"BABEȘ-BOLYAI" UNIVERSITY, CLUJ-NAPOCA
Faculty of Biology and Geology
Doctoral School of Integrative Biology

DOCTORAL THESIS

-SUMMARY-

PhD Student:
Cruceriu Daniel

PhD Supervisor:
Prof. dr. Rakosy-Tican Elena

Cluj-Napoca
2020

"BABEȘ-BOLYAI" UNIVERSITY, CLUJ-NAPOCA
Faculty of Biology and Geology
Doctoral School of Integrative Biology

Pharmaceutical plant extracts in breast cancer management

Calendula officinalis, Solanum chacoense and *S. bulbocastanum:* biochemical profile, selective anti-tumor activity and associated molecular effects

-DOCTORAL THESIS SUMMARY-

PhD Student:
Cruceriu Daniel

PhD Supervisor:
Prof. dr. Rakosy-Tican Elena

Cluj-Napoca
2020

Table of contents
- Summary -

CHAPTER I - Breast cancer: epidemiology and status quo in the current clinical practice 3

CHAPTER II – Plant-derived compounds in breast cancer management 6

1. Plant-derived compounds in the convetional therapy of breast cancer *6*

2. Plant-derived compounds in the complementary and alternative medicine in breast cancer *7*

3. Plant-derived compounds in breast cancer prevention: concepts of nutrigenomics *8*

CHAPTER III - *Calendula officinalis:* a medicinal herb with anti-tumor proprieties 10

1. The phytochemical profile of *C. officinalis* *10*

2. *C. officinalis* in cancer management *11*

CHAPTER IV - *Solanum spp.*: wild species with nutrigenomic potential in cancer 13

1. The phytochemical profile of *S. chacoense* and *S. bulbocastanum* *14*

2. The anti-tumor activity of *Solanum spp.* *14*

Aim and objectives of the thesis 15

CHAPTER V – Materials and methods 16

1. Preparation of the plant extracts *16*

2.1. Plant material and culture conditions 16

2.2. Extract preparation by the ultrasound-assisted extraction technique 16

3. Analysis of the biochemical profile of the extracts *17*

3.1. Identification and quantification of phenolic compounds and alkaloids, by HPLC-PDA/ESI-MS 17

3.2. Identification and relative quantification of the volatile compounds, by ITEX/GC-MS 17

4. Assessment of the biological activities of the extracts *18*

4.1. Cell lines and culture conditions 18

4.2. Assessment of the anti-tumor activity of the extracts by the MTT assay 18

4.3. Assessment of the molecular effects triggered by the extracts, by RT-qPCR 19

CHAPTER VI – Results 20
1. The phytochemical profile of the extracts *20*
1.1. The phytochemical profile of the *C. officinalis* extracts 20
1.1.1. The polyphenolic content of the *C. officinalis* extracts 20
1.1.2 The volatile compounds content of the *C. officinalis* extracts 21
1.2 The phytochemical profile of the *S. chacoense* extracts *24*
2.1.1. The polyphenolic content of the *S. chacoense* extracts 24
2.1.2. The alkaloid content of the *S. chacoense* extracts 24
2.1.3. The volatile compounds content of the *S. chacoense* extracts 24
3. The anti-tumor activity of the extracts on breast cancer *27*
3.1. The anti-tumor activity of the *C. officinalis* extracts 27
3.2. The anti-tumor activity of the *Solanum spp.* extracts 28
4. Molecular effects triggered by the extracts in breast cancer cells *29*
4.1. Molecular effects triggered by *C. officinalis* extracts 29
4.2. Molecular effects triggered by *Solanum spp.* extracts 29
CHAPTER VII – Discussion 32
1. *C. officinalis*: species with potential in breast cancer management *32*
1.1. The phytochemical profile of the *C. officinalis* extracts 32
1.2. The selective anti-tumor activity of the *C. officinalis* extracts 33
1.3. The mechanisms of action of the *C. officinalis* extracts 34
2. *S. chacoense* and *S. bulbocastanum*: species with potential in breast cancer prevention *35*
2.1. The phytochemical profile of *S. chacoense* extracts 35
2.2. The selective anti-tumor activity of the *Solanum spp*. extracts 36
2.3. The mechanisms of action of the *Solanum spp.* extracts 37
Conclusions 39
References 40

Keywords: *Calendula*, *Solanum*, polyphenols, terpenes, alkaloids, antioxidant activity, breast cancer, selective cytotoxicity, proliferation, apoptosis

CHAPTER I - Breast cancer: epidemiology and status quo in the current clinical practice

Breast cancer represents the most common malignant tumor among women, with over 2 million people being diagnosed with this pathology in 2018. Regarding its mortality rates, breast cancer caused 626,000 deaths globally that year, representing 24.2% of the total cancer deaths among the female population (Bray et al., 2018).

In the current clinical approach, the diagnosis and prognosis of breast cancer mainly involve the determination of the tumor stage, the tumor grade and the status of some biomarkers known for their prognostic value or as therapeutic targets. Based on these clinical parameters, the therapeutic strategy will be established, being specific for each patient. Breast cancer is generally classified as carcinoma in situ and invasive carcinoma, depending on the infiltration of the tumor cells into the adjacent tissues, classes which in turn can be divided into ductal carcinoma and lobular carcinoma, depending on the location of the primary tumor (Mastropasqua and Viale, 2017).

The tumor stage is most often determined using the TNM staging system, depending on the size of the tumor (T), the number of invaded axillary lymph nodes (N) and the presence of metastases in other organs (M) (Koh and Kim, 2019). The TNM system indicates the stage in which a tumor is at the time of diagnosis, but fails to establish the aggressiveness of the tumor cells, an extremely important factor in terms of the evolution of the disease. The histological grade of the tumor represents the degree of differentiation of the tumor cells as compared to the normal cells of the same tissue and is most often established using the Nottingham grading system. The Nottingham grade is determined according to three histological parameters: nuclear polymorphism (indicator of the cell structure), mitotic index (indicator of the proliferation capacity) and presence of tubular epithelial formations (indicator of the tissue architecture) in the tumor tissue (Dalle and et al., 2008).

Although the tumor stage and grade are important elements of breast cancer diagnosis that influence the therapeutic strategy (especially from the point of view of surgery and radiotherapy), the decision regarding the chemotherapeutic adjuvant/ neoadjuvant treatment is based mainly on the

status of some biomarkers with prognostic value and therapeutic target potential. Of these, the most commonly used tumor markers are the hormone receptors for estrogen (ER) and progesterone (PR), the tyrosine kinase receptor Her2/ Neu (ERBB2) and the proliferation marker Ki67 (Nounou et al., 2015). Based on the expression level of these four categories of molecules, breast cancer was divided into four general molecular subtypes: luminal A, luminal B, Her2 positive (Her2 +) and triple negative (Onitilo et al., 2009). The classification of a tumor in one of these molecular subtypes provides information regarding the evolution and progression of the disease in the body on one hand, and regarding the tumor response to therapy, on the other. Thus, based on the classification of breast cancer into molecular subtypes, the patient-specific treatment regimen will be established.

The conventional treatment of breast cancer consists of surgery to remove the tumor, doubled by radiotherapy on the one hand and systemic therapy (chemotherapy, hormonal therapy and targeted therapy) on the other, depending on the clinical characteristics of the patient (Dhankhar et al., 2010). In the case of localized mammary tumors, surgical intervention is performed, most often by lumpectomy (breast conservation operation) (Matsen and Neumayer, 2013). Surgery may be preceded by neoadjuvant treatment to reduce tumor size. Subsequent to tumor extirpation, adjuvant therapy is most often used, in order to minimize the chances of cancer metastasis and relapse (Matsen and Neumayer, 2013). The therapeutic decision regarding the systemic therapies to be administered to breast cancer patients is made based on the tumor's molecular subtype.

The clinical responses of breast cancer patients are largely dependent on the molecular subtype of the tumor, both due to the characteristic phenotype of each of them and due to the more or less limited therapeutic options available (Fig. 1). Thus, the luminal A subtype, with a less aggressive phenotype, a reduced proliferation capacity and with hormone therapy available in the clinic, has the best clinical response, with 5-year survival rates over 90%. At the opposite end are the triple negative breast cancers, which belong to the most aggressive molecular subtype, in which there is no targeted therapy in current clinical practice.

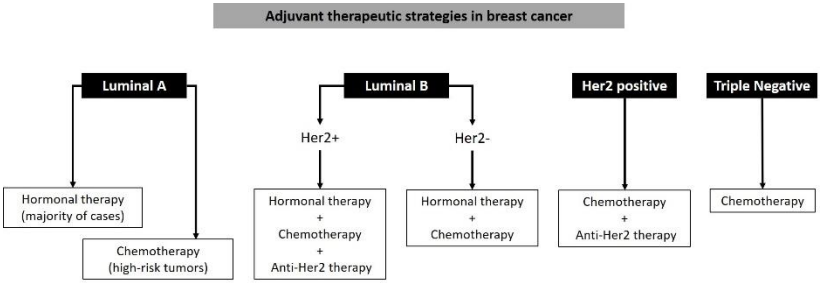

Fig. 1 Therapeutic options in breast cancer, depending on the molecular subtype (adapted from Nounou et al., 2015).

Corroborating all these data, it is evident that conventional breast cancer treatment is far from being effective. Even though there are a several targeted therapeutic options in the current clinical practice, they are limited to certain molecular subtypes, whereas tumor resistance to these treatments is still a common phenomenon (Chun et al., 2017). At the same time, the lack of targeted therapies in triple negative breast cancer, as well as the treatment inefficiency in the case of advanced stage mammary tumors, increase the chances of therapeutic failure (Ismail-Khan and Bui, 2010). Thus, metastatic breast cancer is almost universally fatal in first 5-10 years from the time of diagnosis, a fact that has remained valid for the last 30 years (Tevaarwerk et al., 2013).

In this context, an important part of the efforts of the scientific community is focused on identifying new compounds with anti-tumor action, in order to improve the treatment effectiveness and to reduce the side effects of the conventional therapy. One of the most important sources of such bioactive compounds with anti-cancer potential is represented by plants.

CHAPTER II – Plant-derived compounds in breast cancer management

1. Plant-derived compounds in the convetional therapy of breast cancer

Plants are a primary source of natural compounds, especially secondary metabolites for modern oncology. Over 3000 plant species have been identified over time with anti-tumor properties (Tariq et al., 2017), the mechanisms of induction of cell death caused by natural compounds being extremely diverse (Gali-Muhtasib et al., 2015). Between 1940 and 2014, 49% of the oncological drugs approved for use as part of the chemotherapy grids were naturally occurring compounds (Newman and Cragg, 2016), proving the impact of these plant constituents in the conventional therapy of cancer. However, of the total 250,000 existing plant species, only 10% have been tested for their pharmacological proprieties so far (Iqbal et al., 2017).

The interest in integrating plant compounds into the conventional cancer therapy emerged after the 1950s, when vinca alkaloids and podophyllotoxins were discovered. These compounds represent two categories of secondary metabolites isolated from plants, that are characterized by strong anti-tumor activity (Cragg and Newman, 2005). Subsequently, many other compounds of plant origin with anti-tumor potential have been identified, the most important classes being taxanes and camptothecins, compounds that have been included in conventional chemotherapy grids since the 1990s (Safarzadeh et al., 2014). Thus, an overwhelming proportion of the oncological drugs used today are naturally occurring. Of the total of 65 such drugs approved between 1981-2002 for cancer treatment, 48 are from natural sources (Wang et al., 2012). Furthermore, out of the 121 oncological drugs approved by the Food and Drug Administration (FDA) used in the U.S. in 2013, 90 were initially isolated from plant organisms (Safarzadeh et al., 2014). Of these, two classes of plant-derived compounds are generally used in the treatment of breast cancer: vinca alkaloids and taxanes (Iqbal et al., 2017)

All these data demonstrate the major impact of plant compounds in the treatment of breast cancer, as part of the conventional therapy in current clinical settings. At the same time, it is underlined the potential of the plant constituents in the development of new effective therapies, considering that

only 10% of all plant species have been tested for their pharmacological properties so far (Iqbal et al., 2017).

2. Plant-derived compounds in the complementary and alternative medicine in breast cancer

Complementary and Alternative Medicine (CAM) includes all human health practices that are not an integral part of the conventional health system, but are used by many patients for the purpose of completing their care (Eisenberg et al. 1998). Over 49% of the patients diagnosed with cancer after the year 2000 use at least one such CAM-associated product during or after completion of their conventional treatment (Horneber et al., 2012). The most commonly used form of CAM among cancer patients is the use of different extracts, formulas or supplements of plant origin, grouped under the generic name of herbal preparations (Molassiotis et al., 2006).

