**SCUOLA DI DOTTORATO**
**UNIVERSITÀ DEGLI STUDI DI MILANO BICOCCA**

**SCHOOL OF MEDICINE AND SURGERY**
**PHD PROGRAM IN NEUROSCIENCE CYCLE XXIX**

# EVALUATION OF ANTITUMORAL EFFECTS OF HIBISCUS SABDARIFFA ON MULTIPLE MYELOMA CELLS

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**ACADEMIC YEAR 2015-2016**

# Abstract

*Hibiscus sabdariffa* (HS) is a plant of the Malvacee family commonly cultured in tropical and sub-tropical countries. It is mainly known as ingredient for the preparation of cold drink called Karkadè. Calices and leaves of HS plant are also used in folk medicine thanks to their antioxidant and anti-inflammatory properties. In recent years, HS has also gained great interest as a possible antitumoral agent.

In the present PhD project, we evaluated the antitumoral effects of HS against multiple myeloma cells *in vitro*. Multiple myeloma is the most frequent hematological malignancy worldwide. In recent years, new drugs have increased the survival expectancy of patients. Despite this, new therapeutic approaches are necessary, especially for high multiple myeloma heterogeneity and for relapsed or refractory multiple myeloma.

The project was organized in three distinct phases:

1- **Evaluation of antitumoral effects of HS against RPMI 8226 human multiple myeloma cells.** We demonstrated by MTT and Trypan blue assays that a total HS extract (HSE) and one of its fraction obtained by liquid-liquid extraction (HSEC) were able to impair cell viability of human multiple myeloma cells RPMI 8226 in a dose and time dependent manner. HSE cell viability reduction was due to a cytostatic action, while HSEC was more cytotoxic and induced a caspase dependent apoptosis. Moreover, both HSE and HSEC impaired cell migration and invasion of RPMI 8226 cells in a Boyden chamber assay. We also demonstrated in *in vitro* model of neurotoxicity (dorsal root ganglia model) that HSE and HSEC concentrations used in our experiments were not neurotoxic. In RPMI 8226 cells autophagy and proteasome activity were impaired by both HSE and HSEC. MAPK p38 activation was observed in the first 6h of treatment, while ERK 1 and ERK 2 activation occurred between 16 and 48h.

2- **Evaluation of combinations between Bortezomib (BTZ) and HSE or HSEC against RPMI 8226 multiple myeloma cells.** We evaluated several combinations between BTZ and HSE or HSEC (simultaneous treatment, not-simultaneous treatment and pretreatment) using MTT assay to assess their effectiveness. Among all evaluated combinations, only the 24h BTZ 1nM pretreatment followed by HSE or HSEC treatment resulted more effective than BTZ, HSE or HSEC single treatment in reducing cell viability. This combination was not neurotoxic in the dorsal root ganglia model.

3- **Isolation and characterization of HSEC molecules responsible of the antitumoral effect.** Using a bioguided method, we isolated several fractions from HSEC. Fractions were obtained by flash column chromatography or by resin use. Molecular characterization was performed by HPLC, NMR or mass spectrometry, while biological activity was screened in human multiple myeloma RPMI 8226 cells by MTT and proteasome activity assay. We obtained three fractions with a first isolation process that keep their activity on RPMI 8226 cells, but that are not completely pure. In a second purification process, we isolated

a highly pure fraction with a significant activity on RPMI 8226 cell viability. We also characterized this fraction by both a molecular and biological point of view.

In conclusion, we demonstrated the antitumoral effect of HS *in vitro* against RPMI 8226 multiple myeloma cells. We also found a new therapeutic combination between BTZ and HS that enhanced their antitumoral effect when compared to single treatments. Moreover, we also isolated one of the molecules that are involved in the antitumoral effect of HS.
The results of my PhD project showed that HS could be a promising chemotherapeutic agent, but further studies are still needed.

# Chapter 1: **Introduction**

**Fig.1 – *Hibiscus sabdariffa* calices**

## 1.1 Hibiscus sabdariffa

*Hibiscus sabdariffa* L. (HS) is a plant of the Malvacee family commonly cultivated in tropical and sub-tropical counties of both hemispheres. Main producers of HS are China, Thailand, Mexico, Egypt, Senegal and Tanzania.

### Plant features

HS is a perennial plant and it grows in shrub form up to the height of 2-2.5m. HS is planted in May and blooming take place between September and October. The collection of calices for commercial use occurs in November and December.

### Commercial use

The main use of HS plant is to produce bast fibers, a replacement of jute and used to make carpets and fabrics.
Red flowers of HS are mainly exported in USA and Europe where they are used to produce dyes for food industry.

HS leaves, flowers and calices are edible and contain high amount of nutrients (Table 1). Therefore, they are often used as salad ingredients or to produce jams (thanks to the natural presence of pectin at 3%), syrups, juices and liquor extracts.
Probably, one of the most popular use for HS is Karkadè. It is a cold drink made from an infusion of calices in boiling water (Ross, 2003).

| | | | | | |
|---|---|---|---|---|---|
| Proteins | 4.71g | Carotene | 0.029mg | Sodium | 96.66mg |
| Fat | 2.01g | Thiamine | 0.117mg | Potassium | 49.35mg |
| Carbohydrates | 68.75g | Riboflavin | 0.277mg | Calcium | 12.65mg |
| Fiber | 4.69g | Niacin | 3.765mg | Magnesium | 38.65mg |
| Water | 7.60g | Ascorbic Acid | 16.67mg | Phosphorus | 36.30mg |

**Table 1 – Nutritional values of HS –** Values are referred to 100g of dried calices (Adanlawo and Ajibade, 2007).

### HS in folk medicine

In India, Mexico and some African countries, HS is often used in folk medicine. Infusion of leaves or calices seems to have different and important curative properties. HS is particularly used as antibacterial, antihypertensive, antipyretic and diuretic (Ross, 2003).

## 1.1.1 HS extracts

To date, many techniques are used to produce HS extracts and there is not a completely standardized method. First of all, the extract can be obtained from leaves or from dried calices. Moreover, during the extraction protocol several conditions can be changed, such as type and concentration of solvents, temperature and time of the process. In this way, several types of extract can be obtained, with different characteristics and molecular content.

### Extraction from calices of HS

Commonly, calices from HS are dried twice before the extraction. The first drying is done under sunlight, while the second one at 40°C. Subsequently, calices are pulverized and subjected to the extraction protocol. There are three main extraction methods:

- **Cold water extract:** the used solvent is water and it is kept at 25°C temperature. This method requires 6-18 hours of extraction, but allows to preserve the molecular content of the extract.
- **Hot water extract:** even in this method, water is used as solvent, but in this case, it is heated to a temperature of 80°C. This temperature allows the reduction of extraction time but it could alter some molecules of the extract.
- **Ethanol extract:** in this extraction method, ethanol at different concentrations (20, 50, 100%) is used as solvent.

At the end of the extraction process, the mixture is filtered and dried to be stored or used (Prenesti *et al.*, 2007).

