100 μM allicin. The results with P <0.05 were considered statistically significant (* P <0.05, ** P <0.01 and *** P <0.001) (Țigu et al., 2020).

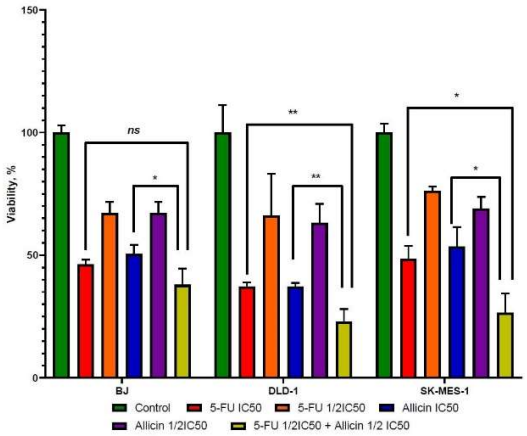


Figure 13. Investigation of cell viability for cells treated with the combination of 5-FU and allicin compared to single-treated groups. This comparison indicates that combination therapy is more effective against tumor cells compared to individual treatments. NS - insignificant; Control - untreated group; 5-FU IC_{50} - group treated with 5-FU at IC50; 5-FU 1/2 IC_{50} - group treated with half of the IC_{50} for 5-FU; Allicin IC_{50} - group treated with allicin IC50; Allicin 1/2 IC_{50} - group treated with half IC_{50} for allicin; 5-FU 1/2 IC_{50} + Allicin 1/2 IC_{50} - the group with the combined treatment between the halves of IC_{50}. The results with $P < 0.05$ were considered statistically significant (* $P < 0.05$, ** $P < 0.01$) (Țigu et al., 2020).

1.15.1.2. Morphological analysis

For a complete toxicity assessment, induced by the combined treatments, the cell morphology was investigated using the triple staining technique developed in the cell therapies laboratory of MedFuture - UMF Cluj-Napoca (Țigu et al., 2020; Budișan et al., 2019). Mitochondrial networks were labeled red using Mitotracker as a marker, the cytoskeleton was labeled green by binding phalloidin-FITC to actin filaments, and the nucleus was labeled blue using 100 μM DAPI.

Figure 14 shows the changes induced by the treatments. Co-treatment has a pronounced antiproliferative effect, cell death being induced in all three cell lines used. In the case of BJ cells, cell damage is mainly attributed to 5-FU. These cells have a much lower division rate compared to tumor cells because the cell regeneration has been greatly affected.

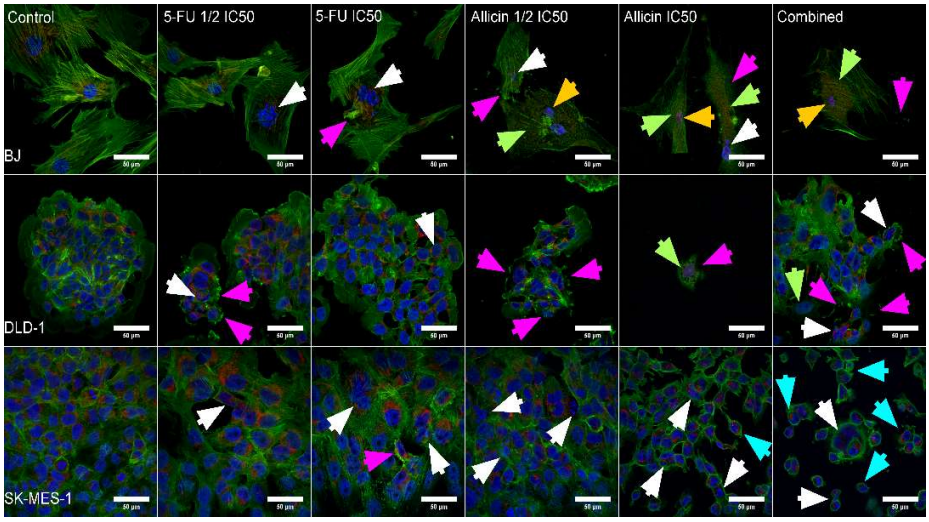


Figure 14. Investigation of morphological changes by confocal fluorescence microscopy. Cellular architecture is highlighted using Mitotracker-Red (red - mitochondrial networks), phalloidin-FITC (green - actin filaments) and DAPI (blue - nucleus). Co-treatment has a cytotoxic effect on all lines examined. Following administration of the combination therapy, cell populations for DLD-1 and SK-MES-1 are reduced, in addition the spaces between cells are enlarged. The normal shape of the cells (control group) is changed, becoming slightly round in the case of treatments. Legend: white arrows - destruction of the nucleus; orange arrows - nuclear shrinkage; magenta arrows (pink) - damage to the cytoskeleton; green arrows - decreased mitochondrial activity; cyan arrows (light blue) - AVD; BJ - normal fibroblasts; DLD-1 - colorectal cancer cells; SK-MES-1 - lung cancer cells; , 5-FU ½ IC50 - group treated with 5-FU IC50, 5-FU IC50 - group treated with 5-FU IC50, Allicin ½ IC50 - group treated with half IC50 for allicin, Allicin IC50 - group treated with allicin at IC50 , Combined - group treated with the combination of IC50 halves (Țigu et al., 2020).

DLD-1 cells showed a reduction in population in all treated groups, but especially in the allicin-treated group. At the IC50 dose, allicin significantly reduced the number of cells, and in the case of co-treatment, cellular changes were suggested that suggest the onset of apoptosis. Cells treated with half the dose of IC50 showed cellular changes attributed to cellular stress.

In the case of lung cancer cells, nucleus damage was observed especially in the allicin-treated group, and cells treated with the therapeutic combination of the two doses at half of the IC50s, a decrease in cell volume was observed (Apoptotic volume decrease - AVD). This is observed only in the lung line after co-treatment. Very few cells had AVD in the allicin group. In the case of normal cells, a decrease in mitochondrial activity was observed as a result of allicin treatment and co-treatment; a specific aspect of the normal line was the reduction of the core shrinkage. In the case of DLD-1, cytoskeleton damage was more intense compared to SK-MES-1 and BJ, but the characteristics of apoptosis were less visible.

The results obtained are confirmed by the existing literature. The synthesized allicin inhibited the migration and formation of colonies, and in combination with 5-FU the inhibitory effect was much more pronounced. The most visible effects were the induced morphological changes, the degradation of the cytoskeleton and the change in the shape of the cells indicating the onset of cell death. In the case of pulmonary adenocarcinoma cells, the effects were more visible. Apoptotic volume decrease (AVD) is a specific aspect of this cell line, not being observed in DLD-1. AVD has been reported as a sign of apoptosis (Gruhlke et al., 2019).

It is also visible that 5-FU and allicin, in combination, produce morphological changes differently: cytoskeleton damage and nuclear damage in the case of DLD-1, AVD, which indicates the onset of apoptosis, in the case of SK-MES-1.

In the images representing SK-MES-1, inhibition of cell growth and cell division and large spaces between cells are observed, following the administration of 5-FU treatment. Allicin treatment produced changes in cell architecture, and the treated cells became spherical and had morphological aspects, such as AVD, which indicates the onset of apoptosis. The results indicate that both the individual treatments and the therapeutic combination affected the morphology of the tumor cells.

1.15.1.3. Flow cytometry analysis

The flow cytometry test with Annexin V / P.I was used to evaluate the apoptosis / necrosis rate. The results indicate that allicin and 5-FU act as stimulators of early apoptosis in normal cells (Figure 15), while for DLD-1 and SK-MES-1 the cell death profile was changed to late apoptosis and necrosis (Figures 16 and 17). Co-treatment was very effective in inducing cell death, especially in the case of DLD-1, while in the case of SK-MES-1, the ratio of viable cells to cells in cell death was higher, probably due to the inability to detect SK-MES-1 cells that died before they could be labeled, these aspects are confirmed by morphological analyzes where SK-MES-1 shows numerous round cells.

Cell death can be induced by cellular programming such as apoptosis or autophagy or by necrosis (Song et al., 2015). In this study, we investigated aspects that allow us to identify the type of cell death induced by 5-FU and allicin; there were differences between the two types of cancer. In the case of SK-MES-1, cell death was dependent on caspases, being a programmed cell death by apoptosis; morphological aspects and Western blot analyzes confirmed the activation of

Caspase 3 and the release of cytochrome C from mitochondria (Figure 20 - subchapter dedicated to Western blot analysis). In the case of DLD-1, cell death was induced by caspase-independent mechanisms, being a cell death that did not require the initiation of apoptosis.

The results obtained, after the administration of the combined treatment, suggest a state of late apoptosis in the case of both cell lines, compared to the normal cells that were in the state of early apoptosis. This proves that toxicity was latently induced in the normal line compared to tumor lines.

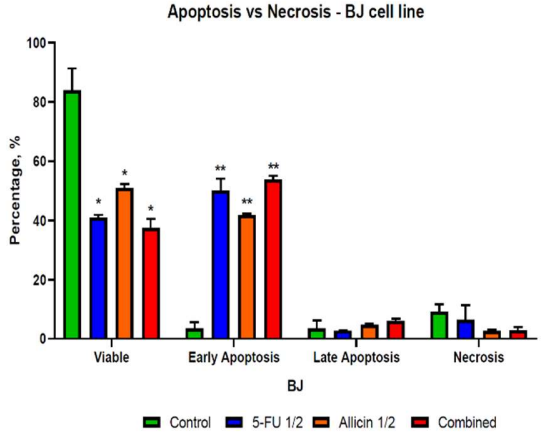


Figure 15. Presentation of the distribution of fibroblast cell populations, after 24 hours of treatment. There is a change in the profile of populations following treatments, significantly increasing the percentage of cells in apoptosis over time (Țigu et al., 2020).

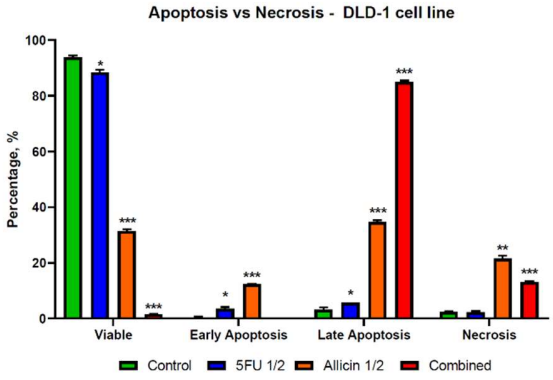


Figure 16. Presentation of the distribution of DLD-1 colorectal adenocarcinoma cell populations. Population change towards late apoptosis and necrosis can be observed at co-treatment and allicin treatment, while 5-FU has very little reduced cell viability (Țigu et al., 2020).

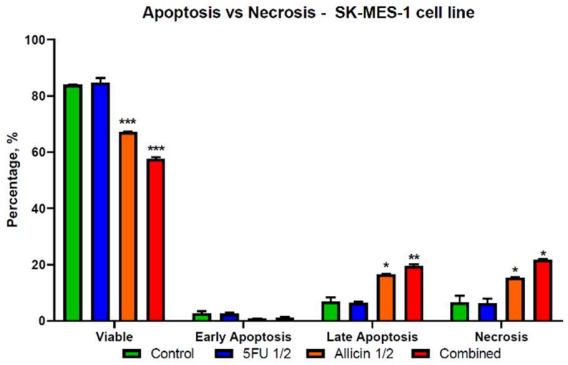


Figure 17. Presentation of the distribution of SK-MES-1 pulmonary adenocarcinoma cell populations. The change in the population is not drastic, compared to DLD-1, but there is a change in the profile towards late apoptosis and necrosis in allicin-containing treatments (Țigu et al., 2020).