Herbal preparations have the potential to increase both the lifespan and the quality of life of oncological patients, with multiple studies suggesting the adjuvant and palliative role they may play (Efferth et al., 2007; Seely and Oneschuk, 2008). These complementary medicine methods based on plant compounds would control the symptoms associated with the conventional treatment, increase the sensitivity of tumor cells to the action of chemotherapy, decrease the cytotoxicity of conventional compounds on normal cells, and improve the body's immune responses (Helyer et al. 2006; Navo et al., 2004). However, the vast majority of data supporting the benefits of herbal preparations come from preclinical, *in vitro* and *in vivo* studies, which cannot be transposed automatically to patients. For human subjects, the efficacy of these complementary therapies is most often based on empirical evidence and particular case studies, whereas large-scale clinical trials are very few (Liao et al., 2013).

Both the beneficial and harmful effects of herbal preparations consumed concomitantly with conventional cancer treatment are largely due to the pharmacodynamic interactions between the compounds involved (Cheng et al., 2010). Thus, even though the plant-derived compounds can potentiate the effects of the chemotherapy, they can also interfere with the anti-tumor activity of oncological drugs (Aung et al., 2017). This phenomenon appears because the additional compounds alter the pathways responsible for the metabolization of the chemotherapies, and thus decrease the tumor exposure

to their action (Enioutina et al., 2017). Therefore, the use of herbal preparations by oncological patients is not always effective (Saxe et al., 2008) and / or safe (Hu et al., 2005).

In view of all these data, herbal preparations seem increasingly used by breast cancer patients, although their efficacy and safety are not always proven. The dual potential of herbal preparations in breast cancer therapy, doubled by the increased confidence in their efficiency among patients, demonstrates the need for the in-depth study of the pharmacodynamic interactions between them and conventional chemotherapeutic compounds. At the same time, the lack of clear scientific evidence on the efficiency and safety of using such herbal preparations, independently or in combination with a specific conventional regimen, makes the subject to be without a consensus in the scientific community.

3. Plant-derived compounds in breast cancer prevention: concepts of nutrigenomics

More and more recent studies demonstrate the important role played by plant bioactive compounds in the prevention of cancer, the plant nutrients being able to modulate the expression of some essential genes in tumorigenesis and in cancer progression (Braicu et al., 2017; Kotecha et al., 2016). Thus, dietary habits influence the risk of cancer, many components of the diet modifying cellular processes relevant to the initiation, promotion and progression of the tumor in the human body (Nicastro et al., 2012; Turati et al., 2015). Breast cancer is one of the types of cancer whose incidence is influenced by environmental factors, including nutrition and diet (Davis, 2007).

The study of cancer prevention revolves around identifying compounds that could have a positive impact against cell transformation in the early stages of oncogenesis (Sapienza and Issa, 2016). A number of plant-derived compounds have the potential to oppose the initiation and promotion of cancer, affecting the initiated cells or triggering other anti-cancer physiological responses of the organism. Thus, various phytoconstituents may induce the detoxification of carcinogens by activating specific metabolic pathways (Royston and Tollefsbol, 2015), but also by enhancing immune surveillance leading to the elimination of the transformed cells (Luis Espinoza et al., 2013). In initiated cells, plant-derived compounds can

increase DNA stability, both by preventing degradation and by inducing the repair of the genetic material (Ferguson et al., 2004). At the same time, many plant constituents are capable of inducing substantial epigenetic changes in transformed cells, which will oppose tumor initiation and promotion (Kotecha et al., 2016).

However, the beneficial effects of nutrients in cancer prevention depend not only on the bioactive compounds themselves, but also on the genetic predisposition of each individual. Thus, the variation in the incidence of cancer in human populations with similar eating habits can be explained from the point of view of the genetic and epigenetic particularities of each individual, which influence the susceptibility to the beneficial action of the diet (Ardekani and Jabbari, 2009). In this context, nutrigenomics in cancer, a science that studies the impact of nutrients on the cellular genome, has developed in recent years, emphasizing how diet-induced epigenetic changes influence tumorigenesis by modulating the expression of genes relevant to cancer initiation and promotion.

In this context, it is emphasized the need to test the plant-derived nutrients from the perspective of the epigenetic effects induced in the initiated cells and to identify those components of the diet that would have a potential protective role against the initiation, promotion and progression of cancer. At the same time, biological barriers that oppose the anti-tumor effects, such as the reduced bioavailability of some nutrients, their low absorption level or the too low concentrations of the active compounds in foods will require increased attention in order to overcome them.

CHAPTER III - *Calendula officinalis*: a medicinal herb with anti-tumor proprieties

[Chapter III, in an adapted form, was published as: Cruceriu D, Balacescu O, Rakosy-Tican E, 2018. Calendula officinalis: potential roles in cancer treatment and palliative care. Integrative Cancer Therapy 17(4):1068-1078]

Calendula officinalis (*Asteraceae* family), popularly called the pot marigold, is a species commonly used in traditional medicine. Various herbal preparations obtained from flowers and leaves of this species are popularly used as anti-spasmodic and anti-inflammatory remedies, in the treatment of wounds, minor burns, irritations and other rashes (Mehta et al., 2012). The extracts of *C. officinalis* are characterized by various pharmacological activities, the most important medicinal properties reported in the literature being the anti-inflammatory, antioxidant (Frankic et al., 2009), antibacterial (Goyal and Mathur, 2011), antifungal (Gazim et al., 2008), and immunostimulatory (Varlijen, 1989) activities. The biological activity of the extracts is due to the biochemical profile, especially the secondary metabolites.

1. The phytochemical profile of *C. officinalis*

The most important classes of compounds found in the organs of *C. officinalis* are polyphenols, terpenes, coumarins and quinones (Ashwlayan et al., 2018; Khalid and Da Silva, 2012). Phenolic compounds, including phenolic acids and flavonoids, are found in large quantities in all above-the-ground organs of this species. Terpenoids are found both in extracts obtained with less polar solvents and in the fraction of volatile compounds of *C. officinalis* preparations, terpenes representing an important part of the plant-derived essential oils.

Given the diversity of constituents in the plant preparations obtained from *C. officinalis*, the premise that this species could have potential in cancer management is also outlined. Polyphenols and volatile terpenes are classes of compounds known for their anti-tumor activity, being phytoconstituents capable of inhibiting cell proliferation and inducing apoptosis (Fantini et al., 2015; Nichenametla et al., 2006). Moreover, three compounds directly isolated from *C. officinalis*, lutein and two glycosylated triterpenes, were

tested for anti-tumor effects and have been shown to be cytotoxic in various tumor pathologies.

2. *C. officinalis* in cancer management

The anti-tumor effects of *C. officinalis* extracts both *in vitro* and *in vivo* in animal models have been demonstrated for the first time more than 25 years ago (Boucaud-Maitre et al., 1988). Numerous other studies have subsequently succeeded in completing these data, various plant preparations obtained from the leaves, flowers and roots of this species being characterized by anti-tumor action. The current state of knowledge regarding *C. officinalis* preparations in terms of their anti-tumor effects *in vitro* and *in vivo*, and also of their potential in palliative care of oncological patients is presented in figure 2.

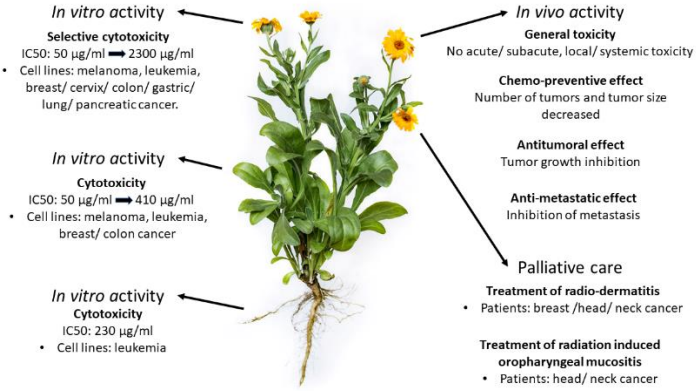

Fig. 2 General aspects of the anti-tumor activity of *C. officinalis* extracts. Flower extracts: activity *in vitro, in vivo* and in palliative care; leaf extracts: *in vitro* activity; root extracts: *in vitro* activity (Cruceriu et al., 2018).

Herbal extracts of *C. officinalis* have been shown to be characterized by anti-tumor activity in *in vitro* systems on numerous cancer cell lines. Moreover, in specific cases, these preparations were also selective in the anti-tumor action. The vast majority of studies were performed on extracts obtained from flowers of this species, although a 2012 study suggests that the root preparation would be superior (Wegiera et al., 2012). The preparations obtained in distilled water by infusion or maceration are characterized by reduced cytotoxic action, even if Jimenez-Medina et al. (2006) manage to

increase the performance of this type of extract by laser activation. The extracts obtained in methanol are superior to those in water from the perspective of the anti-tumor action exerted on the cell lines (Miguel et al., 2016). From the point of view of the mechanisms of action, *C. officinalis* extracts appear to have both cytotoxic and cytostatic effects in tumor cells. The decrease in the levels of cyclin expression following treatment suggests that the cell cycle is hampered, whereas the activation of caspases underlines the likelihood of apoptosis induction (Jimenez-Medina et al., 2006).

The i*n vivo* activity of *C. officinalis* extracts obtained from flowers has been investigated over the last 20 years from the perspective of their general toxicity, anti-genotoxic/ chemo-preventive effects, anti-tumor action and anti-metastatic capacity in different animal models. Herbal preparations obtained from *C. officinalis* flowers do not induce acute and subacute (Silva et al., 2007) and neither systemic nor local (Jimenez-Medina et al., 2006) toxicity, at the doses needed to treat cancer. Both ethanolic (Barajas-Farias et al., 2006) and methanolic extracts (Ali et al., 2014) obtained from *Calendula* flowers have chemo-preventive effects on chemically induced carcinogenesis in model animals. In addition to the ability to prevent cancer, *C. officinalis* extracts also possess anti-tumor actions (Jimenez-Medina et al, 2006). Another ethanolic extract from the flowers of the same species increased the lifespan by 43% and inhibited the lung metastasis of melanoma in *Calendula*-treated mice (Preethi et al., 2010).

In addition to the anti-tumor action of *C. officinalis* extracts, several recent clinical studies suggest that herbal preparations obtained from the flowers of this species could become relevant resources in the palliative care of oncological patients with breast, head or neck cancer, whose therapeutic regimen include radiotherapy (Pommier et al., 2004; Sharp et al., 2013).

Therefore, herbal preparations obtained from the organs of *Calendula officinalis*, have potential in cancer management, both in prevention and therapy, as well as in palliative care. The diversity of secondary metabolites, especially polyphenols and terpenes that are characterized by cytotoxic and cytostatic action on cancer cells, makes *Calendula* extracts to have anti-tumor activity, *in vitro*. At the same time, the fact that the biological activity is maintained in animal models, as anti-genotoxic, anti-tumor and anti-metastatic action, increases the potential of using this species in the prevention and treatment of cancer.

CHAPTER IV - *Solanum spp*.: wild species with nutrigenomic potential in cancer

Potato (*Solanum tuberosum*), as a cultivated species, is one of the most important vegetable sources of food for the world's population, being ranked 4th in terms of consumption, after wheat, rice and corn (FAO - Food and Agriculture Organization of the United Nations, 2018). Thus, *S. tuberosum* plays an essential role in ensuring access to food for the world's population, especially in underdeveloped countries, taking into consideration the rapid growth of the global birth rates (International Potato Center, 2018).

However, *Solanum tuberosum* is susceptible to attack by various consumers/ parasites/ pathogens (insects, nematodes, fungi, bacteria, viruses) and has low resistance to abiotic environmental factors, which cause serious crop losses (Haldar et al., 2006). The main cause for which the cultivated potato species has lost its resistance characteristics is human intervention, through monoculture, artificial selection to increase productivity and asexual propagation through tubers. In this context, the scientific community has proposed the reintegration of those important features, which are involved in the response to various environmental factors, in the potato genome. Thus, wild potato species, such as *S. chacoense* and *S. bulbocastanum*, have been used as genetic resources in potato breeding programs (De Haan and Rodriguez, 2016; Rakosy-Tican et al., 2019).

S. bulbocastanum is used in potato breeding due to its resistance to *Phytophthora infestans* (Lokossou, 2010; Rakosy-Tican et al., 2015), whereas *S. chacoense* due to its resistance to the Colorado beetle and bacterial attacks (Chen et al., 2013; Molnar et al., 2017; Rakosy-Tican et al., 2019). The resistance of these wild potato species to various pathogens is largely based on phytochemical constituents, secondary metabolites present in different organs, such as glycoalkaloids, having repellent and/ or toxic effects on pests (Mweetwa et al., 2012). Not coincidentally, such compounds that are part of the phytochemical profile of different wild potato species, such as solamargine (Liu et al., 2004; Shiu et al., 2007), α-chaconine or α-solanine (Friedman, 2015), also have anti-tumor properties.