### 1.1.2 HS molecules

Mass spectrometry studies have identified some molecules that are present in HS and that are probably responsible for its curative effects (Ramirez-Rodrigues *et al.*, 2011). Some of the identified molecules (Table 2) will be discussed in detail in next paragraphs.

| | | | |
|---|---|---|---|
| Anthocyanins | Protocatechuic acid | β-sitosterol | Caffeoylquinic acid |
| Gallic acid | Tartaric acid | Ascorbic acid | Ergosterol |
| Citric acid | Ossalic acid | Gossypitrin | Luteolin |
| Malic acid | Eugenol | Myricetin | |

**Table 2 – Molecules of HS extracts** (Ramirez-Rodrigues *et al.*, 2011).

### 1.1.3 Anthocyanins

Anthocyanins are natural pigments contained in many varieties of fruits and vegetables. They confer blue and purple colors to berries, grapes, apples, red cabbage and cereals. Anthocyanins are often found in nature in the glycosylated form, due to the formation of a O-glyosidic bond between the molecule and a sugar (glucose, galactose, rhamnose, xylose) (Fig. 2).

All different possible combinations confer to anthocyanins several chemical and pharmacological features. Their regular consumption could reduce the risk of cardiovascular diseases, diabetes, arthritis and cancer. These properties are mainly due to antioxidant and anti-inflammatory effect of the molecules, but more studies are needed to confirm this hypothesis (Wang and Lin, 2000; Karlsen et al., 2007).

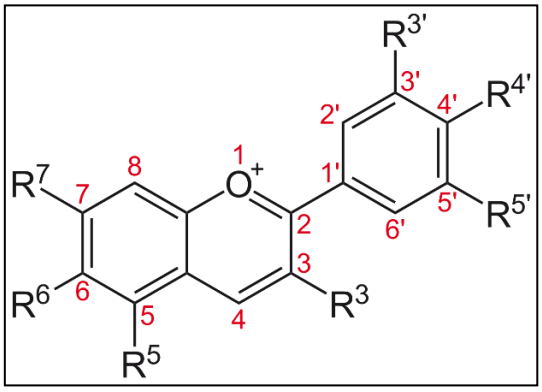
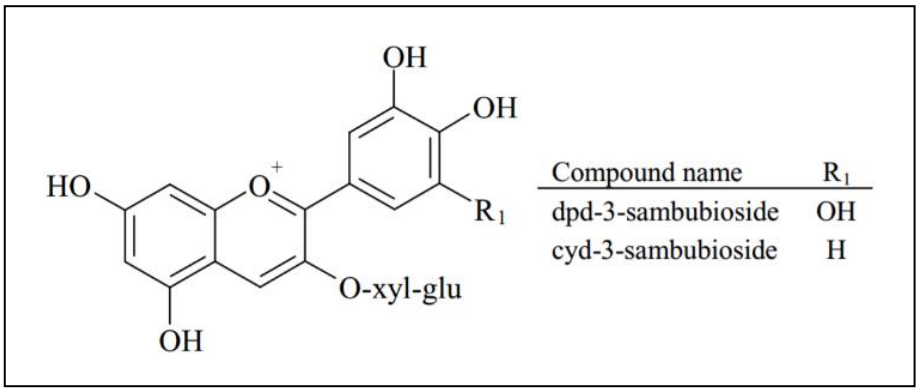



**Fig. 2 – Basic molecular structure of Anthocyanin**

#### Delphinidin and Cyanidin

Delphinidin-3-sambubioside and Cyanidin-3-sambubioside are the main anthocyanins present in HS extracts (Fig. 3) (Ramirez-Rodrigues *et al.*, 2011). Delphinidin is the most abundant anthocyanin in HS extracts, but it is commonly found also in other fruits and flowers. It is a highly water soluble molecule, but it is easily degraded by hydrolysis or at temperatures above 40°C. Some studies have demonstrated the effectiveness of Delphinidin as antioxidant (Wang and Lin, 2000) and as antitumoral (Hou et al., 2005; Wang et al., 2008). Cyanidin is the second most abundant anthocyanin in HS extracts, but the plant with the highest content is probably the elder. This molecule has also important antioxidant and antitumoral effects (Wang and Lin, 2000; Tsuda et al., 1996).




| Compound name | $R_1$ |
|---|---|
| dpd-3-sambubioside | OH |
| cyd-3-sambubioside | H |

**Fig. 3 - Structure of delphinidin- and cyanidin-3-sambubioside** (Ramirez-Rodrigues *et al.*, 2011).

### 1.1.4 Protocatechuic acid

Protocatechuic acid is a phenolic compound commonly isolated from HS flowers, but it can also be found in onions, some wheat varieties, prunes, grapes and nuts (Fig. 4). The main known effects of protocatechuic acid are antioxidation and anti-inflammation, thanks to its action as scavenger, the inhibition of ROS producer enzymes and the prevention of DNA oxidative damage and lipoperoxidation (Yoshino and Murakami, 1998; Liu *et al.*, 2002; Valentova *et al.*, 2003; Yan *et al.*, 2004).

Protocatechuic acid has been extensively studied in animal models of chemical induced cancer. It was effective against oral, colon, pancreas, liver, lungs, skin and breast cancers (Tanaka *et al.*, 1993; Kawamori *et al.*, 1994; Tseng *et al.*, 1998; Nakamura *et al.*, 2000; Suzuki *et al.*, 2003).

Cells from breast cancer, gastric adenocarcinoma, lung cancer and leukemia have been used to study *in vitro* the molecular mechanisms of the molecule (Lee and Yang, 1994; Hudson *et al.*, 2000; Tseng *et al.*, 2000; Kampa *et al.*, 2004). Antiproliferative effect seems mainly associated with the reduction of ROS, which are activators of NF-kB and AP1 (activator protein 1) pathways. These proteins are involved in cell cycle progression and apoptosis regulation. Apoptosis seems induced by the activation of phosphorylation regulatory pathways of RB and Bcl-2 (Tseng *et al.*, 2000).

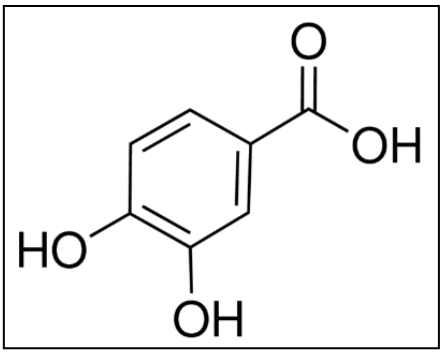



**Fig. 4 – Protocatechuic acid**

### 1.1.5 Gallic acid

Gallic acid is a phenolic acid of natural origin with important antioxidant and antitumoral effects (Fig. 6). It can also be found in other vegetables and naturals products such as tea and oak leaves, wine and mango. Gallic acid is often used by food and pharmaceutical industry as antioxidant additive (Masaki *et al.*, 1997).

Some *in vitro* studies also demonstrated a significant antitumoral effect of esterified derivate of gallic acid against melanoma and lymphoma cells (Serrano *et al.*, 1998; Locatelli *et al.*, 2009, 2013).

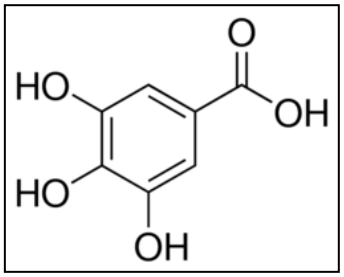
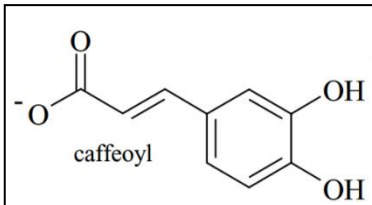



**Fig. 6 – Gallic acid**

### 1.1.6 Caffeoylquinic acid

Caffeoylquinic acid is an ester formed by caffeic acid and quinic acid. In HS extracts all three major isoforms of caffeoylquinic acid have been identified (Fig. 7).