1.15.1.4. Wound healing assay

Migration is a fundamental feature of the tumor cell. Inhibition of the migration mechanism is a therapeutic target, to limit the development of tumors. The effect of allicin and 5-FU on migration was evaluated, both independently and in combination, and the results obtained after 24 hours of treatment are shown in Figure 18.

In this study, certain effects of allicin were highlighted as a single treatment, but also as an adjuvant in combination with 5-FU. Allicin inhibited migration and stimulated the initiation of cell death in the case of tumor cell lines of the colon and lung, like the data mentioned in the literature (Chu et al., 2012; Bat-Chen et al., 2010). The beneficial effects of allicin are attributed to the thiol group, which has the potential to degrade microtubules by interacting with tubulin polymerase. By inhibiting tubulin polymerization, cell migration and polarization are inhibited and thus cell division is significantly reduced (Xiang et al., 2018; Gruhlke et al., 2016).

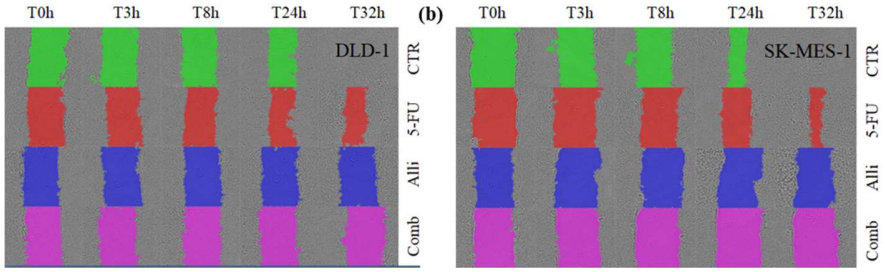


Figure 18. Investigation of tumor cell migration after 24 hours of treatment. (A) DLD-1 cells show high sensitivity to allicin and combination therapy, while 5-FU had limited effect on migration. (B) SK-MES-1 cells show high sensitivity to allicin and co-treatment, while 5-FU has not been able to completely inhibit migration (Țigu et al., 2020).

1.15.1.5. Colony assay

The ability of colonies to form is a property of tumor cells to develop new metastatic sites from a single cell, in most cases a circulating tumor cell. These newly formed colonies will generate metastatic sites, distant from the primary site of the tumor, making treatment difficult. Inhibition of this mechanism and the simultaneous control of several properties of tumor cells (eg migration, proliferation) may be a therapeutic direction to help patients in advanced stages of tumors.

Inhibition of colony formation in lung and colorectal tumor cells was investigated after administration of allicin, 5-FU, and combination therapy for 24 h, and the results are shown in Figure 19.

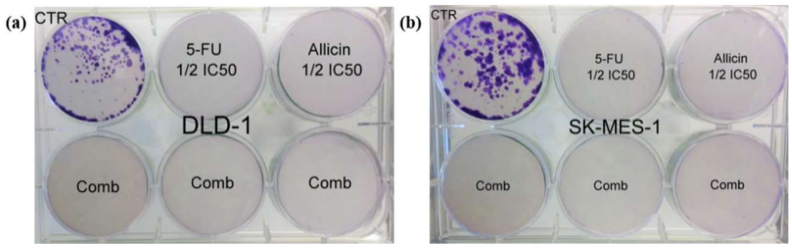


Figure 19. Investigation of colony formation after 24 hours of treatment. (A) DLD-1; (B) SK-MES-1, colony inhibition was confirmed for all treatments applied (Țigu et al., 2020).

1.15.1.6. Western blot

To confirm the previous results and to validate the cell death induced by allicin, 5-FU and the therapeutic combination, the expression level of some key proteins in the apoptotic pathways, cytochrome C (CytC) and caspase 3 (Casp3) were evaluated. Thus, CytC was evaluated as an indicator of the initiation of intrinsic apoptosis, and Casp3 as an effector of the apoptosis mechanism, being also a molecule linking the intrinsic and extrinsic pathway.

GAPDH was used as a normalizer, and the samples were processed in duplicate, the results being shown in Figure 20.

Apoptosis is a mechanism that can be activated by oxidative stress and exposure to xenobiotics (Xu et al., 2014), but also by proapoptotic agents, such as cytostatic or adjuvant compounds. Both apoptotic pathways, the intrinsic and the extrinsic pathway, converge in the activation of caspase 3, the intrinsic pathway being modulated by the release of cytochrome C (CytC). In the case of the extrinsic apoptotic pathway, external stimuli activate caspase 8 through

cell death receptors, and then caspase 3 is activated, which is the key protein for performing cell death by apoptosis. There are also cross-talk interactions between the intrinsic and extrinsic apoptotic pathways, such as the interaction between caspase 8 and Bid (Fulda et al., 2010; Bortner and Cidlowski, 2007).

The results are consistent with other publications and demonstrate that allicin has antitumor potential (Chen et al., 2018; Lichota and Gwozdzinski, 2018; Petrovic et al., 2018) against two types of tumor cells, and 5-FU combined with allicin has similar effects to the synergistic antitumor effect obtained against liver cancer cells (Zou et al., 2016).

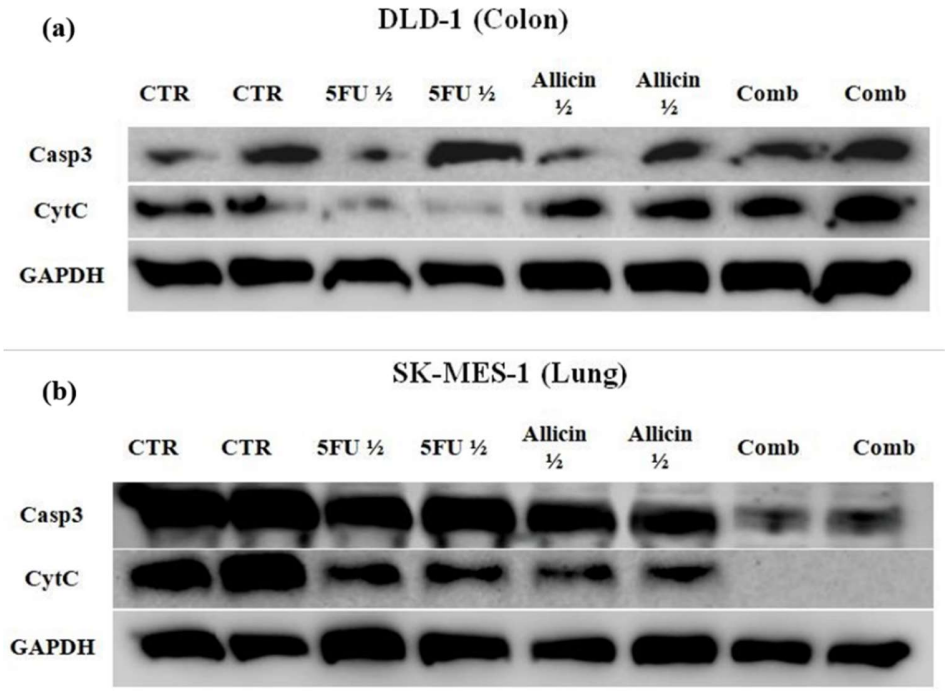


Figure 20. Western blot analysis. (a) Determinations for DLD-1 cell line, GAPDH used as control for Caspase 3 and cytochrome 3. (b) Determinations for SK-MES-1 cell lines, molecular analysis as for DLD-1 cell line (Țigu și colab., 2020).

1.16. *Allium sativum* extract (garlic)

Due to its chemical composition, *A. sativum* is a very good antibacterial, antifungal, anti-inflammatory and antitumor agent (Pârvu et al., 2019; Balea et al., 2018; Bhandari, 2012); the organosulfur compounds in the composition of garlic are responsible for most of the biological properties (Matysiak et al., 2015).

2.2.1. Chemical composition

By using the high-performance liquid chromatography technique coupled with mass spectrometry (HPLC-MS), important data on the chemical composition of the hydroalcoholic extract of garlic were obtained. For the determination of phenolic compounds, from hydroalcoholic extracts, an analysis method was developed within the analytical chemistry laboratory of the Faculty of Chemistry and Chemical Engineering, UBB Cluj-Napoca (Table 3 and Table 4).

Table 3. Elution time, analytic method characteristics and determination of alliin and allicin concentrations in analyzed samples (n=4)

No.	Compound	$t_{elution}$ (min)	R^2	LOD (µg/mL)	*A. sativum* (µg/mL)*
1	Alliin	3.77	0.9999	5.8	1410±50
2	Allicin	15.40	0.9999	14.1	380±15

*LOD - limit of detection, R^2 - coefficient of determination for the calibration curve (at six concentrations). The indicated intervals represented as mean ± standard deviation (n = 4). * Data on A. sativum are published (Pârvu et al., 2019).*

Table 4. Elution time, analytic method characterization and determination of phenolic acids and flavonoids in extract

No.	Compound	$t_{elution}$ (min)	R^2	LOD (µg/mL)	*A. sativum* (µg/mL)*
1	Gentisic acid	8.13	0.9997	3.4	60±5
2	Chlorogenic acid	9.15	0.9995	4.6	65±5
3	4-hydroxybenzoic acid	10.31	0.9999	2.3	25±3
4	Rutin	11.39	0.9998	2.7	-
5	Isoquercitrin	11.91	0.9999	2.2	-
6	p-Coumaric acid	12.38	0.9999	1.9	44±4
7	Quercitrin	12.72	0.9998	2.7	-
8	Ferulic acid	12.85	0.9999	2.0	-
9	Quercetin	16.35	0.9997	3.3	-
10	Kaempferol	19.77	0.9998	2.7	-

*LOD - limit of detection, R^2 - coefficient of determination for the calibration curve (at six concentrations). The indicated intervals represented as mean ± standard deviation (n = 4). * Data on A. sativum are published (Pârvu et al., 2019).*

2.2.2. Onychomycosis characterization and fungi molecular identification

The nail was analyzed from a patient with a double associated infection, *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* (Figure 21); for the identification of fungi, the colonies obtained on SDA were characterized. Colonies of *M. guilliermondii* have a white

mucilaginous appearance, and *R. mucilaginosa* has a mucilaginous appearance and a red color (Figure 21B).

Figure 21. Nails affected by onychomycosis (A); B. White colonies (of *M. guilliermondii*) and red colonies (of *R. mucilaginosa*) obtained from infected nail fragments and cultured on SDA medium (Pârvu et al., 2019).