1. The phytochemical profile of *S. chacoense* and *S. bulbocastanum*

Data related to the biochemical profile of the wild *Solanum* species are extremely scarce in the literature and are far from exhaustive. The two classes of secondary metabolites that characterize the *Solanaceae* species are polyphenols, especially phenolic acids and alkaloids. Regarding the glycoalkaloid content, *S. bulbocastanum* and *S. chacoense* show elevated levels of α-solanine and α-chaconine in the leaves, and high amounts of solamargine and solasonine in the tubers (Distl and Wink, 2009). Regarding their polyphenolic content, chlorogenic acid is the compound that is found in the highest abundance in the organs of these two species (Hale et al., 2008; Navarre et al., 2011).

2. The anti-tumor activity of *Solanum spp.*

The plant extracts obtained from the wild species *S. chacoense* and *S. bulbocastanum* have been little investigated in terms of their anti-tumor properties. Only two plant extracts obtained from *S. chacoense* (Mamone et al., 2011; Mongelli et al., 1999) were tested for their anti-tumor activity, both preparations being characterized by cytotoxic activity. No study investigated the anti-tumor properties of extracts obtained from *S. bulbocastanum*. Even though the complete extracts of these two species of interest have not been intensively studied, the alkaloids specific to the *Solanaceae* family, α-solanine, α-chaconine or solamargine have been extensively analyzed in terms of their anti-tumor effects. All of these compounds are proven to be cytotoxic and/ or cytostatic in various tumor pathologies, including breast cancer (Friedman, 2015).

In this context, the plant extracts obtained from the wild *Solanum* species could be relevant sources of bioactive constituents with anti-tumor activity that could be included in the conventional therapeutic grids. Moreover, due to the intense use of these species in breeding programs, the new varieties obtained could be characterized by high contents of such nutrients with anti-tumor activity. Thus, these newly cultivated plants could play a role in preventing cancers through nutrition and diet. At the same time, given the importance of the potato as a plant food source for the human population, the impact of these new varieties with high contents of compounds with nutrigenomic properties in cancer would be significant.

Aim and objectives of the thesis

Aim of the thesis

Evaluation of the anti-tumor potential of five plant extracts obtained from flowers and leaves of *C. officinalis*, leaves of *S. bulbocastanum* and leaves and tubers of *S. chacoense*, against three cell lines belonging to the luminal and triple negative subtypes of breast cancer.

Objectives of the thesis

Objective 1. Biochemical characterization of the methanolic extracts of *C. officinalis* and *S. chacoense* obtained by ultrasound-assisted extraction (UAE), regarding their content of polyphenols, volatile compounds and alkaloids by spectrophotometric methods, HPLC and MS.

Objective 2. Assessment of the biological activity of the extracts obtained from *C. officinalis*, *S. chacoense* and *S. bulbocastanum* in terms of antioxidant activity by the ABTS, FRAP and CUPRAC methods, and of the selective anti-tumor activity on the breast cancer cell lines MCF7, MDA-MB-231 and HS578T compared to the healthy HUVEC cell line, by the MTT method.

Objective 3. Identification of some molecular mechanisms of action of the extracts from *C. officinalis, S. chacoense* and *S. bulbocastanum* in MCF7 cell line, based on the gene expression evaluation of 14 genes involved in apoptosis and cell proliferation, by RT-qPCR.

CHAPTER V – Materials and methods

1. Preparation of the plant extracts

2.1. Plant material and culture conditions

C. officinalis plants were obtained from seeds germinated in soil, and subsequent cultivation *ex vitro*, under controlled laboratory conditions. The species authentication was carried out in the Herbarium of the "Babes-Bolyai" University, Romania (CL Herbarium; specimen authentication voucher 668431). The leaves and flowers were dried in the dark and further powdered.

The seeds of *Solanum chacoense* Bitt., Accession PI 458310 were obtained from the National Plant Germplasm System of the United States of America (NPGS, Sturgeon Bay, WI, USA), whereas *S. bulbocastanum* Accession GLKS-31741 were provided by the Institute of Plant Genetics and Crop Plant Research, Germany [Gross Lüsewitz Potato Collections (GLKS) of the IPK Gene Bank, Leibniz, Germany]. The two species were grown *in vitro*, under laboratory conditions, optimized for potato species. Subsequently, the explants were acclimatized *ex vitro* in soil, being cultivated under controlled laboratory conditions. The plant material, consisting of leaves from both species and tubers harvested from *S. chacoense*, was dried in the dark and further powdered.

2.2. Extract preparation by the ultrasound-assisted extraction technique

Starting from the plant material collected from the three species included in the study, five plant extracts were prepared: *C. officinalis* - flowers; *C. officinalis* - leaves; *S. bulbocastanum* - leaves; *S. chacoense* - leaves; *S. chacoense* - tubers. Regardless of the plant material used, the protocol for obtaining the extracts was the same, being based on the ultrasonication technique, in 70% methanol. The dried plant material (5 g) harvested from each of the three species was macerated in 70% methanol (50 mL) and ultrasonicated in three successive cycles, with a Sonics Vibra-cell sonicator (VC750, Sonics). The obtained suspension was left to macerate for another 24h, at room temperature, in the dark. Subsequently, the homogenate was centrifuged, and the supernatant containing the dissolved vegetable compounds was collected and filtered. The solvent from this crude methanolic extract was evaporated under reduced pressure, at 40°C, in a rotary vacuum evaporator (Laborota 4000 Efficient, Heidolph Instruments

GmbH), until a powder was obtained. Half of the powder obtained for each extract was homogenized in 100% dimethyl sulfoxide (DMSO) to be used in *in vitro* cell culture assays, and the other half was redissolved in 70% methanol to be used in the determination of the biochemical profile of the extracts.

3. Analysis of the biochemical profile of the extracts

3.1. Identification and quantification of phenolic compounds and alkaloids, by HPLC-PDA/ESI-MS

The HPLC-PDA analysis of the four extracts of interest was performed on an Agilent 1200 device, coupled with an SPD-M20 UV-VIS PDA (DAD) detector. The mobile phase consisted of: solvent A - bidistilled water and 0.1% acetic acid/ acetonitrile (99/1) v/v; Solvent B - acetonitrile and 0.1% acetic acid. Chromatograms were monitored at 340 nm.

For the mass spectrometric analysis, a type 6110, quadruple mass spectrometer (Agilent Technologies), coupled with an ESI ionizer was used. The experimental data were acquired in full scan mode, between 280-1000 m/z. The identification of the compounds and the assignment of the corresponding peaks on chromatograms was performed based on the retention time (Rt), the UV-VIS absorption spectrum and the mass spectrum specific to each compound of the analyzed plant extracts, in comparison with a series of commercial standards.

3.2. Identification and relative quantification of the volatile compounds, by ITEX/GC-MS

The volatile compounds were extracted from the gaseous (evaporated) phase of the extracts using an AOC-5000 Combi PAL autosampler (CTC Analytics) equipped with an ITEX-II syringe (ITEX-2TrapTXTA, Tenax TA 80/100 mesh) and directly desorbed in the GC. GC-MS analysis was performed on a GCMS QP-2010 mass spectrometer coupled with gas chromatography (Shimadzu Sci. Instruments). The gas used to create the gas stream with a role in the transport of volatile compounds was helium, with a flow rate of 1 mL/ min, at a ratio of 1:20. Detection in the MS was performed on a quadruple spectrometer. The experimental data were acquired in full scan mode, in the range 40-450 m/ z.

The identification of the volatile compounds was performed by comparing the results obtained for each compound with the mass spectra contained by the libraries available online (NIST27 and NIST147), taking into account a similarity of at least 85%. At the same time, the retention times obtained for each compound were compared with the retention times available in two online databases, www.pherobase.com and www.flavornet.org. The relative quantification of each volatile compound identified was estimated as a fraction of its integrated ion area from the total ion chromatograms (TIC) area (100%).

4. Assessment of the biological activities of the extracts

4.1. Cell lines and culture conditions

Four human cell lines were used in this study: MCF7, MDA-MB-231, HS578T and HUVEC. All cell lines were obtained from the European Collection of Authenticated Cell Cultures. MCF7 cells were grown in MEM medium, MDA-MB-231 cell line in RPMI-1640 medium, HS578T line in DMEM medium, and HUVEC cells in EGM medium. All cell lines were maintained at 37 ℃, in a humidified atmosphere (90%) containing 5% CO2, in the incubator.

4.2. Assessment of the anti-tumor activity of the extracts by the MTT assay

The anti-tumor activity of the five extracts was determined by the MTT assay (Sigma-Aldrich) on MCF7, MDA-MB-231 and HS578T breast cancer cell lines and on the HUVEC line of normal endothelial cells, according to the manufacturer's protocol. In short, 2 x 10^4 cells/ well were seeded into 96-well plates. The treatment was added after 24 hours, in nine successive concentrations (50-1000 µg/ ml). After 48h of incubation of the cells with the plant extracts under normal cell culture conditions, the supernatant was removed and 100 µL/ well of MTT solution was added. After an additional 1h incubation in the dark at 37 ℃, the MTT solution was replaced with 150 µL of 100% DMSO (Carl Roth GmbH), and the samples' absorbance was measured at 570 nm (Synergy HTX, BioTek).

Cell viability was calculated as the fraction of viable cells in the treated samples compared to the untreated control cells, based on the obtained absorbances. The IC_{50} values for each extract on each cell line were calculated in GraphPad Prism Version 5 software (GraphPad Software). The selectivity of the extracts in the anti-tumor activity was determined by

calculating the specific selectivity coefficients for each cell line, by comparing the IC_{50} values of each extract on the normal HUVEC line to the IC_{50} values of each extract on each breast cancer cell line.

4.3. Assessment of the molecular effects triggered by the extracts, by RT-qPCR

The molecular effects of the five extracts obtained starting from the plant material harvested from *C. officinalis*, *S. chacoense* and *S. bulbocastanum* were determined on the MCF7 cell line by quantifying the relative expression levels of 14 genes considered of interest, by RT-qPCR. The 14 genes evaluated (*BCL2*, *BAX*, *BID*, *BBC3*, *PMAIP*, *TP53*, *TP53INP1*, *CASP3*, *CASP7*, *CCND1*, *NFkB*, *STAT3*, *ZMAT3* and *DRAM1*) encode proteins that are highly relevant in cell proliferation or apoptosis, most of them being considered molecular markers of these processes in cancer.

Regarding the experimental design, cells belonging to the MCF7 cell line were seeded in 12-well plates, at a density of 2×10^5 cells/ well. After 24h from seeding, the cells were treated with each of the five extracts at the concentration corresponding to IC_{50}, in technical duplicates. After an additional 48h, the cells were lysed with TriReagent lysate solution (Sigma-Aldrich), and total RNA was extracted by the classical technique with phenol-chloroform. RNA quantity and quality were evaluated using the nanodrop (NanoDrop 1000, ThermoScientific) and the bioanalyzer (Agilent Technologies). Complementary DNA (cDNA) synthesis, starting from 500 ng of total RNA from each sample, was performed using the RevertAid First Strand cDNA Synthesis Kit (# 1622, ThermoScientific). Evaluation of the relative expression levels of the genes of interest was carried out by RT-qPCR in the TaqMan system, using the TaqMan Master LightCycler kit (Roche), on a LightClycler 480 type qPCR apparatus (Roche). The relative expression level of the genes of interest was calculated according to the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

CHAPTER VI – Results

*[Parts of Chapters V, VI and VII, in an adapted form, were published as: **1**. Cruceriu D, Diaconeasa Z, Socaci S, Socaciu C, Rakosy-Tican E, Balacescu O, 2020. Biochemical profile, selective cytotoxicity and molecular effects of Calendula officinalis extracts on breast cancer cell lines. Notulae Botanicae Horti Agrobotanici 48(1):24-39; **2**. Cruceriu D, Diaconeasa Z, Socaci S, Socaciu C, Balacescu O, Rakosy-Tican E, 2020. Extracts of the wild potato species Solanum chacoense on breast cancer cells: biochemical characterization, in vitro selective cytotoxicity and molecular effects. Nutrition and Cancer, online first: https://doi.org/10.1080/01635581.2020.1761407]*

1. The phytochemical profile of the extracts

The phytochemical profiles of the extracts of *C. officinalis* and *S. chacoense* were evaluated in terms of their content of polyphenols and volatile compounds, the data in the scientific literature suggesting their presence in the biochemical composition of these species (Ashwlayan et al., 2018; Caruso et al., 2018; ., 2013), and the anti-tumor potential of such constituents (Dhifi et al., 2016). At the same time, the presence of alkaloids, compounds with recognized cytotoxic activity, was evaluated in *S. chacoense* extracts (Friedman, 2015).

1.1. The phytochemical profile of the C. officinalis extracts

1.1.1. The polyphenolic content of the C. officinalis extracts

Among the phenolic constituents, 14 compounds were individually identified in the extract obtained from *C. officinalis* flowers, whereas in the leaf extract only 12 such compounds were present (Table 1). Both extracts were dominated by flavonols, including multiple derivatives of quercetin and isorhamnetin. In addition to flavonols, both extracts were characterized by the presence of phenolic acids, including hydroxybenzoic and hydroxycinnamic acids and coumarins (Table 1).