Caffeoylquinic acid is a molecule with mainly antioxidants and antihypertensive effects (Zhao *et al.*, 2006, 2008). Furthermore, it also acts at intestinal level, by regulating the production of some hormones involved in glucose uptake, such as insulin (Clifford *et al.*, 2003).




| Compound name | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| quinic acid | OH | OH | OH |
| 3-caffeoylquinic acid | caffeoyl | OH | OH |
| 4-caffeoylquinic acid | OH | caffeoyl | OH |
| 5-caffeoylquinic acid | OH | OH | caffeoyl |

**Fig. 7 – Caffeoylquinic acid** (Ramirez-Rodrigues *et al.*, 2011)**.**

### 1.1.7 Pharmacologic properties of HS

Main pharmacologic properties of HS have been evaluated in animals or in some clinical trials in humans. The most important results are reported in Table 3.

| | *Event* | *Mechanism* | *Ref* |
|---|---|---|---|
| *Blood pressure* | Reduction of systolic and diastolic blood pressure (humans) | Inhibition of angiotensin-converting enzyme<br>Increased synthesis of nitric oxide | (Herrera-Arellano *et al.*, 2004; Ali *et al.*, 2005; McKay *et al.*, 2010; Ojeda *et al.*, 2010) |
| *Smooth muscle* | Relaxing of aortic and iliac artery (animal cells)<br>Contraction of uterus and diaphragm (animal cells) | Modulation of calcium channels | (Ali *et al.*, 1991; Obiefuna *et al.*, 1992; Salah *et al.*, 2002) |
| *Antipyretic, anti-inflammatory, antibacterial and antifungal* | Tested in animals | Not known | (Guerin and Reveillere, 1984; Afolabi *et al.*, 2008; Fullerton *et al.*, 2011) |
| *Kidney* | Increased diuresis and excretion of sodium and potassium (humans and animals) | Aldosterone modulation in the endothelium of kidney glomeruli | (Cáceres *et al.*, 1987; Kirdpon *et al.*, 1994; Aguwa *et al.*, 2004; Alarcón-Alonso *et al.*, 2012; Jiménez-Ferrer *et al.*, 2012; Mojiminiyi *et al.*, 2012) |
| *Cholesterol and obesity* | Reduction of total cholesterol, LDL and triglycerides (humans)<br>Reduction of obesity (animals) | Inhibition of Cholesterol and triglycerides synthesis<br>Reduction of gastric and pancreatic lipases, thermogenesis stimulation, inhibition of lipids accumulation and inhibition of adipogenic differentiation | (Kim *et al.*, 2003, 2007; Carvajal-Zarrabal *et al.*, 2005; Hirunpanich *et al.*, 2006; Farombi and Ige, 2007; Gurrola-Díaz *et al.*, 2010; Kuriyan *et al.*, 2010; Peng *et al.*, 2011) |
| *Liver and oxidative stress* | Steatosis reduction (animals)<br>Reduction of hepatic injuries from oxidative stress induced by chemical agents (animals) | Inhibition of SREBP-1 and PPAR-γ<br>Antioxidant properties | (Tseng *et al.*, 1997; Amin and Hamza, 2005; Kim *et al.*, 2007; Lee *et al.*, 2012; Villalpando-Arteaga *et al.*, 2013) |

**Table 3 – Pharmacological properties of HS**

### 1.1.8 Antitumoral effects of HS

The antitumoral effects of HS have been evaluated *in vitro* in different cancer cell lines.

#### Gastric cancer

The effects of an aqueous HS extract on gastric cancer cells have been demonstrated by Lin and co-workers *in vitro* (Lin *et al.*, 2005). The extract, obtained with 95°C water, could reduce in a dose and time dependent manner the viability of human AGS gastric cancer cells. $IC_{50}$ was 2.5mg/ml after 24h of treatment and its toxicity was probably due to apoptosis induction. The main pathway involved in HS effect was MAPKs JNK/p38. In addition, researchers observed a reduction of anti-apoptotic proteins (Bcl-2 and Mcl1), an increased translocation from cytoplasm to mitochondria of pro-apoptotic proteins (Bax and t-Bid), an increased cytochrome c release and a consequent increased caspase 8, 9 and 3 activation

#### Leukemia

The antitumoral effect of HS against leukemia cells HL-60 *in vitro* was confirmed both by Chang and Tseng research groups (Tseng *et al.*, 2000; Chang *et al.*, 2005). Protocatechuic acid and anthocyanins were the main responsible of HS effect. Protocatechuic acid was acting mainly by inactivating RB and downregulating Bcl-2 expression. Anthocyanins instead influenced p-38 and c-Jun pathways, which culminate with the activation of caspases and the release of cytochrome c from mitochondria.

#### Breast Cancer

A cold-water HS extract was effective in reducing viability of human breast adenocarcinoma MCF-7 cells (Khaghani, 2011). The mechanism was both estrogen dependent and independent. In the first case, some unidentified molecules of the extract could bound and inhibit estrogen receptor inducing apoptosis. In the second case, the molecules contained in the extract, ergosterol and β-sitosterol, were acting independently from estrogen receptor in inhibiting cell proliferation and inducing apoptosis (Subbiah and Abplanalp, 2003; Ju *et al.*, 2004).

#### Cervical cancer

Antitumoral effects of both cold and hot aqueous extracts of HS were evaluated on human cervical cancer Hela cells *in vitro*. Extracts induced a reduction of cell proliferation and apoptosis. The mechanisms that led to these alterations are still not entirely clarify (Olvera-García *et al.*, 2008).

### 1.1.9 HS toxicity

HS toxicity in humans are still poorly investigated, but first clinical trials have not shown relevant side effect (Herrera-Arellano *et al.*, 2004; Ali *et al.*, 2005; Peng *et al.*, 2011).

Currently, the only detailed studies of *in vivo* HS toxicity were carry out in animal, in particular mice and rats (Onyenekwe *et al.*, 1999; Akindahunsi and Olaleye, 2003; Fakeye *et al.*, 2009; Sireeratawong *et al.*, 2013). However, results obtained by different research groups are never

totally consistent and show high variability. This is probably due to large differences between types of HS extracts used and ways of administration.

Researchers often observed weight loss (Fig. 8) and severe diarrhea in animals chronically treated with high doses of HS. In some cases, animals also suffered from a mild form of muscular dystrophy and an increased liver activity (especially aspartate and alanine aminotransferase and creatinine). These altered conditions often caused the death of the animals.

Some milder effects induced by chronic administration of HS were the reduction of erythrocyte count, the decrease of cholesterol level and slightly variations of kidney and spleen tissues.

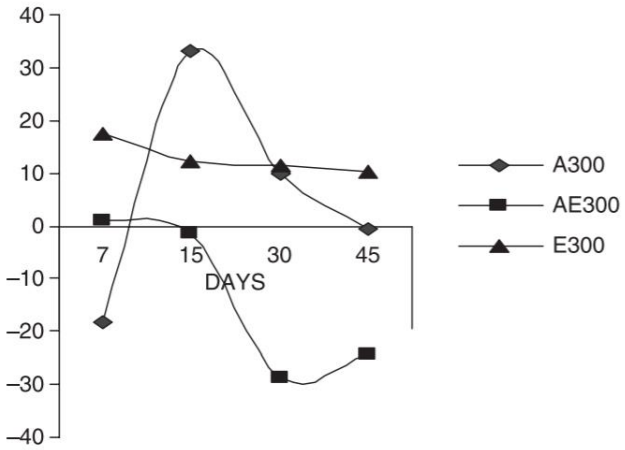



**Fig. 8 – Body weight changes of rats –** Rats were treated with 300mg/kg HS aqueous (A300), 50% ethanol (AE300) or ethanol (E300) extracts (Fakeye *et al.*, 2009).