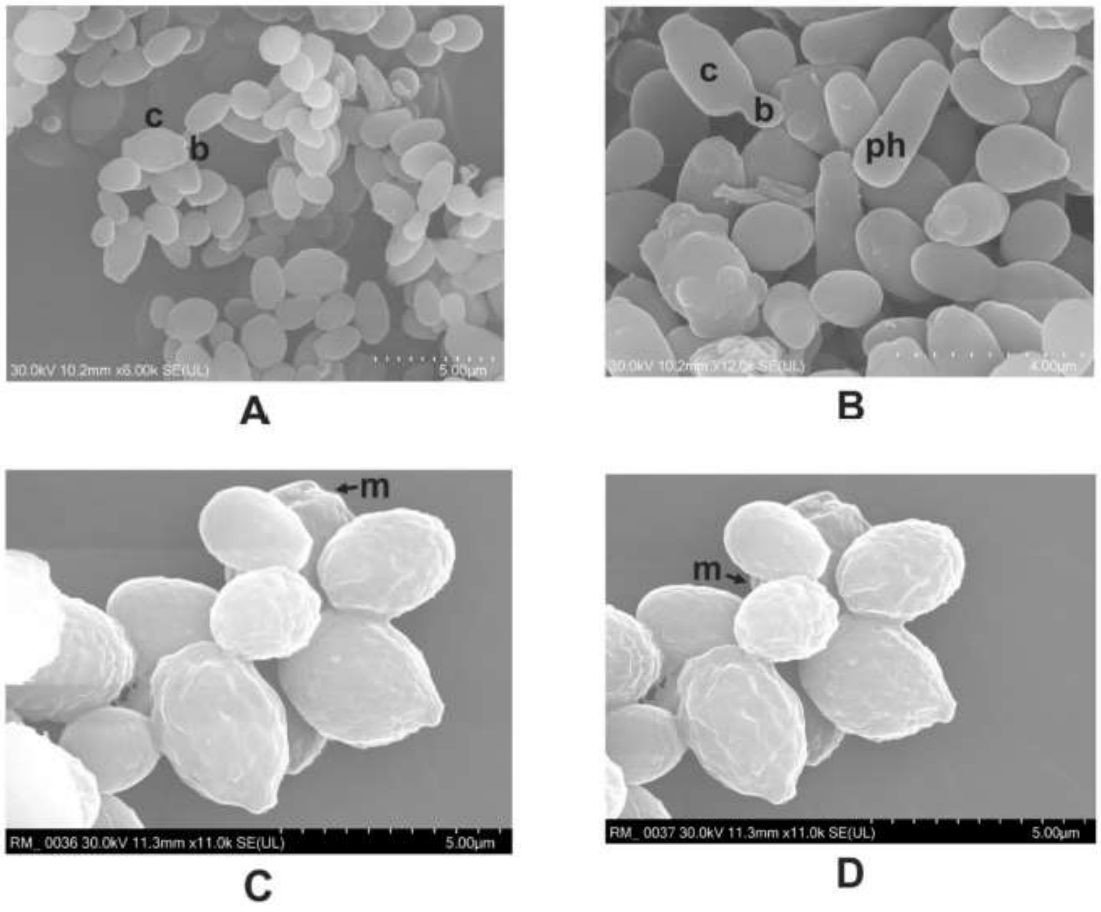


Figure 22. Electron scanning microscopy shows *M. guilliermondii* (A, B) and *R. mucilaginosa* (C, D). Legend: b, buds; c, cell; m, mucilage; ph, pseudohypha (Pârvu et al., 2019).

Morphological characterization was performed using electron scanning microscopy (SEM). *M. guilliermondii* cells have a spherical or sub-spherical shape of approximately 2.0-4.0 x 4.0-6.5 μm, with branches and pseudohyphae, having a fine appearance, but also dense blastoconids (Figure 22 A-B). SEM images for *R. mucilaginosa* show cells with a spherical-oval appearance with dimensions of 2.5-6.5 x 6.5x14.0 μm, with carotenoid pigments and without the formation of hyphae, in addition the mucilage that envelops the colonies of this fungus is very visible (Figure 22 D).

2.2.3. Antifungal effects

A. sativum inhibited the germination and growth of *M. guilliermondii* fungus on the agar medium. After three days of incubation, white mucilaginous colonies of 11-12 mm were present in the control sample, and in the treated samples a reduction in the size of the colonies was observed; the effect was directly proportional to the concentration of garlic extract added to the medium (Figure 23).

On SDA, with 4% extract, the colonies showed a diameter of 9-10 mm, while at 8% extract, the size was reduced to 3-4 mm. In addition, no colony was identified at 12% extract (120 mg / mL). The maximum inhibitory effect of naphthifine hydrochloride was 55-56%, which corresponds to a concentration of 5% (500 μg / mL) in the case of *M. guilliermondii* isolate. Also, 55-56% inhibition was obtained at 3% and 4%, respectively, against the colony of M. guilliermondii (Figure 23; Table 5).

In the case of *R. mucilaginosa*, the control colonies had a mucoid appearance and a diameter of 12-13 mm after 72 hours of incubation on agar. The colonies were highlighted by their red coloration, due to the carotenoid content. On the medium with SDA, the garlic extract reduced the diameter of the colonies of *R. mucilaginosa*. At 4% extract, the colonies were reduced to 10-11 mm in diameter, and at 8% they reached 7-8 mm. The inhibitory effect of the extract against R. mucilaginosa was 22.5% in 4% and 90% in 12% concentration (Figure 24, Table 6*)*.

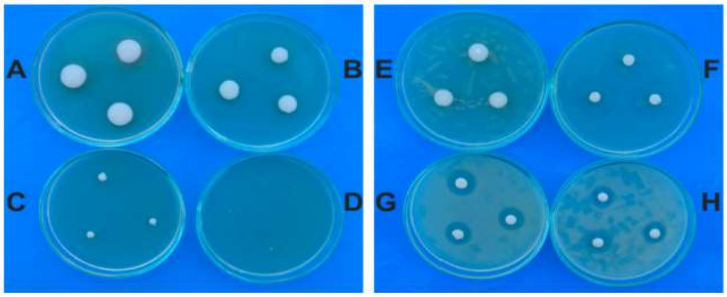


Figure 23. Colonies of *M. guilliermondii* on SDA: (A) Control; (B) 4% garlic extract; (C) 8% garlic extract; (D) 12% garlic extract; (E) Naphthifine 0.5%; (F) Naphthifine 1%; (G) Naphthifine 3%; (H) Naphthifine 5% (Pârvu et al., 2019).

The ultrastructural characteristics of *R. mucilaginosa* control cells highlight a dense lamellar cell wall and electron, plasmalemma, endoplasmic reticulum, mitochondria, lipids and glycogen accumulated in the cytoplasm, but also a spherical-oval nucleus.

In the cytoplasm of young cells, numerous small and uneven lipid granules were found, while in older cells large granules were found, some even fusing (Figure 26 A-D). Garlic extract caused irreversible damage to *R. mucilaginosa*, such as loss of structural integrity and germination capacity. Cell death was induced by precipitation of cytoplasmic contents and destruction of cellular organs and by alteration of the nucleus (Figure 26 E-F).

Table 5. The *in vitro* effect of *A. sativum* and naphthifine extract on *M. guilliermondii* fungus, determined by the agar dilution method (Pârvu et al., 2019)

Garlic extract (%)	**Colony diameter (mm)[a]**	**P[a]**	**Naphthifine solution (%)**	**Colony diameter (mm) [b]**	**P[b]**
C	11.66±0.81	0	C	11.66±0.81	0
2	11.16±0.40	4.28±0.40	0.5	10.0±0.63	14.23±0.63
4	9.66±0.51	17.15±0.51	1	8.33±0.51	28.55±0.51
6	6.66±0.51	42.88±0.51	2	6.83±0.83	41.42±0.83
8	3.50±0.54	69.98±0.54	3	5.16±0.40	55.74±0.40
10	1.33±0.51	88.59±0.51	4	5.16±0.40	55.74±0.40
12	0	100	5	5.16±0.40	55.74±0.40

[a]the effect of garlic extract; [b] the effect of naphthifine; C control (20% EtOH); P mycelium growth inhibition - results expressed as mean ± standard deviation for 6 experiments.

At TEM, control cells of *M. guilliermondii* show cell wall, plasmalemma, vacuoles, mitochondria and nucleus; these organs are very visible (Figure 25 A-D). The distinct layers of the cell wall and adjacent to the plasmalemma are observed in the dense electron field (Figure 25 C).

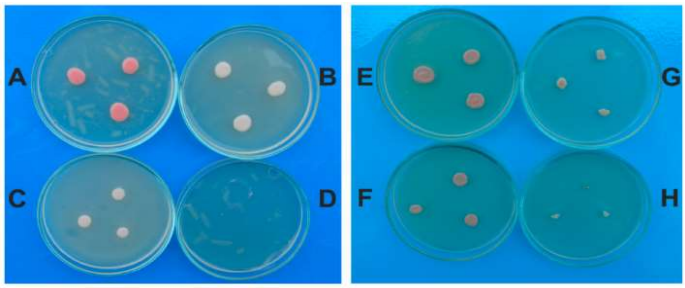
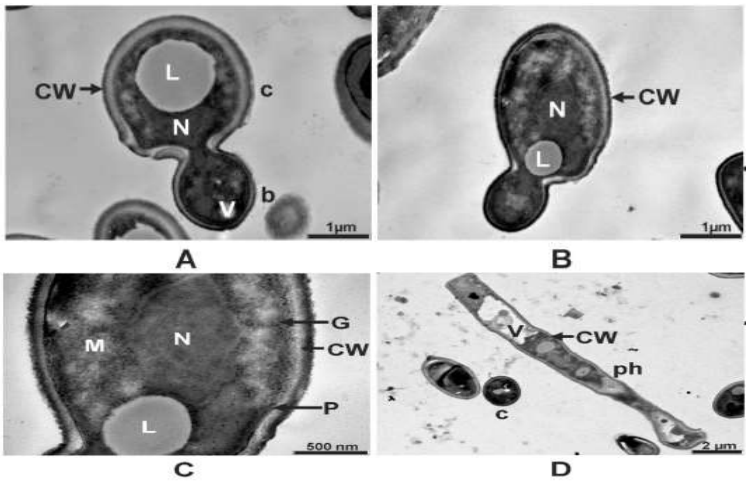


Figure 24. Colonies of *R. mucilaginosa* on SDA: (A) 4% garlic extract; (B) 6% garlic extract; (C) 8% garlic extract; (D) 10% garlic extract; (E) Control; (F) naphthifine 0.1%; (G) naphthifine 1.5%; (H) naphthifine 3% (Pârvu et al., 2019).

Tabelul 6. The in vitro effect of *A. sativum* and naphthifine extract on *R. mucilaginosa* fungus, determined by the agar dilution method (Pârvu et al., 2019)

Garlic extract (%)	Colony diameter (mm)[a]	P[a]	Naphthifine solution (%)	Colony diameter (mm) [b]	P[b]
C	13.33±0.51	0	C	13.33±0.51	0
2	12.16±0.75	8.77±0.75	0.1	12.83±0.40	3.75±0.40
4	10.33±0.51	22.50±0.51	0.5	10.5±0.54	21.23±0.54
6	9.0±0.63	32.48±0.63	1.0	7.83±0.40	41.26±0.40
8	7.33±0.57	45.01±0.57	1.5	6.33±0.51	52.51±0.51
10	4.0±0.63	69.99±0.63	2.0	3.66±0.81	72.54±0.81
12	1.33±0.51	90.02±0.51	2.5	1.33±0.51	90.02±0.51
14	0	100	3.0	0	100

[a]the effect of garlic extract; [b] the effect of naphthifine; C control (20% EtOH); P mycelium growth inhibition - results expressed as mean ± standard deviation for 6 experiments.



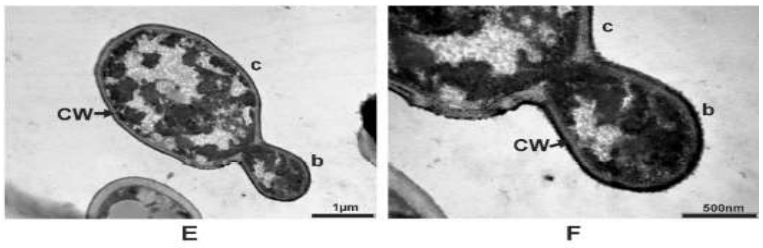


Figure 25. Changes produced by garlic extract (12%) in the ultrastructure of *M. guilliermondii* fungus, examined at TEM: (A-B) longitudinal section through a bud and a cell; (C) details of the longitudinal section through the cell; (D) pseudophyte longitudinal section (ph) and cross section through a cell; (E) changes produced by the extract: longitudinal section through the cell; (F) longitudinal section through the cell (detail). Legend: b, buds; c, cell; CW, cell wall; G, glycogen; L, lipid; M, mitochondria; N, nucleus; P, plasmalemma; V, vacuole (Pârvu et al., 2019).