The major constituents identified in the extract obtained from *C. officinalis* flowers were chlorogenic acid, among phenolic acids, and quercetin-3-O-glucosyl-rhamnosyl-glucoside, isorhamnetin-3-O-galactoside isorhamnetin-3-O-glucosyl-rhamnoside and isorhamnetin-7-O-rhamnoside from the flavonol fraction. The most important polyphenolic constituents

identified in the extract obtained from the leaves of this species were dihydroxybenzoic acid, isoquercetin and isorhamnetin-3-O-glucosyl-rhamnoside (Table 1).

Comparing the polyphenolic profile of the two *C. officinalis* extracts, several relevant differences should be emphasized. The extract obtained from *C. officinalis* flowers is characterized by higher amounts of polyphenols compared to the one obtained from leaves. The only class of phenolic compounds that is present in greater quantity in the leaf extract is the class of hydroxybenzoic acids, their concentration being 57% higher in this extract. In contrast, hydroxycinnamic acids have a twice higher concentration in the flower extracts. In terms of the flavonol content, the flower extract proved to be superior again, containing 188% more compounds belonging to this subclass. Moreover, two flavonols, isorhamnetin and isorhamnetin-3-O-glucoside were identified only in the extract obtained from *C. officinalis* flowers. Last but not least, scopoletin-7-O-glucoside, a compound that is part of the class of coumarins, had a concentration more than three times higher in the flower extract compared to the one obtained from *C. officinalis* leaves (244% difference).

1.1.2 The volatile compounds content of the C. officinalis extracts

A total of 24 volatile compounds were identified in the extract obtained from *C. officinalis* flowers, while the extract from the leaves of the same species contained only 16 such compounds (Table 2). The volatile constituents identified in the extracts belong to several important classes of secondary metabolites, such as monoterpenes, sesquiterpenes, ketones or volatile aldehydes.

Comparing the volatile biochemical profile of the two extracts from *C. officinalis*, several relevant differences are highlighted. The relative abundance of sesquiterpenes in the flower extract (38.07%) is much higher than in the leaf extract (4.65%). Several compounds belonging to the class of volatile aldehydes were identified only in the flower extract (Table 2). Corroborating these data, the extract from *C. officinalis* flowers may be considered superior in terms of the volatile compound diversity, as compared to that obtained from the leaves.

Table 1. Identification and quantification of phenolic compounds in the extracts of *C. officinalis* leaves and flowers. R_t – retention time; $[M+H]^+$- molecular ion; UV λ_{max} - wavelengths of maximum absorption in the visible region.

Class of compounds	Peak No.	R_t (min)	$[M+H]^+$ (m/z)	UV λ_{max} (nm)	Tentative identified compound	Quantification (µg/mL extract) *C. officinalis* flowers	Quantification (µg/mL extract) *C. officinalis* leaves
Hydroxybenzoic acids (BA)	1	3.05	**156,** *139*	240	Dihydroxybenzoic acid	366.89	576.00
TOTAL BA[1]						**366.89**	**576.00**
Hydroxycinnamic acids (HBA)	2	9.87	**355**	320	3-Caffeoylquinic acid (Neochlorogenic acid)	458.61	321.11
	3	11.85	**355**	320	5-Caffeoylquinic acid (Chlorogenic acid)	1261.26	386.57
	11	17.00	**517**	330	3,5 Dicaffeoylquinic acid (Isochlorogenic acid A)	842.42	365.29
TOTAL HBA[2]						**2562.29**	**1072.97**
Flavonols (FL)	4	13.93	**757**,*303*	360, 260	Q-3-O-rhamnosyl-rhamnosyl-glucoside	401.66	103.05
	5	14.68	**773**, *303*	355, 255	Q-3-O-glucosyl-rhamnosyl-glucoside	1165.46	423.18
	6	15.34	**611,** *303*	360, 250	Q-3-O-rutinoside (Rutin)	374.52	402.98
	7	15.77	**478,** *317*	350, 250	I-3-O-galactoside	1273.79	106.05
	8	16.00	**465,** *303*	361, 251	Q-3-O-glucoside (Isoquercetin)	243.65	591.78
	9	16.15	**478,** *317*	350, 250	I-3-O-glucoside	634.70	nd
	10	16.53	**624**, *317*	350, 260	I-3-O-glucosyl-rhamnoside	1866.82	767.72
	12	17.31	**479,** *317*	362, 352	I-7-O-rhamnoside	1727.26	375.56
	14	22.92	**317**		Isorhamnetin	300.53	nd
TOTAL FL[3]						**8728.98**	**2985.54**
Coumarins (CM)	13	18.07	**355,** *193*	358, 261	S-7-O-glucoside	740.59	215.22
TOTAL CM[3]						**740.59**	**215.22**

[1] expressed as µg gallic acid /mL
[2] expressed as µg chlorogenic acid /mL
[3] expressed as µg rutin /mL

Q-Quercetin
I-Isorhamnetin
S-Scopoletin

nd-not detected

Table 2. Identification and relative quantification of volatile compounds in the extracts of *C. officinalis* flowers and leaves. R_t – retention time; Relative abundance - relative percentage (%) of total peaks area.

Class of compounds	Peak. No.	R_t (min)	Tentative identified compound	Relative abundance (%)	
				C. officinalis flowers	*C. officinalis* leaves
(Mono-) Terpenes (MT)	8	14.30	p-Cymene	0.35	0.96
	9	14.45	Limonene	0.32	0.38
	15	17.80	Cosmene	nd	0.64
	16	18.20	Alloocimene	0.8	2.6
TOTAL MT				**1.47**	**4.58**
(Sesqui-) Terpenes (ST)	23	24.60	Alpha-Cubebene	0.59	nd
	24	25.60	Copaene	1.01	nd
	25	29.50	Gamma-Muurolene	3.15	0.73
	26	30.40	Alpha-Muurolene	8.81	3.92
	27	31.10	Delta-Cadinene	24.51	nd
TOTAL ST				**38.07**	**4.65**
Aldehydes	5	12.15	Benzaldehyde	3.37	1.6
	10	15.00	Benzeneacetaldehyde	0.62	0.26
	7	13.60	Octanal	0.28	nd
	14	17.10	Nonanal	0.42	nd
	20	20.30	Decanal	0.22	nd
TOTAL				**4.91**	**1.86**
Ketones	11	15.70	Acetophenone	8.89	7.08
	18	19.00	Propiophenone	nd	0.2
	21	22.70	2-Chloroacetophenone	0.87	nd
TOTAL KT				**9.76**	**7.28**
Esters	2	5.90	Methyl isovalerate	0.33	nd
	3	7.05	Butyl acetate	30.45	69.91
	4	10.80	Methyl hexanoate	0.1	nd
	13	16.70	Methyl benzoate	4.64	3.6
	19	19.90	Methyl Salicylate	0.31	nd
	22	23.00	Benzeneacetic acid, .alpha.-oxo-, methyl ester	1.12	nd
TOTAL				**36.95**	**73.51**
Others	1	4.80	Isobutylaldehyde dimethyl acetal	0.37	0.67
	6	12.70	1,1-Dimethoxyhexane	7.24	6.08
	12	16.50	2-Methyl-1-phenylpropene	nd	0.56
	17	18.70	Benzoic Acid	1.22	0.83
TOTAL				**8.83**	**8.14**

nd – not detected

1.2 The phytochemical profile of the S. chacoense extracts

2.1.1. The polyphenolic content of the S. chacoense extracts

Regarding the polyphenol classes identified in the extracts of *S. chacoense*, the phenolic acids proved to be the majority of the constituents. The phenolic acids identified in the two extracts of *S. chacoense* belong to the classes of hydroxybenzoic and hydroxycinnamic acids. Four phenolic acids were identified in the leaf extract, whereas two additional phenolic acids (caffeic and ferulic acid) were found in the tuber extract (Table 3).

Phenolic acids were found in higher quantities in the tuber extract in comparison to the one obtained from *S. chacoense* leaves, with 67% for hydroxybenzoic acids and 77% for hydroxycinnamic acids (Table 3). Thus, the extract obtained from *S. chacoense* tubers might be considered superior to the one from the leaves of the same species, both in terms of phenolic compound diversity and concentration.

2.1.2. The alkaloid content of the S. chacoense extracts

Four alkaloids specific to the *Solanaceae* family were identified in high concentrations in the extract obtained from the leaves of *S. chacoense* (Table 4). These compounds were not present in significant quantities in the extract from the tubers of the same species. Regarding the classes of compounds to which they belong, the identified alkaloids are both solasodines (solasodine derivatives) and solanidanes (solanidine derivatives).

2.1.3. The volatile compounds content of the S. chacoense extracts

A number of 18 volatile compounds were identified in the leaf extract, while the extract from tubers contained 21 such compounds. These constituents belong to the classes of terpenes, aldehydes, fatty alcohols, esters and ketones (Table 5). The terpenes, aldehydes and ketones identified had higher relative abundances in the tuber extract (5.15%, 20.79% and 21.47%), in comparison to the leaves extract (2.01%, 6.06% and 12.35%). Compounds such as limonene, octane, nonanal, and several acetophenones had much higher relative abundances in the tuber extract, and constituents such as decanal, dodecanal and fatty alcohols were present only in this extract. The only class of compounds whose relative abundance was higher in the leaf extract was the class of esters, butyl acetate accounting for 53.7% of the total volatile compounds identified in this extract.

Table 3. Identification and quantification of phenolic acids in the extracts of *S. chacoense* leaves and tubers. R_t – retention time; $[M+H]^+$ - molecular ion; UV λ_{max} - wavelengths of maximum absorption in the visible region.

Class of compounds	Peak No.	R_t (min)	$[M+H]^+$ (m/z)	UV λ_{max} (nm)	Tentative identified compound	Quantification (µg/mL extract) *S. chacoense* leaves	Quantification (µg/mL extract) *S. chacoense* tubers
Hydroxybenzoic acids (BA)	1	3.05	**156,** *139*	240	Dihydroxybenzoic acid	91.20	152.48
TOTAL BA[1]						**91.20**	**152.48**
Hydroxycinnamic acids (HBA)	2	10.66	**355**	320	3-Caffeoylquinic acid (Neochlorogenic acid)	183.74	69.06
	3	11.35	**355**	320	4-Caffeoylquinic acid (Cryptochlorogenic acid)	214.60	77.56
	4	11.85	**355**	320	5-Caffeoylquinic acid (Chlorogenic acid)	271.10	242.32
	5	13.57	**181**	320	Caffeic acid	nd	431.25
	8	16.88	**195**	322	Ferulic acid	nd	364.92
TOTAL HBA[1]						**669.43**	**1185.11**

[1] expressed as µg chlorogenic acid /mL nd-not detected

Table 4 Identification and quantification of alkaloids in the extracts of *S. chacoense* leaves and tubers. R_t – retention time; $[M+H]^+$ - molecular ion; UV λ_{max} - wavelengths of maximum absorption in the visible region.

Class of compounds	Peak No.	R_t (min)	$[M+H]^+$ (m/z)	UV λ_{max} (nm)	Tentative identified compound	Quantification (µg/mL extract) *S. chacoense* leaves	Quantification (µg/mL extract) *S. chacoense* tubers
Solasodines	6	14.77	**414**	350, 290	Solasodine	331.403	nd
	7	16.02	**868**	370,320,230	Solamargine	451.394	nd
Solanidanes	9	17.29	**868**	370,320,230	α-Solanine	573.333	nd
	10	19.56	**852**	420,310,240	α-Chaconine	657.238	nd
TOTAL Alkaloids[1]						**2013.37**	**-**

[1] expressed as µg chlorogenic acid /mL nd-not detected

Table 5 Identification and relative quantification of volatile compounds in the extracts of *S. chacoense* leaves and tubers. R_t – retention time; Relative abundance - relative percentage (%) of total peaks area.