## 1.2 Bortezomib

Bortezomib (BTZ), also known as Velcade® (its commercial name), is an antineoplastic drug commonly used for multiple myeloma treatment. It is a proteasome inhibitor with a high activity against multiple myeloma cells. Even if BTZ is effective, it presents several side effects which sometimes lead to dose reduction or total discontinuation of the therapy

### 1.2.1 BTZ history

BTZ was the first proteasome inhibitor to be tested and used in humans. The molecule was synthesized for the first time in 1995 by Myogenix laboratory. Thanks to excellent results obtained *in vitro* and to the success of first small phase I clinical trials, BTZ was admitted to most important and extensive phase II and III clinical trials already in 1999. In 2003, the US Food and Drug Administration approved for the first time its use in patients with multiple myeloma. In 2005 the approval was extended to patients with relapsed or refractory multiple myeloma and in 2008 it was also approved for the treatment of multiple myeloma patients that never received any other drug (www.cancer.gov, 2013).

### 1.2.2 Molecular structure

BTZ is a pyrazine and boronic acid derivate. It is a dipeptide composed by Pyrazinoic acid, Phenylalanine, Leucine and a boronic Acid (Fig. 9).

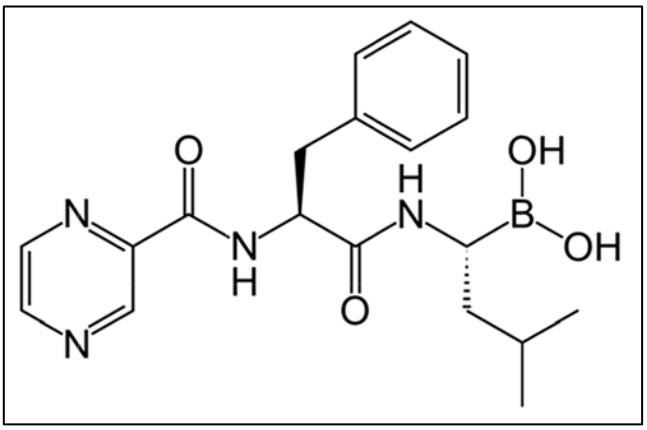



**Fig. 9 – BTZ structure**

### 1.2.3 Action mechanism

Antitumoral effect of BTZ is due to its activity as proteasome inhibitor. Boron atom of BTZ molecule interacts with proteasome 26S catalytic site and inhibits its ability to degrade abnormal or misfolded proteins (Buac *et al.*, 2013).

Proteasome is a multiprotein complex composed principally by one catalytic subunit (proteasome 20S) and by two regulatory subunit caps (proteasome 19S). The subunits together form proteasome 26S (Fig. 10).

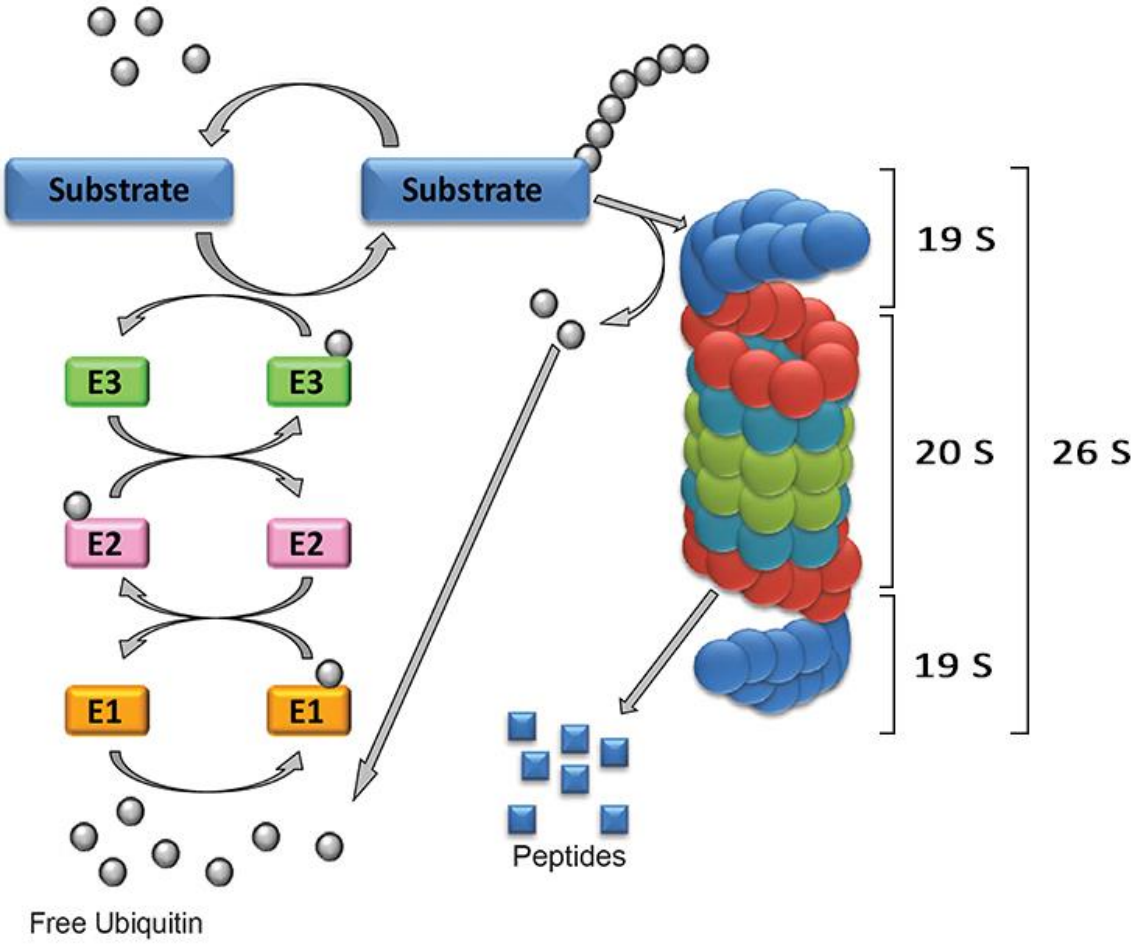



**Fig. 10 - Schematic representation of ubiquitination process and proteasome structure** (Massaly *et al.*, 2014).

Proteins, that should be degraded by proteasome, are recognized thanks to the presence of a 76aa peptide called ubiquitin. Ubiquitin is bound and activated by E1 enzyme (activator of ubiquitin) and subsequently it is transferred to an E2 enzyme (conjugator of ubiquitin). Then, ubiquitin is transferred to an E3 enzyme (ubiquitin ligase) and finally to the target protein (Massaly *et al.*, 2014).

Up to date, it is not completely clear, how BTZ induces cell death by proteasome inhibition. One of the most reliable hypothesis is that proteasome inhibition induces apoptosis. In cancer cells, proteasome degrades pro-apoptotic proteins and enhances the resistance to apoptosis. Inhibition of proteasome by BTZ increases the number of pro-apoptotic proteins and causes cell death (Wu *et al.*, 2016). Furthermore, several pathways are altered by proteasome inhibition. One of the most important is NF-kB, involved in cancer survival and proliferation. Proteasome inhibition also inhibits the activation of NF-kB and its effects on cancer cells (Fig. 11) (Fuchs, 2013).