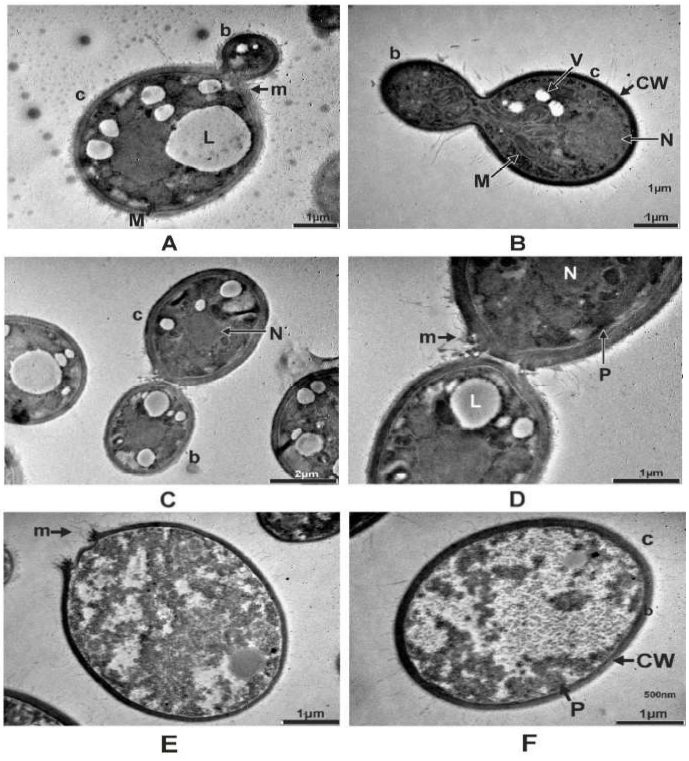


Figure 26. Changes produced by garlic extract (14%) in the ultrastructure of the fungus *R. mucilaginosa* examined at TEM: (A, B, C) longitudinal section through buds and a cell; (D) longitudinal section through bud and cell, in detail; longitudinal section (E) and cross section through the cell (F) with modifications produced by the extract. Legend: b, buds; c, cell; CW: cell wall; ER, endoplasmic reticulum; G, glycogen; L, lipids; M, mitochondria; m, mucilage; N, nucleus; P, plasmalemma; V, vacuole (Pârvu et al., 2019).

The ultrastructural changes induced by the extract can be correlated with what is induced when administering antifungal treatments, indicating the possibility of using garlic extract as a

treatment for dermatophytic fungi such as *Candida* (Li et al., 2016). In general, compounds with antioxidant potential also can act as antimicrobial agents (Esposito et al., 2019), which significantly reduce the level of oxidative stress. In vitro testing of antioxidant activity was performed by ABST and DDPH tests. The effect is attributed to sulfur compounds, which may be involved in electron transfer, helping to restore redox balance, and the content of allicin and alliin is high and confirmed by phytochemical analysis.

2.2.4. Antioxidant effect

The *in vitro* antioxidant effect of *A. sativum* extract was investigated using bleaching assays with 2,20-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrilhydrazyl (DPPH). Antioxidant activity was more intense when the ABST bleaching method was used, compared to DPPH (Figure 27). The antioxidant effect of garlic is very well documented, due to its chemical composition rich in substances with antioxidant potential (Shang et al., 2019; Jang et al., 2018; Sallam et al., 2004).

Extract treatments significantly reduced TOS ($p < 0.01$) and OSI ($p < 0.01$), but without significantly altering TAR, for all dilutions. Allicin had a similar effect in terms of TOS compared to the extract, but diclofenac did not have a very intense effect in inhibiting inflammation (Table 7).

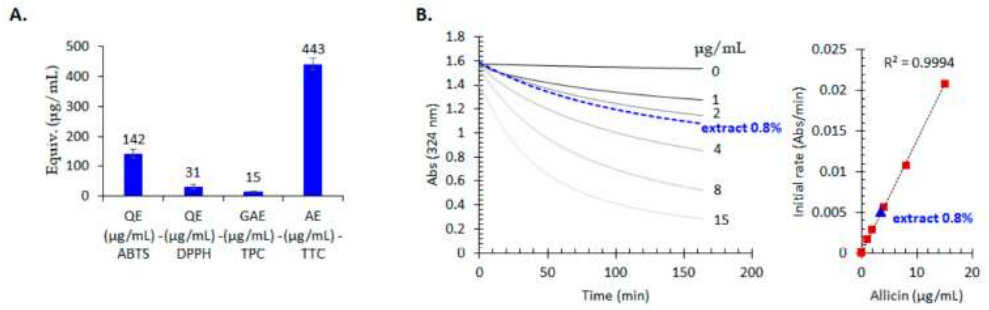


Figure 27. Evaluation of the antioxidant effect in vitro and determination of the total thiol content. (A) In vitro antioxidant effect evaluated by: ABST and DPPH tests and expressed in quercetin equivalents (QE); determination of the total phenol content (TPC) with Folin-Ciocâlteau reagent and expressed as gallic acid equivalents (GAE); total thiosulfinate content (TTC) expressed in allicin (AE) equivalents, n = 3; the error was calculated based on the standard deviation. (B) Kinetic determination of the total thiosulfinate content of the extract used of 0.8% using the method described by Miron et al. (Miron et al., 2002), and allicin standards (0-15 µg / mL) and calibration curve (Pârvu et al., 2019).

Nitrites and serum nitrates were significantly increased in the INFL group compared to the control group. Experimental variants with 100% and 50% garlic extract contributed to a significant reduction of NOx, but the 25% extract was less effective. Allicin was a good inhibitor of NOx synthesis, and the effect was comparable to that obtained after administration of the extract at concentrations of 100% and 50%, but with a much better effect than that obtained after administration of diclofenac (Table 7). . Total thiols (SH) were decreased in the INFLAM group, and treatments with allicin and diclofenac induced a significant increase in SH. Garlic extract, at a concentration of 50%, had a lower SH stimulating effect, compared to the extract at a concentration of 100% and 25% (Table 7).

Tabelul 7. Serum biomarkers for *in vivo* oxidative stress(Pârvu și colab., 2019)

Lot	TOS (µmol H_2O_2 equiv/L)	TAR (mmol trolox equiv/L)	OSI	NOx (µmol/L)	MDA (µmol/mL)	SH mmol (THS/mL)
Control	38.22±4.77 a*	1.0897±0.0014 a**	35.04±4.39 a***	52.88±2.60 a***	5.82±0.56 a***	0.67±0.07 a***
Inflammation	66.35±8.49 b**, c*	1.0891±0.0009 b*, c*	60.90±7.81 b**, c**	82.42±0.27 b***, c*	7.50±0.77 b***, c*	0.50±0.08 b**, c*
Diclofenac	48.65±8.36 a**, b*	1.0970±0.0017 b*	44.47±7.59 a***, b*	58.71±5.29 a**, b*	5.75±0.80 a**, b*	0.71±0.11 a**, b*
Allicin	33.76±4.82 a**, c*	1.0884±0.0005 a*, c*	31.02±4.43 a**, c*	40.30±6.12 a***, c*	5.57±0.48 a**, c*	0.75±0.13 a**, c*
***A. sativum* 100%**	33.13±8.06 a**, b*, c*	1.0894±0.0009 a*, b*, c*	30.43±7.39 a**, b*, c*	38.12±7.57 a***, b*, c**	5.54±0.32 a**, b*, c*	0.60±0.09 a**, b*, c*
***A. sativum* 50%**	31.88±6.91 a**, b*, c*	1.0884±0.0009 a*, b*, c*	29.27±6.34 a**, b*, c*	38.15±8.10 a***, b*, c*	5.48±0.63 a**, b*, c*	0.81±0.29 a**, b*, c*
***A. sativum* 25%**	32.25±3.75 a**, b*, c*	1.0884±0.0005 a*, b*, c*	29.63±3.43 a**, b*, c*	53.91±8.83 a**, b*, c*	5.79±0.51 a**, b*, c*	0.62±0.15 a**, b*, c*

*Values are expressed as mean ± standard deviation (SD) at 5 samples. TOS: total oxidative status; ART: total antioxidant reactivity; OSI: oxidative stress index; NOx: nitrites and nitrates; SH: total thiols; MDA: malondialdehyde; a vs INFLAM; b vs Allicin; c vs DICLOFENAC; p <0.05 *; p <0.01 **; p <0.001 ***.*

This study presents, for the first time, a clinical case of onychomycosis with total dystrophic nail, due to a co-infection with *M. guilliermondii* and *R. mucilaginosa*, indicating the antifungal effect of *A. sativum* on the two species of fungi.

The antioxidant effect of *A. sativum* extract is largely attributed to allicin, so the antioxidant effects obtained were compared with allicin. To confirm the antioxidant and anti-

inflammatory effect, a comparison was made with the effect obtained by diclofenac, a non-steroidal anti-inflammatory (Pârvu et al., 2019).

2.2.5. Effects on normal human cells BJ and HaCaT

2.2.5.1. MTT Assay

To investigate the effect of *Allium* extracts on BJ and HaCaT cell proliferation, the MTT test was chosen at 24 h. Thus, according to Figure 28, *A. sativum* inhibited the growth of BJ and HaCaT cells, in a dose-dependent manner, having an IC50 cell growth inhibition index of 0.8841% extract, in culture medium, for BJ (equivalent of 8,841 mg / mL extract) and 1,086% extract in culture medium for HaCaT (equivalent to 10.86 mg / mL extract).

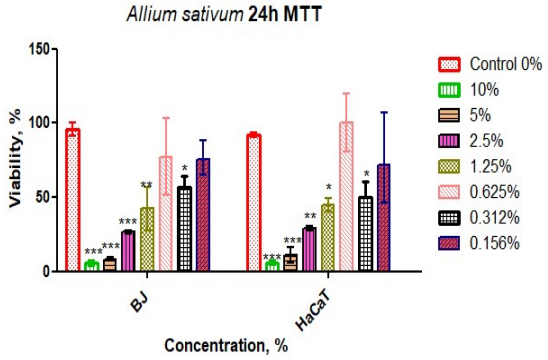


Figure 28. Effect of *A. sativum* extract, on BJ and HaCaT cells, tested by MTT (* P <0.05, ** P <0.01, *** P <0.001 after comparison with the control group). The samples analyzed in triplicate show statistical differences, with pronounced cytotoxic effects of the extract, at concentrations higher than 1.25% in the culture medium (unpublished data).

Compounds that encounter cells induce changes in their behavior, and morphological changes become visible.

To highlight the important cell compartments in the process of apoptosis, the nucleus, mitochondria and cytoskeleton were highlighted fluorescently. Figure 29 shows the inhibitory effect of garlic extract (Bhandari, 2012; Matysiak et al., 2015).

The garlic extract concentration of 100 mg / mL had pronounced inhibitory effects on cell development in both cell lines (Figure 29D and 29H). The concentration of approximately IC50, for both cell lines, the cells show an altered morphology, with the cytoskeleton slightly affected, showing signs of necrosis (Figure 29C and 29G). At concentrations below IC50 (Figure 29B and

29F), it is observed that the morphological changes are minor compared to the control groups (Figure 29A and 29E).