Class of compounds	Peak no.	R_t (min)	Tentative identified compound	Relative abundance (%)	
				S. chacoense **leaves**	***S. chacoense*** **tubers**
Terpenoids	11	14.486	Limonene	1.13	4.09
	12	14.591	Eucalyptol**	nd	1.06
	25	31.087	delta-cadinene	0.88	nd
TOTAL				**2.01**	**5.15**
Aldehydes	4	11.997	2-Heptenal, (E)-	nd	2.26
	5	12.150	Benzaldehyde	4.67	9.39
	10	13.639	Octanal	0.35	1.52
	13	15.029	Benzeneacetaldehyde	0.58	1.19
	14	15.510	2-Octenal, (E)-	nd	1.73
	18	17.082	Nonanal	0.46	1.5
	20	20.336	Decanal	nd	1.1
	23	26.713	Dodecanal	nd	2.1
TOTAL				**6.06**	**20.79**
Fatty alcohols	16	15.940	1-Octanol	nd	2.28
	24	29.460	1-Dodecanol	nd	1.73
TOTAL				**-**	**4.01**
Esters	2	7.048	Butyl acetate	53.7	8.04
	3	10.830	Methyl hexanoate	0.25	nd
	17	16.727	Methyl benzoate	6.89	9.08
	22	23.002	Benzeneacetic acid, .alpha.-oxo-, methyl ester	1.06	3.59
	26	31.216	Methyl laurate**	0.49	nd
TOTAL				**62.39**	**20.71**
Ketones	15	15.743	Acetophenone	10.91	17.01
	21	22.692	2-Chloroacetophenone	1.44	4.46
TOTAL				**12.35**	**21.47**
Others	1	4.796	Isobutylaldehyde dimethyl acetal	1.45	nd
	6	12.291	N-Methylaniline	0.71	nd
	7	12.700	Hexanal dimethyl acetal	9.55	4.12
	8	12.754	Phenol	nd	5.38
	9	13.120	2-pentylfuran	nd	6.49
	19	18.864	Benzoic Acid	3.24	11.9
	27	31.394	Phenylmaleic anhydride	2.23	nd
TOTAL				**17.18**	**27.89**

** - similarity lower than 85% in comparison with NIST27 and NIST147 mass spectra libraries

nd – not detected

3. The anti-tumor activity of the extracts on breast cancer

3.1. The anti-tumor activity of the C. officinalis extracts

Both extracts obtained from the flowers and leaves of *C. officinalis* were characterized by dose-dependent anti-tumor activity (Fig. 3). By comparing the IC_{50} values obtained for the breast cancer cell lines with those characteristics for the healthy HUVEC cell line, both extracts were found to be selective in their anti-tumor activity (Table 6).

Both extracts had the strongest anti-tumor effect on the luminal breast cancer cell line MCF7. However, the extracts can be considered generally selective in their anti-tumor activity, even if the selectivity coefficient of the leaf extract on the HS578T line is below 1. Comparing the results for the two extracts obtained from *C. officinalis*, the flower extract was characterized by stronger anti-tumor activity on all three breast cancer cell lines, compared to that of the leaves.

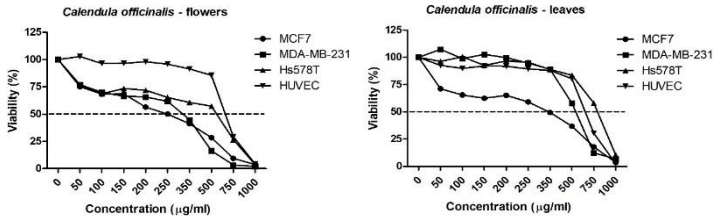

Fig. 3 The anti-tumor activity of the extracts obtained from *C. officinalis* flowers and leaves, at 48h after administration.

Table 6 The IC_{50} concentration ant the selectivity coefficient in the anti-tumor action of the extracts obtained from *C. officinalis* flowers and leaves, at 48h after administration.

Plant extract		Cell line			
		MCF7	**MDA-MB-231**	**HS578T**	**HUVEC**
***C. officinalis* flowers**	**IC_{50} (**µg/mL)	213,4***	386,9***	520,5***	651,4***
	Selectivity coefficient	3,1	1,7	1,3	-
***C. officinalis* leaves**	**IC_{50} (**µg/mL)	252,4***	519,7*	749,4***	631,0***
	Selectivity coefficient	2,4	1,2	0,9	

3.2. The anti-tumor activity of the Solanum spp. extracts

Extracts obtained from *S. bulbocastanum* and *S. chacoense* were characterized by dose-dependent anti-tumor activity (Fig. 4).

The MCF7 luminal breast cancer cell line was found to be most sensitive to the anti-tumor action of these three extracts (Table 7). This cell line was more than twice as sensitive to the extracts obtained from *S. chacoense* and five times more sensitive to the plant preparation from the leaves of *S. bulbocastanum*, as compared to the HUVEC cell line. The lines belonging to the triple negative molecular subtype of breast cancer were more resistant to the action of the extracts obtained from the *Solanum* species.

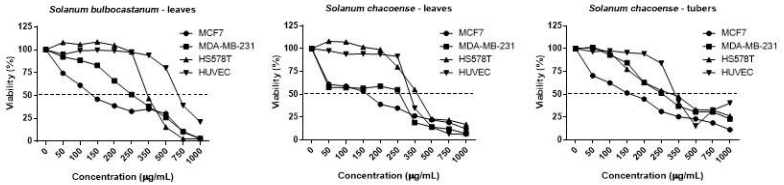

Fig. 4 The anti-tumor activity of the extracts obtained from *S. bulbocastanum* leaves and *S. chacoense* leaves and tubers, at 48h after administration.

Table 7 The IC_{50} concentration ant the selectivity coefficient in the anti-tumor action of the extracts obtained from *S. bulbocastanum* leaves and *S. chacoense* leaves and tubers, at 48h after administration.

Plant extract		Cell line			
		MCF7	**MDA-MB-231**	**HS578T**	**HUVEC**
***S. bulbocastanum* frunze**	**IC_{50} (µg/mL)**	139,1***	273,2***	351,6***	689,9***
	Selectivity coefficient	5,0	2,5	2,0	-
***S. chacoense* frunze**	**IC_{50} (µg/mL)**	132,9***	310,4**	390,7***	328,8***
	Selectivity coefficient	2,5	1,1	0,9	-
***S. chacoense* tuberculi**	**IC_{50} (µg/mL)**	143,2**	203,1***	350,0***	335,9***
	Selectivity coefficient	2,4	1,7	1,0	-

Of the three extracts tested, the one produced from the leaves of *S. bulbocastanum* was the most effective in terms of anti-tumor action. Although empirically, the IC_{50} concentrations of the three extracts for each of the three cancer cell lines are relatively equal, the *S. bulbocastanum* extract differs by much lower toxicity on healthy HUVEC cells (Table 7). Between the two *S. chacoense* extracts, the one obtained from the tubers was superior to the extract obtained from the leaves, with selectivity coefficients over 1.5 in the case of two cell lines (MCF7 and MDA-MB-231).

4. Molecular effects triggered by the extracts in breast cancer cells

4.1. Molecular effects triggered by C. officinalis extracts

The two extracts obtained from the leaves and flowers of *C. officinalis* induced similar molecular effects (Fig. 5). *CCND1, NFkB* and *STAT3* genes, which encode proteins that stimulate cell cycle progression and proliferation, were significantly downregulated in cells treated with both extracts from *C. officinalis*. Also, the gene expression of *BCL2*, one of the most relevant anti-apoptotic genes involved in the intrinsic mechanism of apoptosis induction, was downregulated in the samples treated with both extracts. On the other hand, *BAX* and *BBC3*, pro-apoptotic genes involved in the same intrinsic mechanism of apoptosis induction as *BCL2*, were overexpressed in response to the administration of the two extracts. At the same time, the expression of the *ZMAT3* gene involved in several mechanisms that promote programmed cell death, was increased in MCF7 cells treated with *C. officinalis* extracts.

4.2. Molecular effects triggered by Solanum spp. extracts

The extract obtained from the leaves of *S. bulbocastanum* induced the modulation of six genes among the 14 considered of interest. Thus, both the anti-apoptotic gene *BCL2* and *STAT3* and *CCND1* genes involved in cell proliferation were downregulated in cells treated with this extract. On the other hand, the pro-apoptotic genes *BAX* and *BBC3*, and also *ZMAT3* had increased expression levels in response to the administration of the *S. bulbocastanum* leaves extract (Fig. 6).

In the case of the extracts obtained from the leaves and tubers of *S. chacoense*, the genes *BCL2, BAX, ZMAT3, STAT3* and *CCND1* are modulated in the same sense as in the treatment with the *S. bulbocastanum* leaves extract. In addition to these gene expression changes, both extracts obtained from the

S. chacoense species induce the overexpression of the NFkB transcription factor (Fig. 6).

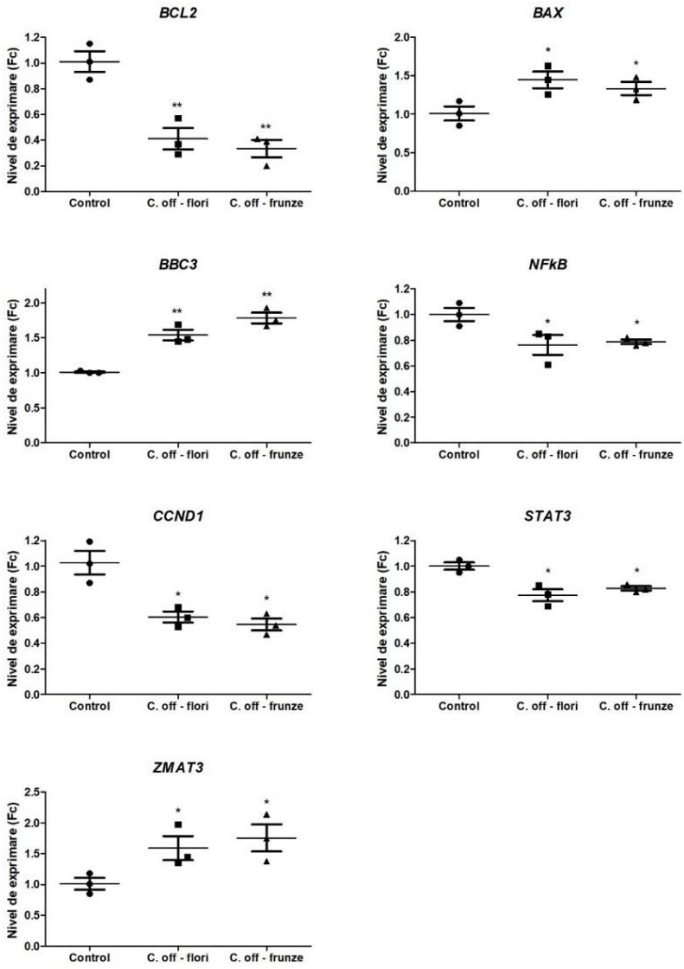

Fig. 5 Gene expression modification (fold change) of the genes involved in proliferation and apoptosis in the MCF7 cell line treated with extracts obtained from *C. officinalis* flowers and leaves, at concentrations equal to IC_{50} values, at 48 hours after administration.

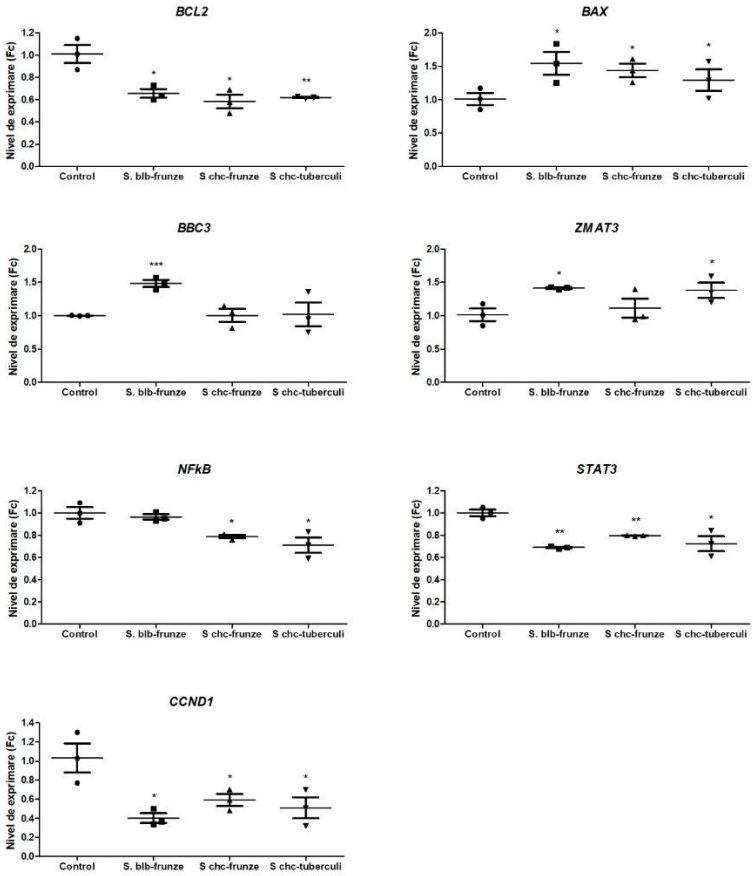

Fig. 6 Gene expression modification (fold change) of the genes involved in proliferation and apoptosis in the MCF7 cell line treated with extracts obtained from *S. bulbocastanum* leaves and *S. chacoense* leaves and tubers, at concentrations equal to IC_{50} values, at 48 hours after administration.