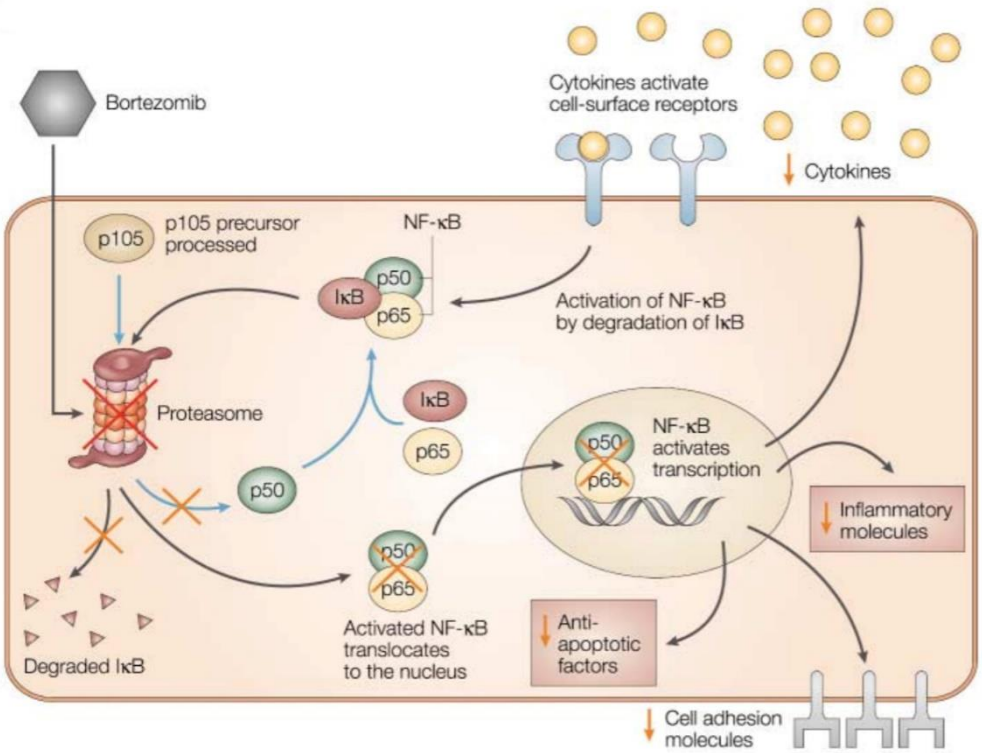



**Fig. 11- NF-kB pathway and BTZ –** In physiological conditions, cytokines activate NF-kB by proteasome degradation of IkB. Free NF-kB translocate to the nucleus where it induces transcription of proteins involved in inflammation, anti-apoptosis, cells adhesion, cell proliferation and survival. Proteasome inhibition by BTZ blocks the degradation of IkB and impairs NF-kB activation. (Nature Reviews Drug Discovery, 2004)

### 1.2.4 Pharmacokinetic

Recommended BTZ dose for multiple myeloma treatment in adults is 1.3mg/m$^2$. BTZ is injected intravenously twice a week for two weeks with a third week of rest. This conclude a 21-day cycle of BTZ treatment.

BTZ pharmacokinetic has been studied in several clinical trials of phase III (Schwartz and Davidson, 2004; Levêque *et al.*, 2007). Maximum plasma peak concentration of BTZ after the first dose ranges from 89 to 120ng/ml and has an elimination half-life of 76-108h. Total body clearance is about 112L/h and body distribution is 1884 L/m$^2$.

BTZ metabolism is mediated by cytochrome P450. BTZ undergoes to a first deboronation which generates about thirteen different deboronated metabolites. These metabolites have no effect on proteasome inhibition and subsequently undergo hydroxylation (Schwartz and Davidson, 2004).

To date, no reliable data on BTZ excretion are available.

### 1.2.5 Pharmacodynamic

BTZ pharmacodynamic is measured by proteasome inhibition in peripheral blood mononucleated cells. BTZ induces a dose dependent inhibition of proteasome and inhibition maximum is observed after 1h of treatment (Fig. 12). Recommended BTZ dose, 1.3 mg/m$^2$, inhibits about 65% of proteasome activity. After 24h of treatment proteasome partially recovers its activity which returns to baseline after 72-96h (Hamilton *et al.*, 2005).

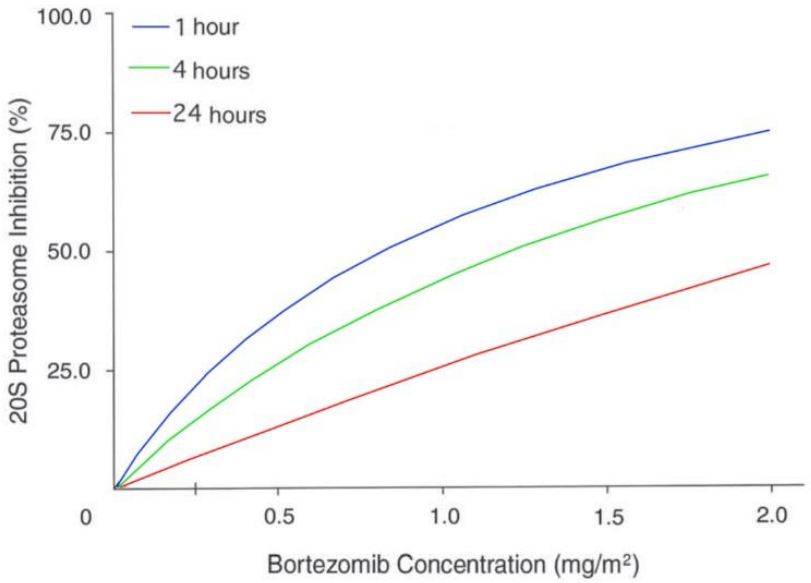



**Fig. 12 - BTZ proteasome inhibition in peripheral blood** (Hamilton *et al.*, 2005)**.**

## 1.2.6 Adverse effects

BTZ prolonged treatment causes several and important adverse effects (Table 4) (Palumbo *et al.*, 2011; Vij, 2011). Among all adverse effects, gastrointestinal, hematologic and peripheral neuropathy are the most frequent and disabling.

| | | *Patients* |
|---|---|---|
| *Nervous system* | Peripheral neuropathy | 49% |
| | Paresthesia and Dysesthesia | 23% |
| | Headache | 22% |
| *Hematologic* | Thrombocytopenia | 34% |
| | Neutropenia | 27% |
| | Anemia | 23% |
| | Leucopenia | 20% |
| | Lymphopenia | 23% |
| *Gastrointestinal* | Nausea | 64% |
| | Diarrhea | 51% |
| | Constipation | 43% |
| *Cardiovascular* | Hypotension | 13% |
| *Musculoskeletal* | Arthralgia | 26% |
| | Pain in limb | 26& |
| | Bone pain | 14% |
| | Myalgia | 14% |
| *Respiratory* | Dyspnea | 22% |
| | Cough | 20% |
| | Infections | 18% |
| *Dermatologic* | Rash | 18% |
| | Herpes Zoster | 12% |

**Table 4 – BTZ adverse effects and frequency in patients.**

### Peripheral neuropathy

Peripheral neuropathy is the most frequent and disabling adverse effect caused by BTZ. In most patients, it is the first cause of BTZ dose limiting or total treatment discontinuation.
BTZ toxicity on peripheral nerves are not completely understood. Several hypotheses reported a possible damage of mitochondria and endoplasmic reticulum of dorsal root ganglia neurons or of Schwann cells. Another theory associates the inhibition of NF-kB, induced by BTZ, with the reduction of nerve growth factor production (Cavaletti and Jakubowiak, 2010; Callander *et al.*, 2014).