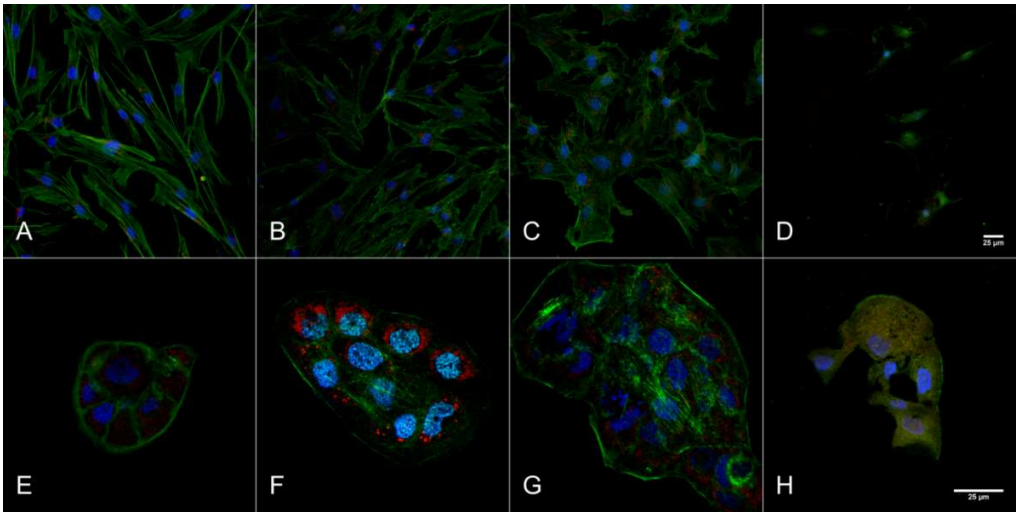


Figure 29. Illustration of the dose-dependent inhibitory effect of garlic extract on BJ and HaCaT cell proliferation, examined with the 40X objective (A-control group, B-0.5% extract, C-dose IC50, D-10% extract) and with 100X immersion objective (E-control group, F-0.1% extract, G-dose IC50, H-10% extract). Cells were labeled with DAPI for nucleus (blue), Mitotracker for mitochondria (red) and Phalloidin-FITC for cytoskeleton (green). The most pronounced cytotoxicity was highlighted in the group with 10% extract, where the cytoskeleton and the nucleus can be hardly observed, and the mitochondrial networks are missing. The concentration of IC50 does not show a pronounced cytotoxic effect, but it induces morphological changes in the cytoskeleton. Morphological characteristics and cell integrity are not affected in cells treated with doses lower than IC50. The images suggest an induction of cell death that ends in necrosis with few signs of apoptosis (unpublished data).

2.2.5.2. Determination of caspase 3, catalase and lactate dehydrogenase

Allium species extracts can induce apoptosis, due to high concentrations of sulfur compounds, which increase the Bax / Bcl-2 ratio and Casp3 activity (Farhadi et al., 2015). *A. sativum* extract induced an increase in Casp3 (Figure 30), but not large enough to be decisive in stating that the cells are in apoptosis.

To confirm cell death, either apoptosis or necrosis, Casp 3 investigation is essential, it is the key molecule in the mechanism of apoptosis, being the common element between the pathway of intrinsic and extrinsic apoptosis. The literature suggests that allicin, the active compound of *Allium* extracts, activates Caspase 3 and induces apoptosis (Blazquez et al., 2006; Park et al., 2005). In addition, for the investigation of necrosis, the analysis of LDH activity may highlight a

membrane degradation, the main feature for this type of cell death (Szychowski et al., 2018; Chan et al., 2013).

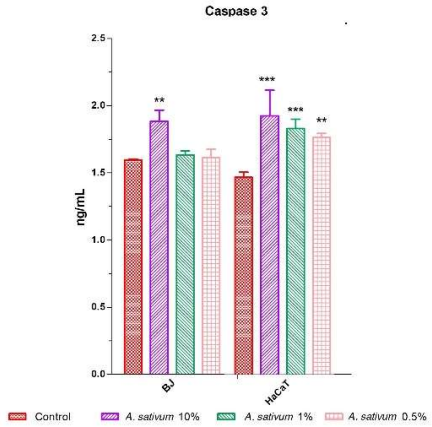


Figure 30. Evaluation of the effect of garlic extract on the activity of caspase 3, on BJ and HaCaT cell lines. The dose of 10% extract, in the culture medium, produced an intensification of the activity of caspase 3 in both cell lines, while the doses of 1% and 0.5% intensified the activity of caspase 3, only in the case of HaCaT line (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control group). Results are expressed as mean ± SD, and statistical significance was determined using the student T test (unpublished data).

Catalase (CAT) is an important enzyme involved in the cellular mechanism of protection against oxidative stress. CAT contributes to the dissociation of hydrogen peroxide into water and molecular oxygen (Nandi et al., 2019; Chelikani et al., 2004). Intensified CAT activity suggests oxidative stress in cells.

The mechanism by which necrosis was induced is difficult to determine, but morphological aspects suggest cellular stress, possibly oxidative stress, although the extract is rich in antioxidant compounds, and the relatively high concentration may have the opposite effect (Syntichaki and Tavernarakis, 2002). Thus, investigation of catalase activity is welcome, as it is the key enzyme involved in the cellular mechanism of protection against oxidative stress, dissociating hydrogen peroxide (Chelikani et al., 2004).

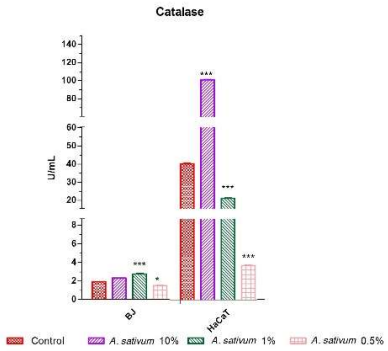


Figure 31. Evaluation of the modulation of catalase activity (CAT), following the administration of garlic extract, in different doses, at non-tumor cell lines (BJ and HaCaT). Intensification of catalase activity, in the case of the BJ cell line, was observed after administration of garlic extract at the IC50 dose. In the case of the HaCaT line, CAT activity was intensified only by the addition of the extract in a concentration of 10%, and at low doses it was significantly reduced compared to the control. Thus, the dual effect of the extracts is observed depending on the dose administered. * P <0.05, ** P <0.01, *** P <0.001 compared to the control group. Results are expressed by mean ± SD, and statistical significance was determined using the student T test (unpublished data).

The investigation of necrosis and apoptosis requires confirmation, so that the determinations by the Annexin V and P.I. by flow cytometry, they are the standard method of confirming cell death and are frequently used for such studies (Țigu et al., 2020; Budișan et al., 2019; Koç et al., 2018).

Compared to 5% DMSO, which is a positive control for necrosis, it was validated that necrosis is dose-dependent induced in normal cell lines.

To draw a conclusion about the effect of extracts on BJ and HaCaT cells, the determination of LDH activity in the cytoplasm of treated cells provides us with valuable information about membrane integrity (Figure 32).

Increased LDH activity indicates membrane destruction and possible necrosis (Szychowski et al., 2018; Faloppi et al., 2015; Chan et al., 2013). LDH activity was intensified (Figure 32), following the administration of garlic extract, in different concentrations, compared to the control group, in the case of both cell lines (BJ, HaCaT).

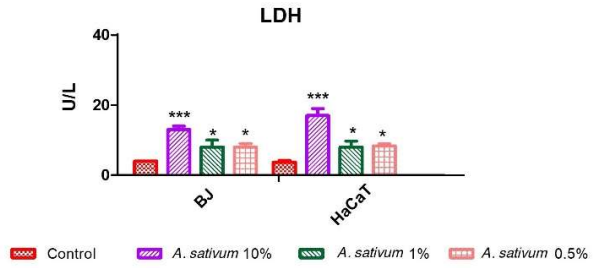


Figure 32. Evaluation of LDH activity, at cell lines (BJ, HaCaT), following the administration of garlic extract. LDH activity was intensified, following the administration of the three doses (10%; 1%; 0.5%) of garlic extract, in the case of both analyzed cell lines (BJ, HaCaT). The intensification of LDH activity is pronounced significantly, at a concentration of 10%, compared to the doses of 1% and 0.5% extract. Thus, the dual effect of the extracts is observed, depending on the dose administered; LDH activity indicates membrane degradation (* P <0.05, ** P <0.01, *** P <0.001 compared to the control group). The expressed results are mean ± SD, and the statistical significance was determined using the student T test (unpublished data).

2.2.5.3. Cell viability determination

The test for determining the type of cell death, with Annexin V and P.I .., is the universally used method. This test determines the percentage of viable, necrotic and apoptotic cells in each sample analyzed at the flow cytometer. In this case, all data obtained by microscopy techniques, feasibility tests and biochemical analyzes are validated by the results obtained in this test, at 24 hours, with treatment (Figure 33).

HaCaT cells have a viability of 95%, in the control group, with only 5% necrotic, while the positive control treated with DMSO 5%, has a viability of 88.6%, with 10.8% necrosis, and 0.6% apoptosis. DMSO is used as an indicator of necrosis, as all previous data suggest that BJ and HaCaT cells are affected by necrosis. Necrosis was induced, in a dose-dependent manner, on HaCaT cells; at 5% garlic extract are 6.1% necrotic cells and 93.9% viable (Figure 33).

Like any other treatment, at a certain concentration, it becomes cytotoxic to the cells in the experiment. *Allium* extracts, especially garlic extracts, are extensively studied to determine their biological properties, such as antioxidant activity (Maldonado et al., 2003; Razo-Rodriguez et al., 2008), anti-inflammatory activity (Lee et al., 2012).), antitumor potential (Wang et al., 2012), antibacterial effect (Lu et al., 2011), but also other potential beneficial effects, due to the chemical composition.

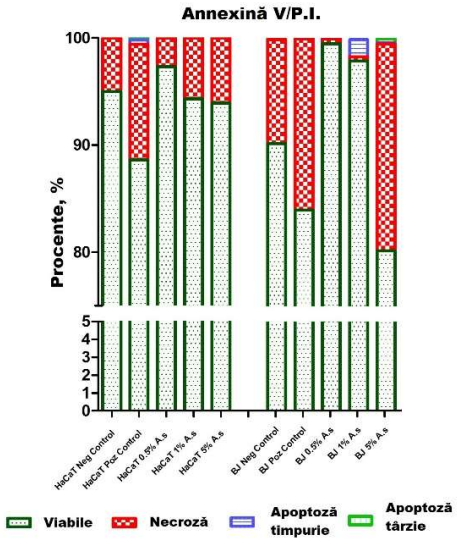
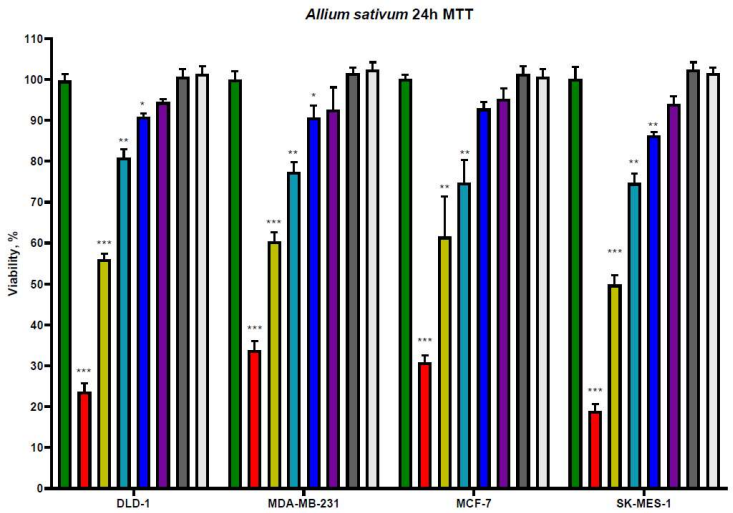


Figure 33. Determination of apoptosis / necrosis ratio, by flow cytometry, using Annexin V and P.I. (I.P), on BJ and HaCaT lines, treated with *A. sativum* extract (5%; 1%; 0.5%). Negative control was not treated, and positive control is treated with 5% DMSO to validate the necrosis indicator (unpublished data).