CHAPTER VII – Discussion

1. *C. officinalis*: species with potential in breast cancer management

1.1. The phytochemical profile of the C. officinalis extracts

In the extract obtained from the flowers of *C. officinalis* 14 phenolic compounds were identified, while the diversity of this class of compounds in the preparation from the leaves of the same species was lower, only 12 phenolic compounds being quantified. Of all these biochemical constituents, 10 have been previously reported in different preparations obtained from the organs of this species (Mehta et al., 2012; Miguel et al., 2016; Olennikov et al., 2017; Rigane et al., 2013). However, four of these, namely dihydroxybenzoic acid, quercetin-3-O-glucosyl-rhamnosyl-glucoside, isorhamnetin-3-O-galactoside and isorhamnetin-7-O-rhamnoside, are reported for the first time as part of the biochemical profile of *C. officinalis*.

Phenolic compounds are among the most important classes of secondary metabolites that are characterized by anti-tumor activity, being able to inhibit cell proliferation and induce apoptosis (Fantini et al., 2015; Nichenametla et al., 2006). Numerous phenolic compounds identified in the *C. officinalis* extracts used in this study, such as chlorogenic acid (Yamagata et al., 2018), quercetin derivatives (Kashyap et al., 2016), isorhamnetin derivatives (Wu et al., 2018) and scopoletin derivatives (Li et al., 2015) are recognized for their anti-tumor properties in the literature.

A total of 28 volatile compounds were identified in the *C. officinalis* extracts, 24 of which were present in the flower preparation and only 16 in the leaves extract. The results regarding the volatile biochemical profile of *C. officinalis* are consistent with previous data, different studies reporting the presence of cymene, limonene, cubebene, copaene, muurolene, cadinene and nonanal in various plant preparations obtained from *C. officinalis* (Gazim et al., 2008; Kaškonien, 2008; Kaškonienė; et al., 2011; Okoh et al., 2007; Petrović et al., 2010). However, this study identifies for the first time the compounds octanal, nonanal, cosmene, alloocimene, propiophenone and chloroacetophenone as part of the biochemical profile of this species.

Volatile compounds, such as monoterpenes and sesquiterpenes, are recognized for their anti-tumor activity, both individually and as mixtures of plant constituents (Dhifi et al., 2016; Greay and Hammer, 2015). Anti-tumor activity was previously reported for several individual volatile compounds

that were identified in the *C. officinalis* extracts used in this study. Such compounds are cadinene (Hui et al., 2015), copaene (Turkez et al., 2014), methyl benzoate and multiple acetophenones (Nakamura et al., 2002).

1.2. The selective anti-tumor activity of the C. officinalis extracts

The methanolic extracts from *C. officinalis* flowers and leaves obtained by UAE yielded better results in terms of anti-tumor activity compared to extracts of the same species prepared by classical methods (Wegiera et al., 2013; Matic et al., 2013; Miguel et al., 2016).

The highest cytotoxicity for both extracts was observed against the MCF7 cell line. MCF7 is a luminal A breast cancer cell line, a less aggressive molecular subtype, which is also often responsive to chemotherapy. On the other hand, MDA-MB-231 and Hs578T are triple-negative, claudin-low breast cancer cell lines, with a phenotype characterized by intermediate to low chemotherapy responsiveness (Holliday and Speirs, 2011). In this context, the differences regarding the specific IC50 values between the MCF7 cell line and MDA-MB-231/ Hs578T were expected.

The selectivity in the anti-tumor action of *C. officinalis* flower extracts has been reported previously, by comparing their cytotoxicity on cancer cell lines with that on immunocompetent mononuclear cells (PBMC - Matic et al., 2013), on swine liver cells (PLP2 - Miguel et al., 2016) and on healthy colon cells (CCD18 - Mouhid et al., 2018). However, no selectivity was found for a methanol extract obtained from *C. officinalis* flowers, by comparing its action on breast cancer cells (T47D) and normal human skin fibroblasts (Matysik et al., 2005). The toxicity of *C. officinalis* extracts on a healthy endothelial cell line (HUVEC) was first evaluated in this study, demonstrating, once again, the selective cytotoxicity of *C. officinalis* flower extract. Moreover, the selectivity of the extract from the leaves of *C. officinalis* in terms of its anti-tumor activity is proven for the first time in the present work.

Comparing the two plant preparations obtained from *C. officinalis*, the flower extract yielded superior results in terms of anti-tumor activity on all three breast cancer cell lines. These results are in agreement with the only other study that compared the cytotoxicity of extracts from *C. officinalis* flowers and leaves on tumor cell lines (Wegiera et al., 2012), which demonstrated lower anti-tumor activity of the leaf extract, with IC_{50} values

within the same range as in the present study. These differences in the anti-tumor activity exerted on the breast cancer cell lines can be explained, at least in part, by the distinct biochemical profile of the two extracts of interest. The flower extract was superior in terms of the total amount of polyphenols, these compounds being known for their anti-tumor activity (Fantini et al., 2015). Furthermore, the diversity of the phenolic compounds from the extract obtained from *C. officinalis* flowers was also richer. Moreover, several volatile compounds recognized for their anti-tumor activity, such as delta-cadinene (Hui et al., 2015) and copaene (Turkez et al., 2014), were identified only in the flower preparation.

1.3. The mechanisms of action of the C. officinalis extracts

C. officinalis extracts exert their cytotoxic activity on cancer cells by induction of apoptosis (Jimenez-Medina et al., 2006; Mouhid et al., 2018; Wegiera et al., 2012) and cell cycle arrest in the G0/G1 phase (Jimenez-Medina et al., 2006). It has been demonstrated that both caspase 3 and caspase 7 are activated (cleaved) at the protein level in response to *C. officinalis* extracts administration, and thus apoptosis is induced in a caspase3/7-dependent manner (Jimenez-Medina et al., 2006; Mouhid et al., 2018). On the other hand, the cell cycle arrest effect of *C. officinalis* extracts on cancer cells is induced by the down-regulation of cyclin D1, D3, A, E and several cyclin-dependent kinases (CDKs) (Jimenez-Medina et al., 2006). In this context, in this study, seven genes involved in the modulation of cell cycle progression and apoptosis were found deregulated in MCF7 cells treated with *C. officinalis* extracts. underlining new molecular effects triggered by these herbal preparations in breast cancer.

BCL2, BAX and *BBC3* genes encode proteins in the BCL2 family capable of homo- and heterodimerization, which function as regulators of apoptosis (Cory and Adams, 2002). According to the data obtained, *BCL2*, one of the most important anti-apoptotic genes in this family, was overexpressed in breast cancer cells treated with *C. officinalis* extracts. On the other hand, pro-apoptotic genes *BAX* and *BBC3* were overexpressed in MCF7 cells in response to the administration of the extracts at concentrations equal to IC_{50} values. Therefore, cytochrome c release from the mitochondria could be stimulated by both tested extracts, and thus apoptosis is most probably implemented through caspase activation, as was shown by previous

reports (Jimenez-Medina et al., 2006; Mouhid et al., 2018). Expression levels of *ZMAT3*, a gene involved in both cell growth and apoptosis signaling (Bersani et al., 2014) were also increased in cells treated with both plant extracts. All of these data suggest the induction of apoptosis by extracts from *C. officinalis*.

Cell proliferation is controlled by multiple signaling networks, several regulatory proteins as cyclins and transcription factors like NFkB or STAT3 being crucial for cell cycle progression. The *CCND1* gene encodes cyclin D1, the regulatory component of cyclin D-CDK4/6 complexes that are responsible for inducing the G1/S transition during the cell cycle, by passing through the R point (Musgrove, 2006). In this study, cyclin D1 was downregulated in MCF7 cells following administration of both extracts obtained from *C. officinalis*. These data are consistent with the results obtained by Jimenez-Medina et al. (2006), who demonstrated the downregulation of cyclin D1 at the protein level after treatment with a laser-activated aqueous extract obtained from the flowers of this species. NFkB is a transcription factor that is activated by a large panel of extra- and intracellular stimuli, whose activation is associated with cell proliferation (Serasanambati and Chilakapati, 2016). STAT3 is a transcription factor that mediates cellular responses to cytokines and growth factors, being involved in the G1-S transition (Levy and Lee, 2002). Its activation induces the expression of key genes in proliferation, such as *CCND1*. The results obtained here demonstrate the downregulation of the transcription factors NFkB and STAT3. All these data highlight important molecular changes in mammary tumor cells induced by *C. officinalis* extracts, which could underlie the cytostatic effects of these plant preparations.

2. *S. chacoense* and *S. bulbocastanum*: species with potential in breast cancer prevention

2.1. The phytochemical profile of S. chacoense extracts

Hydroxycinnamic acids, including derivatives of caffeic and chlorogenic acids, are known to be the main phenolic compounds present in the organs of *S. chacoense* (Hale et al., 2008; Navarre et al., 2011). On the other hand, ferulic acid and hydroxybenzoic acid were first identified in the biochemical composition of this species in this study. Phenolic acids are characterized by substantial anti-tumor activity, with potential in both cancer prevention and

treatment (Saibabu et al., 2015). Individual phenolic acids identified in *S. chacoense* methanolic preparations, such as chlorogenic acid (Yamagata et al., 2018), caffeic acid (Rosendahl et al., 2015) or ferulic acid (Gao et al., 2018) have been identified in previous studies as anti-cancer agents. In this context, the phenolic profile of the extracts from *S. chacoense* contributed substantially to the anti-tumor activity observed on breast cancer cell lines.

Previous studies have quantified high amounts of α-solanine and α-chaconine in tubers and leaves of different wild potato species (Distl and Wink, 2009), including *S. chacoense* (Mweetwa et al., 2012). However, in the tuber extract obtained from 12-weeks old plants of *S. chacoense* accession PI 458310 used in this study, α-solanine and α-chaconine were not present. Besides these two solanidanes, solasodine and its derivate, solamargine were identified in the leaf preparation. These compounds have been previously identified in the organs of some wild *Solanum* species (Distl and Wink, 2009), but never in the leaves of *S. chacoense*. All these alkaloids are recognized in the literature for their anti-tumor activity, being able to induce both cell death and cell cycle arrest (Milner, et al., 2011; Friedman, 2015), thus contributing to the anti-tumor activity of leaf extract.

This study presents for the first time the characterization of the volatile biochemical profile of the extracts obtained from the tubers and leaves of *S. chacoense*. The volatile biochemical profile of the *S. chacoense* species overlaps, as expected, with the volatile composition of the cultivated species *S. tuberosum* (Morris et al., 2010; Mosneaguta et al., 2012), with compounds belonging to the classes of aldehydes, esters, fatty alcohols and ketones being present in both species. However, constituents such as cadinene, 2-chloroacetophenone, isobutylaldehyde dimethyl acetal or phenylmaleic anhydride appear to be specific to this wild species. The individual volatile compounds identified in *S. chacoense* extracts, such as cadinene (Hui et al., 2015), methyl benzoate or acetophenone (Nakamura et al., 2002), are known for their anti-tumor activity.

2.2. The selective anti-tumor activity of the Solanum spp. extracts

The lowest IC_{50} values corresponding to the three extracts from *Solanum* species were obtained on the MCF7 line. Similar to the data obtained for extracts from *C. officinalis*, the best results obtained on this luminal breast

cancer cell line were expected, given that it is less aggressive (Holliday and Speirs, 2011).

Both extracts obtained from *S. chacoense* were selective in the anti-tumor action against the MCF7 cell line with high selectivity coefficients, but only the tuber preparation maintained the selectivity against the MDA-MB-231 line. This study is the first to evaluate the selective anti-tumor action of the extracts obtained from this species. The extract obtained from the leaves of *S. bulbocastanum* yielded the best results in terms of selectivity in anti-tumor activity, with selectivity coefficients up to 5.0 (MCF7 cell line). This study is the first to evaluate the anti-tumor activity in general and selectivity in the anti-cancer action in particular of phytoconstituents of *S. bulbocastanum.*

Comparing the two herbal preparations obtained from the *S. chacoense* species, the extract from the tubers was superior regarding the cytotoxic/ cytostatic effects observed in all three breast cancer cell lines. This was observed although it did not contain glycoalkaloids, compounds thought to be responsible for the anti-tumor activity of extracts from different *Solanum* species (Freidman, 2015). However, the tuber extract was richer in terms of the phenolic compounds content. At the same time, caffeic and ferulic acids, known for their anti-tumor activity (Rosendhl et al., 2015, Gao et al., 2018), were identified only in this preparation. Moreover, the increased diversity of volatile compounds known for their anti-tumor activity (ketones and aldehydes), doubled by their higher relative abundances, were also noted in the biochemical profile of the extract from the tubers, compared to that of the leaves of *S. chacoense*.