### Gastrointestinal

Gastrointestinal side effects include nausea, vomiting, diarrhea and constipation. Even if they are really frequent, only 5% of patients need to completely suspend BTZ treatment. In other cases, a pharmacological support is sufficient to continue the therapy (Colson *et al.*, 2004; Colson, 2015).

### Hematologic

Thrombocytopenia is a frequent consequence of BTZ treatment and occurs after 1 or 2 cycles. It consists in the reduction of platelet count, but the real mechanism that lead to this event is not completely clear. Probably, it is due to a reduced NF-kB dependent thrombopoiesis.
Thrombocytopenia often occurs with neutropenia and leucopenia, but only in rare cases it needs BTZ therapy discontinuation. In most of patients a supportive care is enough to continue the treatment (Colson, 2015).

## 1.3 Multiple myeloma

Multiple myeloma is a hematologic neoplasia characterized by uncontrolled proliferation of plasma cells.

Plasma cells are a component of the immune system with the role to produce antibodies in response to an antigen stimulus. They derive from B lymphocytes after differentiation induced by antigens and CD4+ T lymphocytes (Fig. 12).

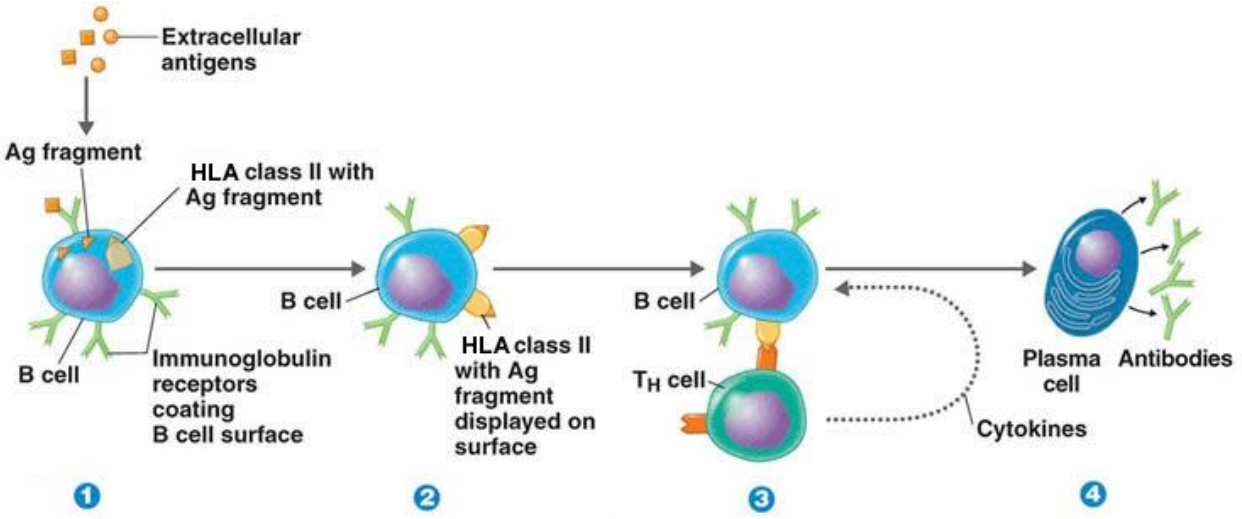



**Fig. 12 - Plasma cells differentiation -** (1) Immunoglobulin receptors on B cell surface recognize and attach to antigen, which is then internalized and processed. Within the B cell a fragment of the antigen combines with HLA class II. (2) HLA class II antigen fragment complex is displayed on B cell surface. (3) Receptor on the T helper cell ($T_H$) recognizes complex of HLA class II and antigen fragment. Cytokines produced by $T_H$ activate the B cell. (4) B cell is activated and begins clonal expansion. Some of the progeny become antibody-producing plasma cells.

### 1.3.1 Multiple myeloma epidemiology

Multiple myeloma represents 1% of all tumors and 10% of hematological cancers. In Italy in 2015, multiple myeloma was newly diagnosed in about 2400 women and 2900 men, with an incidence of 7.6 and 9.8 on 100000 respectively. The number of newly diagnosed case didn't change compared to previously years (www.airc.it, 2015).

### 1.3.2 Risk factors

Etiopathology of multiple myeloma is not yet completely understood and this is due to low frequency of the disease and to low connection with risk factors.

Age is probably one of the most important risk factor because 66% of patients is more than 65 years old and only 1% is less than 40 years old. Also the exposition to some chemical agents (pesticides and petroleum derivate) and ionizing radiation could increase the risk to develop the pathology (Alexander *et al.*, 2007).

Many research also focused their attention on tobacco, alcohol, diet and hormonal factors, but they were often inconclusive and did not demonstrate any correlation with multiple myeloma (Alexander *et al.*, 2007; Becker *et al.*, 2008; Costantini *et al.*, 2008).

### 1.3.3 Multiple myeloma staging

The staging of multiple myeloma is important to understand the progress of the pathology and for treatment options and prognosis. Today there are two main staging system of multiple myeloma: Durie-Salmon and the International staging system (Zhan *et al.*, 2006).

#### Durie-Salmon staging system

This type of staging system is based principally on four factors:

- Quantity of monoclonal antibodies in the blood or urine, which is correlated to the amount of neoplastic plasma cells.
- Quantity of calcium in the blood to determine bones damage.
- X-Ray analysis of bones damage.
- Quantity of hemoglobin in the blood, which indicates how many neoplastic cells are occupying bone marrow.

The system divides patients in three stages, in order of pathology severity.

#### International staging system

This staging system is based on serum levels of β-2 microglobulin and albumin and divides patients in three stages.

### 1.3.4 Pathogenesis

Neoplastic plasma cells differentiate from B lymphocytes and infiltrate bone marrow where they find an advantageous microenvironment. Both multiple myeloma cells and resident cells produce high quantity of IL-6, IL-10, IL-15, TGF-β, LIF and IGF, which promote cell proliferation and adhesion. In a normal bone marrow, plasma cells reach at most 3% of total cell population. In multiple myeloma patients, at least 10% are plasma cells and sometimes all bone marrow population is replaced. When all bone marrow cells are substituted by neoplastic plasma cells, an impairment in physiologic hematopoiesis can occur. It could lead to anemia, leucopenia and thrombocytopenia (Kyle and Vincent Rajkumar, 2006).

Neoplastic plasma cells are morphologically indistinguishable from normal cells and only in some cases they present cytological alterations (Matsui *et al.*, 2004; Bergsagel and Kuehl, 2005).

Multiple myeloma patients often suffer from bone resorption, hypercalcemia and bone fractures. These conditions are caused by the effect of neoplastic plasma cells on osteoclasts and osteoblasts. Plasma cells produce Macrophage Inflammatory Protein 1 and modulate WNT pathway which respectively activate osteoclasts and inhibit osteoblasts (Hjertner *et al.*, 2006).