2.2.6. Antitumoral potential

To evaluate the antitumor potential, *A. sativum* extract was tested on four tumor cell lines (Table 2) (DLD-1, MDA-MB-231, MCF-7 and SK-MES-1), at similar concentrations (of at 10% to 0.156%, in culture medium), with those used to investigate normal cell proliferation (Figure 34).

The values obtained for the IC50 dose are very high, being equivalent to over 1 mg / mL. This aspect does not allow us to evaluate a possible antitumor potential of this extract, because the dose is very high. A possible explanation for this aspect may be the high content of nutrients present in this extract, which favors the development of normal cells, but also tumor cells.



	DLD-1	MDA-MB-231	MCF-7	SK-MES-1
IC50	5.482	6.375	6.131	4.651

Figure 34. Effect of *A. sativum* extract on tumor cells DLD-1, MDA-MB-231, MCF-7 and SK-MES-1, determined by MTT test (* P <0.05, ** P <0.01, * ** P <0.001, following the comparison made with the control group, using the student T test). The analyzed samples, in triplicate, show statistical differences, with pronounced cytotoxic effects of the extract, at concentrations higher than 1.25%, in the culture medium. The most pronounced effect is at a concentration of 10% extract in the culture medium, equivalent to approximately 100 mg extract / mL. IC50 values are relatively close, around 5% extract, in culture medium, equivalent to approximately 50 mg / mL extract (unpublished data).

Garlic is associated with various prophylactic effects and is used as a medicinal plant; the antitumor effect has been investigated in recent decades. In 1990, in the USA, a national program for the investigation of cancer prevention was initiated by studying various foods that may have antitumor effect (Dahanukar and Thatte, 1997); garlic is among the most potent foods that can have antitumor effect, by activating the mechanisms to prevent the uncontrolled development of tumor cells (Bayan et al., 2014). Garlic extract reduced the proliferation of tumor cell lines, used in experiments such as HeLa (cervical cancer), 5637 and J83 (bladder cancer) (Li et al., 2018), SCG-15 (tumor squamous cells) (Szychowski et al., 2018) or contributed to the blockade of MCF-7 (breast cancer) and HepH2 (liver cancer) cells at certain stages of the cell cycle (Shaban et al., 2018).

1.17. *Allium fistulosum* extract (Welsh onion)

2.3.1. Chemical composition

Similar to the investigation of the chemical composition for *A. sativum* extract, *A. fistulosum* extract was analyzed for the phytoconstituents it contains. Four replicates were prepared and analyzed from the Welsh onion extract, and the results were expressed in µg / mL. Welsh onion extract contains 145 µg / mL allicin and 20 µg / mL allicin (Table 8).

A. fistulosum is rich in isoquercitrin, ferulic acid and rutin, and *A. sativum* extract contains large amounts of chlorogenic acid, p-coumaric acid and 4-hydroxybenzoic acid (Table 4). Interestingly, all the compounds identified (phenolic acids and flavonoids) in the Welsh onion extract (Table 9) are not found in the *A. sativum* extract (Table 4).

Table 8. Elution time, characteristics of the analytical method and determination of the concentration of alliin and allicin in the analyzed samples (n=4)

No.	Compound	$t_{elution}$ (min)	R^2	LOD (µg/mL)	*A. fistulosum* (µg/mL)
1	Alliin	3.77	0.9999	5.8	145±15
2	Allicin	15.40	0.9999	14.1	20±5

LOD - limit of detection, R^2 - coefficient of determination for the calibration curve (at six concentrations). The indicated intervals represented as mean ± standard deviation (n = 4).

Table 9. Elution time, analytical method and determination of phenolic and flavonoid acid concentrations in the plant extract

No.	Compound	$t_{elution}$ (min)	R^2	LOD (µg/mL)	*A. fistulosum* (µg/mL)
1	Gentisic acid	8.13	0.9997	3.4	-
2	Chlorogenic acid	9.15	0.9995	4.6	-
3	4-hydroxybenzoic acid	10.31	0.9999	2.3	-
4	Rutin	11.39	0.9998	2.7	215±3
5	Isoquercitrin	11.91	0.9999	2.2	280±3
6	p-Coumaric acid	12.38	0.9999	1.9	-
7	Quercitrin	12.72	0.9998	2.7	95±3
8	Ferulic acid	12.85	0.9999	2.0	230±2
9	Quercetin	16.35	0.9997	3.3	26±3
10	Kaempferol	19.77	0.9998	2.7	30±3

LOD - limit of detection, R^2 - coefficient of determination for the calibration curve (at six concentrations). The indicated intervals represented as mean ± standard deviation (n = 4).

2.3.2. Effects on normal human cells BJ and HaCaT

2.3.2.1. MTT Assay

For *A. fistulosum* extract, the results were analyzed in comparison with those obtained for garlic extract, because it maintains the trend and dose-dependence. IC50 values are different for the two non-tumor cell lines. For the BJ cell line, IC50 has a value of 0.2433% extract in the culture medium, i.e. the equivalent of 2,019 mg / mL extract. Regarding the HaCaT cell line, the value (of IC50) is 0.6197% or 5.144 mg / mL extract (Figure 35). *A. fistulosum* has proven to be a good antifungal and antimicrobial agent, properties supported by sulfur compounds and sterols it contains (Vlase et al., 2013).

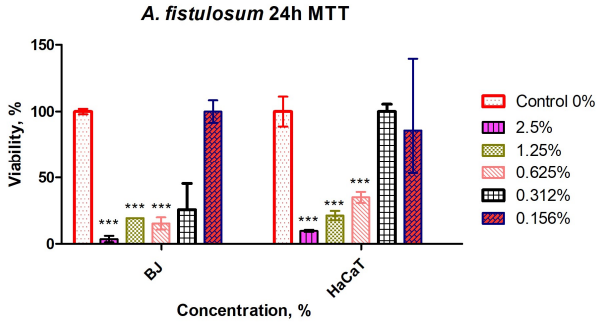


Figure 35. Effect of *A. fistulosum* extract, on BJ and HaCaT cells, determined by MTT test (* P <0.05, ** P <0.01, *** P <0.001 after comparison with the control group). The analyzed samples, in triplicate, show statistical differences, with pronounced cytotoxic effects of the extract, at high concentrations. The limitation was 2.5% extract in the culture medium. At higher concentrations, immediate cell toxicity was observed, most likely due to altered osmolarity (unpublished data).

Figure 36, at a concentration of 83 mg / mL (Figure 36D and 36H), shows the cell death induced at both cell lines in the experiment (concentration equivalent to 10% extract in the culture medium). The cytoskeleton is destroyed, the mitochondrial networks are completely missing, and the nucleus is unclear compared to the control group (Figure 36A and 36E). The dose of IC50 induces changes in mitochondrial networks in HaCaT cells, and in the BJ the cytoskeleton shows slight traces of fragmentation (Figure 36C and 36G). At a concentration of 0.1% (below IC50), the BJ and HaCaT cell lines show no signs of apoptosis or necrosis, and the morphology is like normal (Figure 36B and 36F).

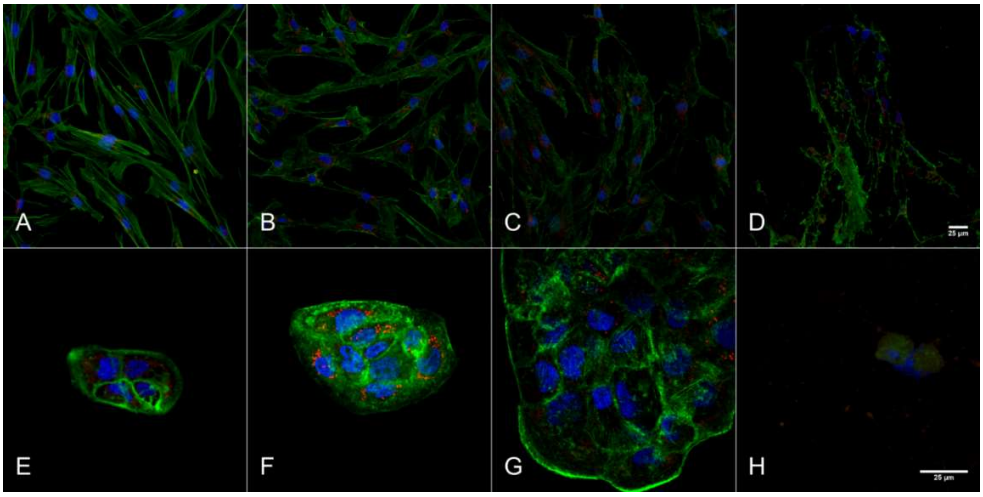


Figure 36. Illustration of the cytotoxic effect of *A. fistulosum* extract, with 40X objective, on BJ cells (A-control group, B-0.5% extract, C-dose IC50, D-10% extract) and with 100X objective with immersion, on HaCaT cells (E-control group, F-0.1% extract, G-dose IC50, H-10% extract). Cells were labeled with DAPI for the nucleus (blue), Mitotracker for the mitochondria (red) and Phalloidin-FITC for the cytoskeleton (green). The most pronounced cytotoxicity was highlighted in the group with 10% extract, where the cytoskeleton and the nucleus can be hardly observed and the mitochondrial networks are missing. At the dose of IC50, small changes in appearance are observed, but are not decisive in cell survival. At doses below IC50, cells have normal morphological features, with highlighted mitochondrial networks and intact cytoskeleton (unpublished data).

2.3.2.2. Determination of caspase 3, catalase and lactate dehydrogenase

Determining the activity of caspase 3 (Casp 3), catalase (CAT) and lactate dehydrogenase (LDH) allows us to assess the cellular stress induced by the Welsh onion extract and to determine whether cell death was due to necrosis or apoptosis. The type of cell death was confirmed by flow cytometry.

Caspase 3 is the determining molecule for performing the process of apoptosis; this is the effector protein that facilitates the link between intrinsic apoptosis and extrinsic apoptosis (Ponder and Boise, 2019; Végran et al., 2011).

During intrinsic apoptosis, caspase 9 is activated after CytC is released from the mitochondria, and then caspase 3 is activated and the apoptosis process is performed. During all this time, caspase 7 is the support element for maintaining the process of intrinsic apoptosis and stimulates cell detachment (Brentnall et al., 2013). Through extrinsic apoptosis, caspase 8 and

caspase 10 activate the executor, and caspase 3 facilitates the apoptosis process, due to external stimuli (Krautwald et al., 2010).

Following administration of *A. fistulosum* extract, an increase in caspase 3 activity was observed: at the HaCaT line, following the administration of the three doses of extract, and in the case of BJ only at the dose of 10%. These results indicate a possible initiation of the apoptosis process, in the case of both cell lines; HaCaT was more visibly affected by this extract (Figure 37).

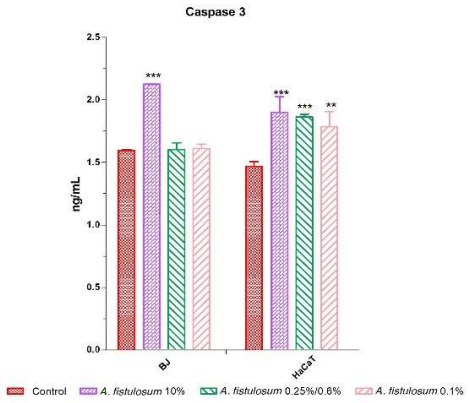


Figure 37. Evaluation of caspase 3 activity in BJ and HaCaT cell lines following administration of *A. fistulosum* extract. The pronounced activation of caspase 3 is visible at a concentration of 10% extract, which is a high enough dose to induce cell death, while doses of IC50 (0.25% for BJ and 0.6% for HaCaT) and 0.1% extract have intensified caspase activity only at the HaCaT cell line (* P <0.05, ** P <0.01, *** P <0.001 compared to the control group). Results are expressed by mean ± SD, and statistical significance was determined using the student T test (unpublished data).