2.3. The mechanisms of action of Solanum spp. extracts

Out of the BCL2 family of apoptosis regulatory proteins, three genes were modulated by the extracts obtained from the *Solanum* species included in the study. The *BCL2* gene, one of the most important anti-apoptotic proteins in this family, was downregulated, and *BAX*, a pro-apoptotic gene with multiple BH domains, was overexpressed following administration of all three extracts. The pro-apoptotic gene *BBC3* was also overexpressed in MCF7 cells treated with the extract obtained from *S. bulbocastanum* leaves. These data suggest that apoptosis induction might be a relevant mechanism by which *S. chacoense* and *S. bulbocastanum* extracts affect breast cancer

tumor cells. The statistically significant overexpression of *ZMAT3* also supports the hypothesis of apoptosis induction by these extracts.

Cyclin D1, a protein involved in cell cycle progression through the R point from phase G1 to phase S, was substantially downregulated following treatment with all three extracts obtained from *Solanum* species. Similarly, *STAT3*, a transcription factor relevant in the transcription of multiple genes involved in proliferation, had low levels of expression following the administration of the three extracts of interest. *NFkB* expression was significantly modulated in the sense of inhibiting cell proliferation only by the extracts from the *S. chacoense* species. Thus, the premise that the extracts obtained from *Solanum* species could hamper the cell cycle progress and cell proliferation is emphasized, underlining the extract's cytostatic potential.

Considering all these data, the molecular basis of the cytotoxic and cytostatic effects of the bioactive compounds from *S. chacoense* and *S. bulbocastanum* is highlighted for the first time.

Conclusions

The methanolic extracts obtained from the flowers and leaves of *C. officinalis* by the UAE technique were rich in polyphenols and volatile compounds, constituents known for their anti-tumor activity. Both leaf and flower extracts from *C. officinalis* were characterized by *in vitro* anti-tumor activity against all three breast cancer lines. Moreover, they were also selective in terms of cytotoxicity against tumor cell lines compared to healthy endothelial cells. Both extracts modulated the expression of several relevant genes involved in cell proliferation and apoptosis, suggesting the extracts' ability to induce cell death and to hamper the cell cycle in tumor cells. These data demonstrate, at least in part, the molecular basis of the previously observed cellular effects, according to which extracts from *C. officinalis* would have both cytotoxic and cytostatic effects. Thus, *C. officinalis* can be considered a relevant source of bioactive compounds with anti-cancer properties, and herbal preparations of this species could be included as products associated with complementary medicine in the treatment and the palliative care of breast cancer patients.

The extracts obtained from the leaves and tubers of *S. chacoense* were rich in phenolic acids and volatile compounds. Additionally, the leaf extract contained four alkaloids specific to the *Solanaceae* family. The two extracts obtained from *S. chacoense* were characterized by anti-tumor activity on all three breast cancer cell lines and were selective against the MCF7 breast cancer cell line. In contrast, the extract obtained from the leaves harvested from *S. bulbocastanum* had significantly lower toxicity on HUVEC healthy cell line and thus much increased selectivity in the anti-tumor action. All three extracts obtained from wild *Solanum* species modulated the expression of genes involved in apoptosis and cell proliferation, suggesting the cytotoxic and also cytostatic activity that would characterize these plant preparations. Therefore, wild *Solanum* species, being intensively used in potato breeding programs, could significantly improve the biochemical profile responsible for the anti-tumor activity of the new potato cultivated varieties, thus participating in the prevention of breast cancer through nutrition, worldwide.

References

1. Ali F, Khan R, Khan AQ, Lateef MA, Maqbool T, Sultana S, 2014. Assessment of Augmented Immune Surveillance and Tumor Cell Death by Cytoplasmic Stabilization of p53 as a Chemopreventive Strategy of 3 Promising Medicinal Herbs in Murine 2-Stage Skin Carcinogenesis. *Integrative cancer therapies* **13(**4):351-367.
2. Ardekani AM, Jabbari S, 2009. Nutrigenomics and cancer. *Avicenna journal of medical biotechnology* **1**(1):9-17.
3. Ashwlayan V, Kumar A, Verma M, Garg V, Gupta S, 2018. Therapeutic Potential of *Calendula officinalis*. *Pharmacy & Pharmacology International Journal* **6**(2):149-155.
4. Aung TN, Qu Z, Kortschak RD, Adelson DL, 2017. Understanding the Effectiveness of Natural Compound Mixtures in Cancer through Their Molecular Mode of Action. *International journal of molecular sciences* **18**(3):45-53.
5. Barajas-Farias LM, Perez-Carreon JI, Arce-Popoca E, Fattel-Fazenda S, Aleman-Lazarini L, Hernandez-Garcia S, Salcido-Neyoy M, Cruz-Jimenez FG, Camacho J, Villa-Trevino S, 2006. A dual and opposite effect of Calendula officinalis flower extract: chemoprotector and promoter in a rat hepatocarcinogenesis model. *Planta medica* **72**(3):217-221.
6. Bersani C, Xu LD, Vilborg A, Lui WO, Wiman KG, 2014. Wig-1 regulates cell cycle arrest and cell death through the p53 targets FAS and 14-3-3sigma. *Oncogene* **33**(35):4407-4417.
7. Boucaud-Maitre Y, Algernon O, Raynaud J, 1988. Cytotoxic and antitumoral activity of Calendula officinalis extracts. *Die Pharmazie* **43**(3):220-221.
8. Braicu C, Mehterov N, Vladimirov B, Sarafian V, Nabavi SM, Atanasov AG, Berindan-Neagoe I, 2017. Nutrigenomics in cancer: Revisiting the effects of natural compounds. *Seminars in cancer biology* **46**:84-106.
9. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A, 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians* **68**(6):394-424.
10. Caruso I, Dal Piaz F, Malafronte N, De Tommasi N, Aversano R, Zottele CW, Scarano MT, Carputo D, 2013. Impact of ploidy change on secondary metabolites and photochemical efficiency in Solanum bulbocastanum. *Natural product communications* **8**(10):1387-1392.
11. Cheng CW, Fan W, Ko SG, Song L, Bian ZX, 2010. Evidence-based management of herb-drug interaction in cancer chemotherapy. *Explore* **6**(5):324-329.
12. Chun KH, Park JH, Fan S, 2017. Predicting and Overcoming Chemotherapeutic Resistance in Breast Cancer. *Advances in experimental medicine and biology* **1026**:59-104.
13. Cory S, Adams JM, 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nature reviews. Cancer* **2**(9):647-656.
14. Cragg GM, Newman DJ, 2005. Plants as a source of anti-cancer agents. *Journal of ethnopharmacology* **100**(1-2):72-79.
15. Dalle JR, Leow WK, Racoceanu D, Tutac AE, Putti TC, 2008. Automatic breast cancer grading of histopathological images. *Conference proceedings : ... Annual International*

Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Annual Conference **2008**:3052-3055.
16. Davis CD, 2007. Nutritional interactions: credentialing of molecular targets for cancer prevention. *Experimental biology and medicine* **232**(2):176-183.
17. De Haan S, Rodriguez F, 2016. Potato origin and production, în: Singh, J, Kaur, L (Eds.), Advances in potato chemistry and technology. Elsevier Inc, London, GB, pp. 1-32.
18. Dhankhar R, Vyas SP, Jain AK, Arora S, Rath G, Goyal AK, 2010. Advances in novel drug delivery strategies for breast cancer therapy. *Artificial cells, blood substitutes, and immobilization biotechnology* **38**(5):230-249.
19. Dhifi W, Bellili S, Jazi S, Bahloul N, Mnif W, 2016. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines* **3**(4):123-141.
20. Distl M, Wink M, 2009. Identification and Quantification of Steroidal Alkaloids from Wild Tuber-Bearing Solanum Species by HPLC and LC-ESI-MS. *Potato Research* **52**(1):79-104.
21. Efferth T, Li PC, Konkimalla VS, Kaina B, 2007. From traditional Chinese medicine to rational cancer therapy. *Trends in molecular medicine* **13**(8):353-361.
22. Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, Kessler RC, 1998. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *Jama* **280**(18):1569-1575.
23. Enioutina EY, Salis ER, Job KM, Gubarev MI, Krepkova LV, Sherwin CM, 2017. Herbal Medicines: challenges in the modern world. Part 5. status and current directions of complementary and alternative herbal medicine worldwide. *Expert review of clinical pharmacology* **10**(3):327-338.
24. Fantini M, Benvenuto M, Masuelli L, Frajese GV, Tresoldi I, Modesti A, Bei R, 2015. In vitro and in vivo antitumoral effects of combinations of polyphenols, or polyphenols and anticancer drugs: perspectives on cancer treatment. *International journal of molecular sciences* **16**(5):9236-9282.
25. Ferguson LR, Philpott M, Karunasinghe N, 2004. Dietary cancer and prevention using antimutagens. *Toxicology* **198**(1-3):147-159.
26. Frankic T, Salobir K, Salobir J, 2009. The comparison of in vivo antigenotoxic and antioxidative capacity of two propylene glycol extracts of Calendula officinalis (marigold) and vitamin E in young growing pigs. *Journal of animal physiology and animal nutrition* **93**(6):688-694.
27. Friedman M, 2015. Chemistry and anticarcinogenic mechanisms of glycoalkaloids produced by eggplants, potatoes, and tomatoes. *Journal of agricultural and food chemistry* **63**(13):3323-3337.
28. Gali-Muhtasib H, Hmadi R, Kareh M, Tohme R, Darwiche N, 2015. Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. *Apoptosis : an international journal on programmed cell death* **20**(12):1531-1562.
29. Gao J, Yu H, Guo W, Kong Y, Gu L, Li Q, Yang S, Zhang Y, Wang Y, 2018. The anticancer effects of ferulic acid is associated with induction of cell cycle arrest and autophagy in cervical cancer cells. *Cancer cell international* **18**:102-109.
30. Gazim ZC, Rezende CM, Fraga SR, Svidzinski TI, Cortez DA, 2008. Antifungal activity of the essential oil from Calendula officinalis L. (asteraceae) growing in Brazil.

Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology] **39**(1):61-63.
31. Goyal M, Mathur R, 2011. Antimicrobial effects of Calendula officinalis against human pathogenic microorganisms. *J Herbal Med Tox,* **5**(1):97-101.
32. Greay SJ, Hammer KA, 2015. Recent developments in the bioactivity of mono- and diterpenes: anticancer and antimicrobial activity. *Phytochemistry Reviews* **14**(1):6.
33. Haldar K, Kamoun S, Hiller NL, Bhattacharje S, van Ooij C, 2006. Common infection strategies of pathogenic eukaryotes. *Nature reviews. Microbiology* **4**(12):922-931.
34. Hale AL, Reddivari L, Nzaramba MN, J. B, J.C. MJ, 2008. Interspecific variability for antioxidant activity and phenolic content among Solanum species. *American Journal of Potato Research* **85**:332-341.
35. Helyer LK, Chin S, Chui BK, Fitzgerald B, Verma S, Rakovitch E, Dranitsaris G, Clemons M, 2006. The use of complementary and alternative medicines among patients with locally advanced breast cancer--a descriptive study. *BMC cancer* **6**:39-48.
36. Horneber M, Bueschel G, Dennert G, Less D, Ritter E, Zwahlen M, 2012. How many cancer patients use complementary and alternative medicine: a systematic review and metaanalysis. *Integrative cancer therapies* **11**(3):187-203.
37. Hu Z, Yang X, Ho PC, Chan SY, Heng PW, Chan E, Duan W, Koh HL, Zhou S, 2005. Herb-drug interactions: a literature review. *Drugs* **65**(9):1239-1282.
38. Hui LM, Zhao GD, Zhao JJ, 2015. delta-Cadinene inhibits the growth of ovarian cancer cells via caspase-dependent apoptosis and cell cycle arrest. *International journal of clinical and experimental pathology* **8**(6):6046-6056.
39. Iqbal J, Abbasi B, Mahmood T, Kanwal S, Ali B, Shah SA, Khalil AT, 2017. Plant-derived anticancer agents: A green anticancer approach. *Asian Pacific Journal of Tropical Biomedicine* **7**(12):21.
40. Ismail-Khan R, Bui MM, 2010. A review of triple-negative breast cancer. *Cancer control : journal of the Moffitt Cancer Center* **17**(3):173-176.
41. Jimenez-Medina E, Garcia-Lora A, Paco L, Algarra I, Collado A, Garrido F, 2006. A new extract of the plant Calendula officinalis produces a dual in vitro effect: cytotoxic anti-tumor activity and lymphocyte activation. *BMC cancer* **6**:119-132.
42. Kashyap D, Mittal S, Sak K, Singhal P, Tuli HS, 2016. Molecular mechanisms of action of quercetin in cancer: recent advances. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **37**(10):12927-12939.
43. Kaškonienė V, Kaškonas P, Jalinskaitė M, Maruška A, 2011. Chemical Composition and Chemometric Analysis of Variation in Essential Oils of Calendula officinalis L. during Vegetation Stages. *Chromatographia* **73**:9.
44. Khalid K, Da Silva J, 2012. Biology of *Calendula officinalis* Linn: focus on pharmacology, biological activities and agronomic practices *Medicinal alnd Aromatic Plant Science and Biotechnology* **6**(1):12-27.
45. Koh J, Kim MJ, 2019. Introduction of a New Staging System of Breast Cancer for Radiologists: An Emphasis on the Prognostic Stage. *Korean journal of radiology* **20**(1):69-82.
46. Kotecha R, Takami A, Espinoza JL, 2016. Dietary phytochemicals and cancer chemoprevention: a review of the clinical evidence. *Oncotarget* **7**(32):52517-52529.