Genetic disorders are often recurring in multiple myeloma cells. Chromosomal aberrations are frequent and are mainly on genes of immunoglobulin high chain (14q23), FGFR (4p16), Cyclin D1 (11q13), Cyclin D3 (6p21) and cMAF (16q23). Point mutations on RAS, BCL-2, RB and p53 are also frequent. All these genetic alterations contribute to a high molecular heterogeneity of the disease (Bergsagel and Kuehl, 2001).

A peculiar feature of multiple myeloma cells is the high production of monoclonal antibodies. These antibodies (also called component M) are often dysfunctional and have altered chemical-

physical features. Sometimes plasma cells also produce incomplete parts of antibodies such as light chains, also called Bence Jones chains (Bergsagel and Kuehl, 2001).

### 1.3.5 Multiple myeloma therapy

Multiple myeloma treatment is constantly evolving and the introduction of new drugs or therapeutic techniques made it possible to raise the average life expectancy from 2-3 to 4-5 years. Chemotherapy is the most used and effective treatment against multiple myeloma. It allows a complete remission in 60-70% of the patients (Rajkumar, 2005; Terpos *et al.*, 2007; Anderson *et al.*, 2008). Most common multiple myeloma drugs are:

- **Bortezomib**
- **Lenalidomide**: a drug with three different effects, anticancer, antiangiogenesis and immunomodulator (Thomas *et al.*, 2007; Benboubker *et al.*, 2014).
- **Melphalan**: it alkylates guanine and impair DNA or RNA synthesis causing cell death (Dimopoulos *et al.*, 2007).
- **Thalidomide**: it acts mainly on NF-kB pathway and consequently it impairs interleukins production and cell proliferation (Rajkumar *et al.*, 2006).

Usually, chemotherapy is followed by autologous stem cell transplantation, which allows bone marrow restoration and supports a complete patient remission. It is recommended in patients under 65 years, because in older patients this therapy is not well tolerated (Mian *et al.*, 2016). Chemotherapy is often supported by other drugs or treatments. For example bisphosphonates are commonly used to prevent bone resorption, while transfusions of red blood cells or erythropoietin cure anemia (Raje *et al.*, 2014).

# 1.4 Autophagy

Autophagy is a cellular process responsible for degradation of cytoplasmic components (proteins and organelles) through a autophagosome-lysosome pathway.

## 1.4.1 Types of autophagy

There are at least three types of autophagy, discriminated especially for the delivery mode to the lysosome:

- Macroautophagy: cytoplasmic materials are sequestered into a double-membrane vesicle (autophagosome) that subsequently fuses with lysosome (autolysosome).
- Microautophagy: cytoplasmic materials are directly absorbed by the surface of lysosomes after division, protrusion and invagination of membrane.
- Chaperone mediated autophagy: protein with a specific peptide motif are directly imported inside the lysosome.

## 1.4.2 Autophagy process

In this PhD project, we focused on macroautophagy (herein referred to as autophagy). The process is divided in different finely regulated stages (Meléndez and Levine, 2009) (Fig. 13):

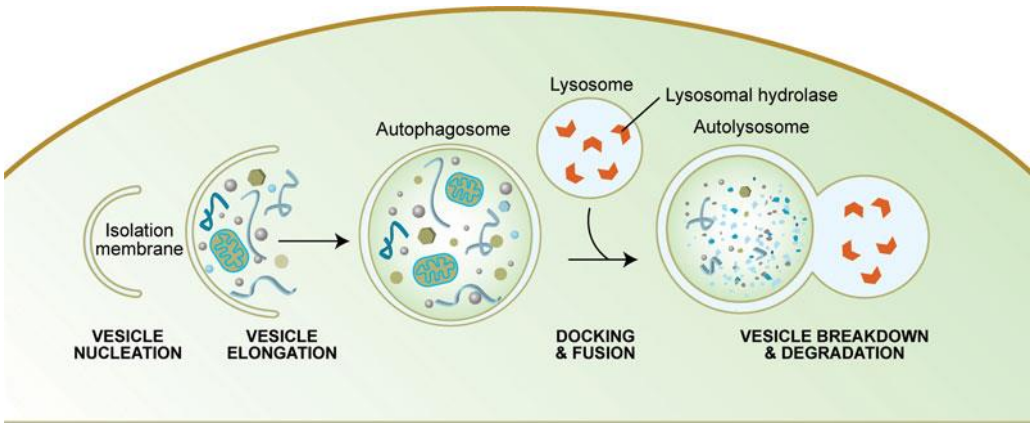



**Fig. 13 – Schematic representation of autophagy process** (Meléndez and Levine, 2009)

1. Vesicle nucleation: in normal conditions, autophagy is inhibited by growth factors, ROS and amino acids sensing, which regulates mTOR and AMPK. These kinases inactivate ULK1 and ULK2 by phosphorylation. When autophagy is induced, for example by nutrient starvation, ULK1 and ULK2 are dephosphorylated and activated. Subsequently, the activate Beclin-1 complex, which has a PI3K class kinase. Activated ULK and Beclin-1 complexes are relocated to autophagy initiation site and the isolation of membrane begins (Funderburk *et al.*, 2010).
2. Vesicle elongation and autophagosome formation: VPS34 phosphorylates the phosphatidylinositol-3-phosphate (PI3P) on vesicle membrane. PI3P acts as docking for WIPI2 protein family and LC3, which contributes to autophagosome closure.

3. Docking and fusion with lysosome: autophagosome and lysosomes recognize each other, dock and fuse with a SNARE and UVRAG dependent process.
4. Degradation: cytoplasmic components inside autolysosome are degraded by lysosome hydrolases and made available for cell recycling.

### 1.4.3 Functions of autophagy

Autophagy is involved in many cellular processes, both physiologic and pathologic:

- Nutrient starvation: autophagy is triggered when cells undergoes nutrient depletion. This allows proteins and organelles degradation and amino acids recycling.
- Repair mechanisms: to date, autophagy is the only process able to degrade and recycle cell damaged organelles.
- Cell death: it is not clear if autophagy is a possible cause or if it prevents cell death (Bincoletto *et al.*, 2013).
- Cancer: autophagy has both tumor suppressor and promotion role, it depends on cell type, activation stimulus and process length. As tumor suppressor, autophagy prolonged activation may lead to apoptosis or necrosis of cancer cells. In some conditions, autophagy can contribute to cancer cell growth. For example, in starvation conditions, it allows protein recycling or degrades pro-apoptotic proteins. Indeed, several researchers reported that chloroquine inhibition of autophagy during chemotherapy can enhance the effectiveness of the treatment (Cui *et al.*, 2013).

## 1.5 Aims

The aim of this PhD project was the *in vitro* evaluation of the antitumoral effects of HS against multiple myeloma cells. We assessed the effectiveness of a total extract of HS and of its fractions obtained with different chemical techniques.
The project was organized in three distinct phases:

1- In the first phase, we evaluated the effects of HSE and of its fractions, obtained by liquid-liquid extraction, on human multiple myeloma cells RPMI 8226. We evaluated the effect on cell viability, apoptosis, cell migration and invasion and neurotoxicity. Finally, to give an overview of the mechanisms responsible of the antitumoral effect of HS, we evaluated the involvement of autophagy, proteasome and MAPKs.