Catalase, an enzyme of the antioxidant system, is crucial for evaluating oxidative stress at the cellular level. CAT activity is directly proportional to the level of hydrogen peroxide (Glorieux and Calderon, 2017; Alfonso-Prieto et al., 2009).

Following the administration of the Welsh onion extract, in the case of the BJ line, CAT showed a reduced activity compared to the control group. Regarding the HaCaT cell line, the 10% dose of *A. fistulosum* extract enhanced CAT activity; Following administration of the IC50 dose and below IC50, CAT activity decreased compared to control (Figure 38).

These results indicate that the dose we choose is very important for the studies, because the very high concentrations of the extract produce imbalances at the cellular level and intensify the oxidative stress.

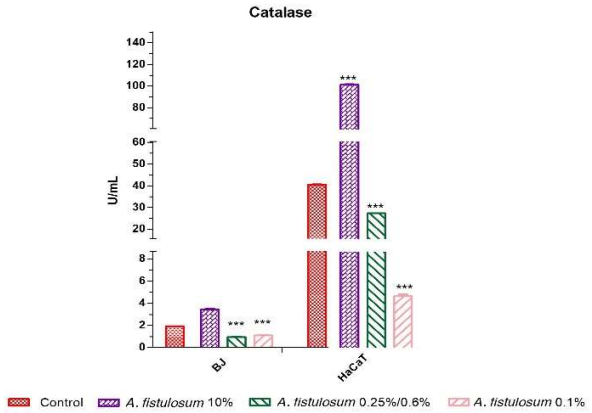


Figure 38. Evaluation of CAT activity, at BJ and HaCaT cell lines, following the administration of *A. fistulosum* extract. In the case of BJ, there is a small increase in CAT activity, following the administration of 10% extract, and in the HaCaT line, the increase in CAT activity is much more pronounced. In both cell lines, doses of IC50 (0.25% for BJ and 0.6% for HaCaT) and below IC50 reduced CAT activity compared to control (* P <0.05, ** P <0.01, *** P <0.001 compared with the control group). Results are expressed by mean ± SD, and statistical significance was determined using the student T test (unpublished data).

Because confocal microscopy images show cytoskeleton degradation at both cell lines (BJ and HaCaT) in a dose-dependent manner, LDH assessment confirms whether cytoskeleton degradation may be associated with membrane degradation. LDH binds to membranes through electrostatic bonds, but can also form bonds through hydrophobic forces (Dym et al., 2000). LDH is a very good indicator for membrane damage, because it is released into the extracellular environment, when the membranes are subjected to various stressors (Kumar et al., 2018; Specian et al., 2016; Benson et al., 2015 ; Kato et al., 2006). Following treatment with Welsh onion extract, both cell lines showed an intensification of LDH activity, even at a concentration of 0.1%. These results indicate that, due to the chemical composition of the extract, cell membranes allow the release of LDH, even at very low doses (of the extract, in the culture medium), compared to the control (Figure 39). Although rich in nutrients, *A. fistulosum* extract can become cytotoxic if the concentration of these substances causes imbalances at the cellular level.

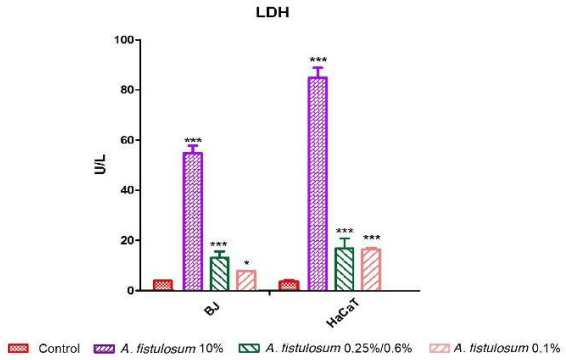


Figure 39. Evaluation of LDH activity, at BJ and HaCaT cell lines, following the administration of *A. fistulosum* extract. LDH activity is significantly intensified, following the administration of 10% extract. In the case of IC50 concentrations (0.25% for BJ and 0.6% for HaCaT) and below IC50, LDH activity is less intense, but significantly more pronounced compared to the control group, in the case of both cell lines (* P <0.05, ** P <0.01, *** P <0.001 compared to the control group). Results are expressed by mean ± SD, and statistical significance was determined using the student T test (unpublished data).

2.3.2.3. Cell viability determination

Cell death can be induced by programmed cellular processes or due to external factors that may affect the structural integrity of cells (Kroemer et al., 2009). The cell death assessment test, by flow cytometry, with Annexin V and I: P: (propidium iodide), is the standard test that highlights the ratio between viable cells and apoptotic or necrotic ones (Figure 40). Annexin V is a small Ca^{2+} ion-dependent protein that binds to phosphatidylserine. Phosphatidylserine is arranged at the periphery of the cell inside the cytoplasmic membrane.

Annexin V can bind phosphatidylserine only when membranes are permeabilized or degraded and phosphatidylserine residues are exposed to the outside of the cell (Crowley et al., 2016). This is characteristic of apoptotic cells, but Annexin V can bind phosphatidylserine residues in necrotic cells as well. To distinguish necrosis, propidium iodide is used, which cannot bind genetic material to apoptotic cells, but only to necrotic ones (Rieger et al., 2011; Schutte et al., 1998).

A. fistulosum induces the death of a small percentage of cells, even at low doses of extract; at doses of IC50 and lower doses, these results require further studies to determine exactly the optimal dose that does not affect normal cells. Doses of 5% extract showed results similar to the positive control, which represents 5% Dimethylsulfoxide, added to the culture medium,

confirming the induction of necrosis. However, the active substances in the composition of the extract are of therapeutic interest, and isolated and purified, may have therapeutic potential against inflammatory diseases and may have antioxidant effect (Braicu et al., 2020).

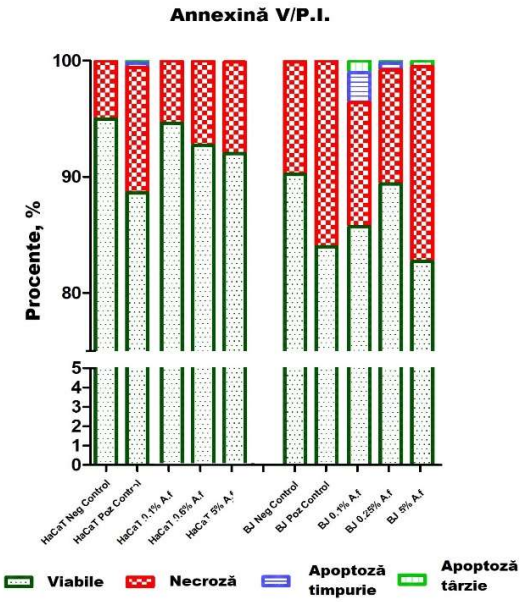


Figure 40. Determination of apoptosis / necrosis ratio, by flow cytometry, with Annexin V and P.I. (I.P), at cell lines, BJ and HaCaT, treated with *A. fistulosum* extract. The negative control was not treated, and the positive control is treated with 5% DMSO to validate the necrosis indicator. The concentrations of the extracts are: 5% for both cell lines; 0.25% for BJ; 0.6% for HaCaT. *A. fistulosum* extract was added in a concentration of 0.1% (unpublished data).

2.3.3. Antitumoral potential

To investigate the antiproliferative potential, the Welsh onion extract was tested on tumor cell lines (DLD-1, MDA-MB-231, MCF-7 and SK-MES-1) by MTT test (Figure 41). For this, the same concentrations were used, as in the evaluation of the antiproliferative effect on normal cells (Figure 35).

Welsh onion extract has a reduced antitumor activity because the maximum dose that could be used to investigate the antiproliferative effect was 2.5% extract in the culture medium. Higher doses induced a change in the pH of the culture medium, so that cell death was induced by a change in the acid-base balance.

The chemical composition of *A. fistulosum* extract indicates high concentrations of phenolic compounds such as rutin (0.215 mg / mL), isoquercitrin (0.280 mg / mL), ferulic acid (0.230 mg / mL), quercetin (0.026 mg / mL) and kaempferol (0.030 mg / mL). The routine phenolic compound has antithrombotic and anti-inflammatory potential (Morling et al., 2018), and isoquercitrin has an anti-inflammatory effect and can be used as an agent to prevent thromboembolism. Also, the phenolic compound isoquercitrin may have antitumor effects, because it acts on the mechanism of lipid peroxidation and on the mechanism of inhibition of inflammation (Orfali et al., 2016). Quercetin acts as an antioxidant, being a good free radical scavenger (Russo et al., 2014). Kaempferol is a product of plant metabolism, being used in alternative medicine, having an antioxidant effect and can play a role of chemopreventive and even antitumor agent (Budișan et al., 2019; Chen and Chen, 2013).

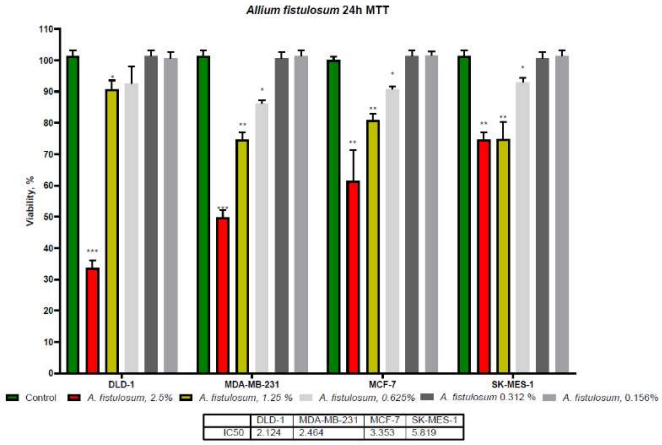


Figure 41. Effect of *A. fistulosum* extract on tumor cells DLD-1, MDA-MB-231, MCF-7 and SK-MES-1, determined by MTT test (* P <0.05, ** P <0.01, * ** P <0.001 after comparison with the control group using the student T test). The analyzed samples, in triplicate, show statistical differences, with pronounced cytotoxic effects of the extract, at high concentrations, and the limitation was 2.5% extract in the culture medium. At higher concentrations, immediate toxicity to cells was observed, most likely due to altered osmolarity. Doses of IC50 are slightly different; the least sensitive cell line was the lung line. Doses of IC50 range from the equivalent of 50 mg / mL extract to approximately 20 mg / mL extract (unpublished data).

3. Conclusions

Based on the results of the research and data from the literature, conclusions can be drawn with important therapeutic applications.

Laboratory-synthesized allicin has antitumor potential on lung (SK-MES-1) and colorectal (DLD-1) cell lines, proven by several current research methods.

The effect of allicin combined with 5-fluorouracil (5-FU) on the two tumor cell lines (SK-MES-1 and DLD-1) is stronger (synergistic) than that of allicin and has been shown by various experimental results: inhibition of formation colonies in tumor cells; inhibition of tumor cell migration; evaluation of cell death by flow cytometry; evaluation of cellular changes caused by treatments by confocal microscopy; confirmation of the initiation of cell death by western blot.

The antiproliferative effect of allicin is a dose-dependent one, and the induction of cell death can follow either the path of necrosis or the path of apoptosis.