47. Levy DE, Lee CK, 2002. What does Stat3 do? *The Journal of clinical investigation* **109**(9):1143-1148.
48. Li L, Zhao P, Hu J, Liu J, Liu Y, Wang Z, Xia Y, Dai Y, Chen L, 2015. Synthesis, in vitro and in vivo antitumor activity of scopoletin-cinnamic acid hybrids. *European journal of medicinal chemistry* **93**:300-307.
49. Liao GS, Apaya MK, Shyur LF, 2013. Herbal medicine and acupuncture for breast cancer palliative care and adjuvant therapy. *Evidence-based complementary and alternative medicine : eCAM* **2013**:437948.
50. Liu LF, Liang CH, Shiu LY, Lin WL, Lin CC, Kuo KW, 2004. Action of solamargine on human lung cancer cells--enhancement of the susceptibility of cancer cells to TNFs. *FEBS letters* **577**(1-2):67-74.
51. Livak KJ, Schmittgen TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**(4):402-408.
52. Luis Espinoza J, Takami A, Trung LQ, Nakao S, 2013. Ataxia-telangiectasia mutated kinase-mediated upregulation of NKG2D ligands on leukemia cells by resveratrol results in enhanced natural killer cell susceptibility. *Cancer science* **104**(6):657-662.
53. Mamone L, Di Venosa G, Valla JJ, Rodriguez L, Gandara L, Batlle A, Heinrich M, Juarranz A, Sanz-Rodriguez F, Casas A, 2011. Cytotoxic effects of Argentinean plant extracts on tumour and normal cell lines. *Cellular and molecular biology* **57**:1487-1499.
54. Mastropasqua MG, Viale G, 2017. Clinical and pathological assessment of high-risk ductal and lobular breast lesions: What surgeons must know. *European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology* **43**(2):278-284.
55. Matsen CB, Neumayer LA, 2013. Breast cancer: a review for the general surgeon. *JAMA surgery* **148**(10):971-979.
56. Mehta D, Rastogi P, Kumar A, K. CA, 2012. Review on Pharmacological Update: Calendula officinalis Linn. *Inventi Impact - Planta Activa* **2012**(4):195-204.
57. Miguel M, Barros L, Pereira C, Calhelha RC, Garcia PA, Castro M, Santos-Buelga C, Ferreira IC, 2016. Chemical characterization and bioactive properties of two aromatic plants: Calendula officinalis L. (flowers) and Mentha cervina L. (leaves). *Food & function* **7**(5):2223-2232.
58. Molassiotis A, Scott JA, Kearney N, Pud D, Magri M, Selvekerova S, Bruyns I, Fernadez-Ortega P, Panteli V, Margulies A, Gudmundsdottir G, Milovics L, Ozden G, Platin N, Patiraki E, 2006. Complementary and alternative medicine use in breast cancer patients in Europe. *Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer* **14**(3):260-267.
59. Mongelli E, Coussio J, Ciccia G, Maestri D, Zygadlo J, 1999. Medicinal species of the *Solanaceae* family: primary screening of cytotoxicity. *Acta Horticulturae* **501**:177-180.
60. Morris WL, Shepherd T, Verrall SR, McNicol JW, Taylor MA, 2010. Relationships between volatile and non-volatile metabolites and attributes of processed potato flavour. *Phytochemistry* **71**(14-15):1765-1773.
61. Mosneaguta R, Alvarez V, Barringer SA, 2012. The effect of antibrowning agents on inhibition of potato browning, volatile organic compound profile, and microbial inhibition. *Journal of food science* **77**(11):1234-1240.

62. Musgrove EA, 2006. Cyclins: roles in mitogenic signaling and oncogenic transformation. *Growth factors* **24**(1):13-19.
63. Mweetwa AM, Hunter D, Poe R, Harich KC, Ginzberg I, Veilleux RE, Tokuhisa JG, 2012. Steroidal glycoalkaloids in Solanum chacoense. *Phytochemistry* **75**:32-40.
64. Nakamura ES, Kurosaki F, Arisawa M, Mukainaka T, Okuda M, Tokuda H, Nishino H, Pastore F, 2002. Cancer chemopreventive effects of constituents of Caesalpinia ferrea and related compounds. *Cancer letters* **177**(2):119-124.
65. Navarre D, Pillai S, Shakya R, Holden M, 2011. HPLC Profiling of phenolics in diverse potato genotypes. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **127**:34-41.
66. Navo MA, Phan J, Vaughan C, Palmer JL, Michaud L, Jones KL, Bodurka DC, Basen-Engquist K, Hortobagyi GN, Kavanagh JJ, Smith JA, 2004. An assessment of the utilization of complementary and alternative medication in women with gynecologic or breast malignancies. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **22**(4):671-677.
67. Newman DJ, Cragg GM, 2016. Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of natural products* **79**(3):629-661.
68. Nicastro HL, Trujillo EB, Milner JA, 2012. Nutrigenomics and Cancer Prevention. *Current nutrition reports* **1**(1):37-43.
69. Nichenametla SN, Taruscio TG, Barney DL, Exon JH, 2006. A review of the effects and mechanisms of polyphenolics in cancer. *Critical reviews in food science and nutrition* **46**(2):161-183.
70. Nounou MI, ElAmrawy F, Ahmed N, Abdelraouf K, Goda S, Syed-Sha-Qhattal H, 2015. Breast Cancer: Conventional Diagnosis and Treatment Modalities and Recent Patents and Technologies. *Breast cancer : basic and clinical research* **9**(Suppl 2):17-34.
71. Okoh OO, Sadimenko AA, Afolayan AJ, 2007. The effects of age on the yield and composition of the essential oils of Calendula officinalis. *Journal of Applied Sciences* **7**(23):3806-3811.
72. Olennikov DN, Kashchenko NI, Chirikova NK, Akobirshoeva A, Zilfikarov IN, Vennos C, 2017. Isorhamnetin and Quercetin Derivatives as Anti-Acetylcholinesterase Principles of Marigold (Calendula officinalis) Flowers and Preparations. *International journal of molecular sciences* **18**(8):225-264.
73. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN, 2009. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clinical medicine & research* **7**(1-2):4-13.
74. Petrović L, Lepojević Z, Sovilj V, Adamović D, V. T, 2010. Composition of Essential Oil Obtained From Tubular, Head and Ligulate Flowers of Calendula officinalis L. by Steam Distillation of Plant Material and CO2 Extracts. *Journal of Essential Oil Research* **22**(2):4.
75. Pommier P, Gomez F, Sunyach MP, D'Hombres A, Carrie C, Montbarbon X, 2004. Phase III randomized trial of Calendula officinalis compared with trolamine for the prevention of acute dermatitis during irradiation for breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **22**(8):1447-1453.

76. Rakosy-Tican E, Lorincz-Besenyei E, Molnar I, Thieme R, Hartung F, Sprink T, Antonova O, Famelaer I, Angenon G, Aurori A, 2019. New Phenotypes of Potato Co-induced by Mismatch Repair Deficiency and Somatic Hybridization. *Frontiers in plant science* **10**:3.
77. Rigane G, Younes SB, Ghazghazi H, Salem R, 2013. Investigation into the biological activities and chemical composition of Calendula officinalis L. growing in Tunisia. *International Food Research Journal* **20**(6):3001-3008.
78. Rosendahl AH, Perks CM, Zeng L, Markkula A, Simonsson M, Rose C, Ingvar C, Holly JM, Jernstrom H, 2015. Caffeine and Caffeic Acid Inhibit Growth and Modify Estrogen Receptor and Insulin-like Growth Factor I Receptor Levels in Human Breast Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **21**(8):1877-1887.
79. Royston KJ, Tollefsbol TO, 2015. The Epigenetic Impact of Cruciferous Vegetables on Cancer Prevention. *Current pharmacology reports* **1**(1):46-51.
80. Safarzadeh E, Sandoghchian Shotorbani S, Baradaran B, 2014. Herbal medicine as inducers of apoptosis in cancer treatment. *Advanced pharmaceutical bulletin* **4**(Suppl 1):421-427.
81. Saibabu V, Fatima Z, Khan LA, Hameed S, 2015. Therapeutic Potential of Dietary Phenolic Acids. *Advances in pharmacological sciences* **2015**:823539.
82. Sapienza C, Issa JP, 2016. Diet, Nutrition, and Cancer Epigenetics. *Annual review of nutrition* **36**:665-681.
83. Saxe GA, Madlensky L, Kealey S, Wu DP, Freeman KL, Pierce JP, 2008. Disclosure to physicians of CAM use by breast cancer patients: findings from the Women's Healthy Eating and Living Study. *Integrative cancer therapies* **7**(3):122-129.
84. Seely D, Oneschuk D, 2008. Interactions of natural health products with biomedical cancer treatments. *Current oncology* **15**(2):109-117.
85. Serasanambati M, Chilakapati SR, 2016. Function of nuclear factor kappa B (NF-kB) in human diseases-a review. *South Ind. J. Biol. Sci.* **2**:368-387.
86. Sharp L, Finnila K, Johansson H, Abrahamsson M, Hatschek T, Bergenmar M, 2013. No differences between Calendula cream and aqueous cream in the prevention of acute radiation skin reactions--results from a randomised blinded trial. *European journal of oncology nursing : the official journal of European Oncology Nursing Society* **17**(4):429-435.
87. Shiu LY, Chang LC, Liang CH, Huang YS, Sheu HM, Kuo KW, 2007. Solamargine induces apoptosis and sensitizes breast cancer cells to cisplatin. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* **45**(11):2155-2164.
88. Silva EJ, Goncalves ES, Aguiar F, Evencio LB, Lyra MM, Coelho MC, Fraga Mdo C, Wanderley AG, 2007. Toxicological studies on hydroalcohol extract of Calendula officinalis L. *Phytotherapy research : PTR* **21**(4):332-336.
89. Tariq A, Sadia S, Pan K, Ullah I, Mussarat S, Sun F, Abiodun OO, Batbaatar A, Li Z, Song D, Xiong Q, Ullah R, Khan S, Basnet BB, Kumar B, Islam R, Adnan M, 2017. A systematic review on ethnomedicines of anti-cancer plants. *Phytotherapy research : PTR* **31**(2):202-264.
90. Tevaarwerk AJ, Gray RJ, Schneider BP, Smith ML, Wagner LI, Fetting JH, Davidson N, Goldstein LJ, Miller KD, Sparano JA, 2013. Survival in patients with metastatic

recurrent breast cancer after adjuvant chemotherapy: little evidence of improvement over the past 30 years. *Cancer* **119**(6):1140-1148.
91. Turati F, Rossi M, Pelucchi C, Levi F, La Vecchia C, 2015. Fruit and vegetables and cancer risk: a review of southern European studies. *The British journal of nutrition* **113**(2):102-110.
92. Turkez H, Togar B, Tatar A, Geyıkoglu F, Hacımuftuoglu A, 2014. Cytotoxic and cytogenetic effects of α-copaene on rat neuron and N2a neuroblastoma cell lines. *Biologia* **69**(7):936-943.
93. Varlijen J, 1989. Structural analysis of rhamnoarabinogalactans and arabinogalactans with immunostimulating activity from Calendula officinalis. *Phytochemistry* **28**:2379-2384.
94. Wang H, Khor TO, Shu L, Su ZY, Fuentes F, Lee JH, Kong AN, 2012. Plants vs. cancer: a review on natural phytochemicals in preventing and treating cancers and their druggability. *Anti-cancer agents in medicinal chemistry* **12**(10):1281-1305.
95. Wegiera M, Smolarz HD, Jedruch M, Korczak M, Kopron K, 2012. Cytotoxic effect of some medicinal plants from Asteraceae family on J-45.01 leukemic cell line--pilot study. *Acta poloniae pharmaceutica* **69**(2):263-268.
96. Wu Q, Kroon PA, Shao H, Needs PW, Yang X, 2018. Differential Effects of Quercetin and Two of Its Derivatives, Isorhamnetin and Isorhamnetin-3-glucuronide, in Inhibiting the Proliferation of Human Breast-Cancer MCF-7 Cells. *Journal of agricultural and food chemistry* **66**(27):7181-7189.
97. Yamagata K, Izawa Y, Onodera D, Tagami M, 2018. Chlorogenic acid regulates apoptosis and stem cell marker-related gene expression in A549 human lung cancer cells. *Molecular and cellular biochemistry* **441**(1-2):9-19.