Following administration of the combination of allicin and 5-fluorouracil (5-FU) the following aspects were highlighted:

- 5-FU acts nonspecifically, having a similar cytotoxic dose, both in the case of lung (SK-MES-1) and colon (DLD-1) tumor lines, and in the case of the normal BJ line;
- allicin acts differently, depending on the cell line exposed to treatment; lung tumor cells are much more sensitive to this treatment compared to colorectal tumor cells;
- administration of combinations of IC_{50} halves produced a decrease in viability compared to 5-FU and allicin at the IC_{50} dose;
- the morphological changes induced by the combined treatment are varied, in the case of the DLD-1 line significant degradations of the cytoskeleton are produced, and the decrease of the cell volume (AVD - apoptotic volume decrease) is a characteristic of the effect of the combined treatment, in the case of the SK-MES-1 line ;
- allicin as an individual treatment and the combination of allicin and 5-FU have an inhibitory effect on cell migration and colony formation, two important characteristics of tumor cells;
- cell death is induced by different mechanisms. In the case of the colorectal tumor line, cell death is non-apoptotic, without activating caspase 3, while in the case of the lung tumor line, cell death was induced by: apoptosis, cytochrome C release and caspase 3 activation.

In the plant extracts of *Allium sativum* (garlic) and *Allium fistulosum* (Welsh onion) were determined quantitatively different phytocompounds (allicin, alline, phenolic acids and flavonoids).

Welsh onion extract has a low amount of alliin and allicin (about 10 times less than in garlic extract) and significant amounts of phenolic compounds, including isoquercitrin and rutin (over 0.2 mg / mL).

The plant extract of *A. sativum* (garlic) was obtained from bulbs and was researched in terms of: antioxidant effect; antifungal effect; cytotoxic effect on normal BJ and HaCaT cells; antitumor potential.

The antioxidant effect of *A. sativum* extract has been determined *in vitro* and *in vivo* and is largely attributed to allicin.

Meyerozyma guilliermondii and *Rhodotorula mucilaginosa* were isolated from a nail with a mixed onychomycosis infection and were grown on SDA culture medium.

In addition to the morphological and cultural characteristics, the confirmation of *M. guilliermondii* and *R. mucilaginosa* fungi was performed by molecular DNA analysis.

The in vitro effect of *A. sativum* extract on *M. guilliermondii* and *R. mucilaginosa* fungi was determined by the agar dilution method and compared with the commercial product naphthifine (Exoderil).

The plant extract of *A. sativum* has a fungicidal effect on the fungi *M. guilliermondii* and *R. mucilaginosa*, at 12% and 14% MIC, respectively, aspects illustrated by the irreversible ultrastructural changes obtained in TEM images (Figures 25 and 26).

A. sativum extract can induce necrosis in human cells of BJ fibroblasts and HaCaT keratinocytes, if administered in high concentrations (eg 100 mg / mL garlic extract), but does not affect cell viability at low doses;

The antitumor potential of *A. sativum* extract was tested in different concentrations (10%; 5%; 2.5%; 1.25%; 0.625%; 0.312%; 0.156%) on DLD-1 tumor cells, MDA-MB -231, MCF-7 and SK-MES-1, and the results obtained confirm that the antitumor effect of garlic extract is determined by allicin.

A. fistulosum extract obtained from leaves was investigated for: effect on normal / non-tumor cell lines (BJ and HaCaT) and tumor lines (DLD-1, MDA-MB-231, MCF-7 and SK-MES-1) modulation of caspase 3, LDH and catalase activity; the ratio of viable cells to those in cell death.

A. fistulosum extract has shown an *in vitro* inhibitory effect on the proliferation of normal BJ and HaCaT cells in a dose-dependent manner; the most pronounced effect was visible at doses of 2.5% and 1.25% extract in the culture medium.

The antiproliferative effect of *A. fistulosum* extract on tumor cell lines (DLD-1, MDA-MB-231, MCF-7 and SK-MES-1) was assessed by a slight decrease in tumor cell viability at doses of 2.5% and 1.25% extract in the culture medium.

The cytotoxic potential of plant extracts of *Allium sativum* and *A. fistulosum* on normal cell lines (BJ and HaCaT) was studied by: MTT test; determination of caspase 3, catalase and lactate dehydrogenase; analysis of induced cell death;

The cytotoxic effects were highlighted, in the two *Allium* extracts, at doses of 10% extract in the culture medium; at doses of IC_{50}, changes in the morphological aspects of BJ and HaCaT cells were observed. The cytoskeleton and nucleus of normal cells were affected at 10% dose, and at IC_{50} doses the cell compartments showed reduced structural changes. The correlation with molecular analyzes, involved in cell death, indicates that the cells followed the path of cell death through necrosis. Cell death triggered in the case of normal cells is of the necrosis type, because membrane integrity is affected and LDH activity is intensified.

The antioxidant effect of *A. sativum* and *A. fistulosum* extracts is dose dependent. At low doses (less than 1%) in the culture medium, CAT activity is normal. At higher doses, CAT activity

is intensified in both *Allium* extracts and in both normal cell lines, indicating induced oxidative stress (very high concentration of extracts).

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Scientific activity

Articole ISI, în calitate de autor principal (prim-autor, co-prim-autor, corespondent sau ultim-autor), în domeniul tezei de doctorat

Original -Țigu A.B, Toma V.-A, Moț A.C, Jurj A, Moldovan C.S, Fischer-Fodor E, Berindan-Neagoe I, Pârvu M. The Synergistic Antitumor Effect of 5-Fluorouracil Combined with Allicin against Lung and Colorectal Carcinoma Cells. *Molecules* **2020**, 25, 1947. doi: 10.3390/molecules25081947 **(IF 3.060)**

Original - Pârvu M, Moţ C. A, Pârvu A. E, Mircea C, Stoeber L, Roşca-Casian O, **Țigu A. B**. *Allium sativum* Extract Chemical Composition, Antioxidant Activity and Antifungal Effect against *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* Causing Onychomycosis. *Molecules* ,**2019**, 24(21), 3958. **(IF 3.060)**

Review - Zimta AA#, **Țigu A.B#**, Muntean M, Cenariu D, Slaby O, Berindan-Neagoe I. Molecular Links between Central Obesity and Breast Cancer. *Int J Mol Sci*. **2019**;20(21):5364. Published 2019 Oct 28. doi:10.3390/ijms20215364 **(IF 4.182)**

Original - Toma VA#, **Țigu A.B#**, Farcaș AD, Sevastre B, Taulescu M, Gherman A. M. R, Roman I, Fischer-Fodor E, Parvu M. New Aspects Towards a Molecular Understanding of the Allicin Immunostimulatory Mechanism via Colec12, MARCO, and SCARB1 Receptors. *Int J Mol Sci*. 2019;20(15):3627. doi:10.3390/ijms20153627 **(IF 4.182)**

Articole ISI, în calitate de co-autor, în domeniul tezei de doctorat

Review - Braicu C, Zanoaga O, Zimta A-A, **Țigu A.B**, Kilpatrick KL, Bishayee A, Nabavi S.M, Berindan-Neagoe Ioana. Natural compounds modulate the crosstalk between apoptosis- and autophagy-regulated signaling pathways: Controlling the uncontrolled expansion of tumor cells, Seminars in Cancer Biology, **2020**. doi.org/10.1016/j.semcancer.2020.05.015 **(IF 9.658)**

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Articole ISI în calitate de co-autor

Review - Desmirean M, Rauch S, Jurj A, Pasca S, Iluta S, Teodorescu P, Berce C, Zimta A.-A, Turcas C, **Țigu A.B**, Moldovan C, Paris I, Steinheber J, Richlitzki C, Constantinescu C, Sigurjonsson O.E, Dima D, Petrushev B,Tomuleasa C. B Cells versus T Cells in the Tumor Microenvironment of Malignant Lymphomas. Are the Lymphocytes Playing the Roles of Muhammad Ali versus George Foreman in Zaire 1974? J. Clin. Med., **2020**, 9, 3412. **(IF 3.303)**

Original – Teodorescu P, Pasca S, Jurj A, Gafencu G, Joelsson J-P, Selicean S, Moldovan C, Munteanu R, Onaciu A, **Țigu A.B**, Buse M, Zimta A-A, Stiufiuc R, Petrushev B, Desmirean M, Dima D, Vlad C, Bergthorsson J.T, Berce C, Ciurea S, Ghiaur G, Tomuleasa C. Transforming growth factor β-mediated micromechanics modulates disease progression in primary myelofibrosis, Journal of Cellular and Molecular Medicine**,** 2020; 00:1-11, **2020,** 10.1111/jcmm.15526, **(IF 4.486)**

Review - Zimta A-A, **Țigu A.B**, Braicu C, Stefan C, Ionescu C, Berindan-Neagoe I. (**2020**) An Emerging Class of Long Non-coding RNA With Oncogenic Role Arises From the snoRNA Host Genes. Front. Oncol. 10:389., **2020**, doi: 10.3389/fonc.2020.00389 **(IF 4.137)**

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Original - Budisan L, Gulei D, Jurj A, Braicu C, Zanoaga O, Cojocneanu R, Pop L, Raduly L, Barbat A, Moldovan A, Moldovan C, **Țigu A. B**, Ionescu C, Atanasov A. G, Irimie A, Berindan-Neagoe I. Inhibitory Effect of CAPE and Kaempferol in Colon Cancer Cell Lines-Possible Implications in New Therapeutic Strategies. IJMS, **2019**, 20(5), 1199.**(IF 4.183)**

Review - Buduru SD, Gulei D, Zimta AA, **Țigu A.B**, Cenariu D, Berindan-Neagoe I. The Potential of Different Origin Stem Cells in Modulating Oral Bone Regeneration Processes. Cells. **2019**, 8(1):29. doi:10.3390/cells8010029 **(IF 4.892)**

Review - Gulei D, Petrut B, **Țigu A.B**, Onaciu A, Fischer-Fodor E, Atanasov AG, Ionescu C, Berindan Neagoe I. Exosomes at a glance - common nominators for cancer hallmarks and novel diagnosis tools. Crit Rev Biochem Mol Biol. **2018**;53(5):564-577. doi:10.1080/10409238.2018.1508276 **(IF 5.279)**

Participare în proiecte de cercetare:

2020-2022 – Researcher in project SEATTLE – Sustaining a young research group in developing a research program by applying" Sleeping Beauty" for developing CAR T cells. **PN-III-P1-1.1-TE-2019-0271, director CSII Dr. Ciprian Tomuleasa.**

2020-2022 – Researcher in project Selenium Biofortification, P1-1.1-TE-2019-1396, director CSIII Dr. Augustin Cătălin Moț.
2020-2021 – Administrative technician in project HE-RO-IS – Strategic cooperation in Hematology. SEE Project, director CSII Dr. Ciprian Tomuleasa.
May 2019 – December 2020 - Researcher in project ARTEMIS - Genomic mapping of population from polluted area with radioactivity and heavy metals to increase national security **– Director Prof. Dr. Berindan-Neagoe Ioana, UMF Cluj-Napoca.**
April 2017 – December 2019 - Researcher in project H2020 – REPROGRAM - Targeting epigenetic REPROGRamming of innate immune cells in Atherosclerosis Management and other chronic inflammatory diseases **– coordinator from UMF Cluj-Napoca Prof. Dr. Iancu Adrian.**
March 2018 – November 2019 - Researcher in project PCE-2016-RECONSIDER - The Effect of Glucocorticoid Therapy on Left Ventricular Remodeling in Acute ST Elevation Myocardial Infarction – **Director Prof. Dr. Iancu Adrian.**
April 2019 – November 2019 – Researcher in project ECHITAS - Increasing the performance of scientific research and technology transfer in translational medicine through the formation of a new generation of young researchers – **Director Prof. Dr. Irimie Alexandru.**
Summer school
1-13 July 2019 – "From Genes To Cells: A Basic Course of Molecular, Cellular and Ultrastructural Biology" – Pisa University, Pisa, Italia. Grade: 29 out of 30 points.

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