2- In the second phase of the project, we evaluated several combinations between BTZ and HS against multiple myeloma cells RPMI 8226 *in vitro*. BTZ is the most used and probably effective drug for multiple myeloma treatment. Despite this, BTZ has several important adverse effects and it is important to find new therapeutic approaches to increase its efficacy. The aim is to find a combination that allows to maintain, or enhance, treatment efficacy, reducing BTZ doses and consequently its side effects. The effectiveness of combinations was evaluated by MTT assay, while neurotoxicity was evaluated by DRGs *in vitro* model.

3- In the third phase of the project we aimed to isolate and characterize one or more molecules responsible of HS effect. Using a bioguided method we isolated and characterized, both from a molecular and a biological point of view, the fractions obtained from HS total extract. MTT and proteasome activity assay were used to screen effective fractions on RPMI 8226 cells.

# Chapter 2: **Materials and methods**

## 2.1 Cell lines

RPMI 8226 cells were cultured in IMDM medium supplemented with 20% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin (Euroclone, Pero, Italy). Cells were maintained at 37°C in an humified incubator with 5% CO2.

RPMI 8226 are human multiple myeloma cells isolated from peripheral blood of a 66 years old male with multiple myeloma. The cells produce and secrete only Ig lambda light chains (but not heavy chains) (Moore and Kitamura, 1968).

## 2.2 HSE and fractions

*Hibiscus sabdariffa* total extract (HSE) is kindly provided by CREA (Research Unit for Floriculture and Ornamental Species) of San Remo. It is solubilized in Phosphate Buffered Saline (PBS, Euroclone, Pero, Italy) at 1g/ml concentration. Subsequently it is diluted at final concentrations directly in culture medium.

First fractioning of HSE was performed using a separatory funnel and liquid-liquid extraction technique (Fig. 14):

1. HSE was dissolved in $H_2O$ and the solution was transferred in the separatory funnel. Ethyl acetate was added and mixed with $H_2O$ solution.
2. After separation of the two phases, ethyl acetate fraction was collected. The procedure was repeated 3-4 times. Ethyl acetate fraction was dried.
3. H2O fraction was added with Butanol and mixed. After separation of the two phases, H2O fraction and Butanol fraction were collected and dried. The procedure was repeated 3-4 times.

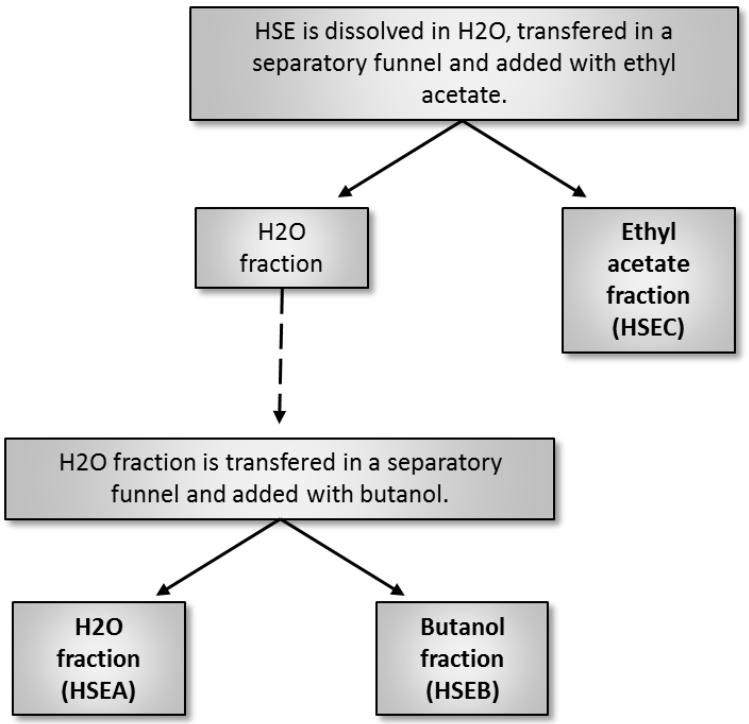


**Fig. 14 - Schematic representation of HSE isolation process.**

We obtained three fractions from HSE: H2O fraction (HSEA), Butanol fraction (HSEB) and Ethyl acetate fraction (HSEC). All fractions, in a solid state, were solubilized in PBS at 1g/ml concentration and subsequently diluted at final concentrations directly in culture medium.

## 2.3 Bortezomib

Bortezomib powder was solubilized in DMSO at 2,6mM concentration. Subsequently it is diluted at final concentrations directly in culture medium.

## 2.4 MTT assay

Cell viability was evaluated with MTT assay. MTT is a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that is converted to the insoluble formazan salt by mitochondrial enzymes. Quantity of produced salt is directly proportional to viability of cells.

RPMI 8226 cells were seeded in 96-well plates at 10000 cell/well density. After 24h cells were treated for further 24, 48 or 72h. Then, a MTT assay was performed. A 5mg/ml of MTT solution (Sigma-Aldrich, S.Louis, MO) was added directly to culture medium to reach a final concentration of 0.5mg/ml. After 4h, plates were centrifuged for 10 minutes at 2000RPM and 4°C. Culture medium was then removed and formazan crystals were solubilized in acidified 2-propanol (2-Propanol added with 1% HCl) (Sigma-Aldrich, S.Louis, MO). Absorbance of the solution was read at 560nm in a microplate reader (Biorad, Hercules, CA).

## 2.5 DRGs neurotoxicity assay

Embryo rat dorsal root ganglia (DRGs) neurotoxicity assay allows the evaluation of the neurotoxicity of a drug or compound *in vitro*.

All experimental procedures were approved by the Ethics Committee for Animal Studies of the University of Milan Bicocca.

Pregnant Wistar rat (Envigo, Udine, Italy) was sacrificed after a deep anesthesia. Embryos were collected in L15 medium (Euroclone, Pero, Italy) and DRG were removed after dissection.

DRGs were seeded in 35mm dishes previously coated with collagen and filled with AN2 medium (MEM (Euroclone, Pero, Italy), 10% Calf Bovine Serum (Euroclone, Pero, Italy), 1.4mM L-Glutamine (Euroclone, Pero, Italy), 0.6% Glucose (Sigma-Aldrich, S.Louis, MO) and 5ng/ml Nerve Growth Factor (NGF, Thermo Fisher, Waltham, MA)) . After 2h, culture medium was removed and replaced with treatment medium. After 24 and 48h, micrographs were taken under a light microscope (Nikon Eclipse TS100) and the length of DRGs neurites was measured with ImageJ software.

## 2.6 Trypan blue vital count

Viable and dead cells were distinguished by Trypan blue vital count assay. Trypan blue is a vital dye that passes through damaged membrane of dead cells. Viable cells with intact membrane are not stained.

RPMI 8226 cells were seeded in 6-well plates at 250000 cell/well density. After 24h cells were treated and after further 24, 48 or 72h they were collected. An aliquot of cells were suspended

in a Trypan blue solution 1:1 (Sigma-Aldrich, S.Louis, MO). Viable and death cells were counted in a Burker haemocytometer under a light microscope (Nikon Eclipse TS100).

## 2.7 Annexin V/Propidium Iodide staining

Annexin V/Propidium Iodide staining was performed to evaluate apoptosis. Apoptosis is characterized by phosphatidylserine translocation on the outer side of the cellular membrane. Annexin V is a phospholipid binding protein with high affinity for phosphatidylserine. Propidium Iodide (PI) is a vital dye commonly used in association with Annexin V to discriminate early and late apoptosis, because it passes the damaged membrane of dead cells.
RPMI 8226 cells were seeded in 6-well plates at 250000 cell/well density and after 24h cells were treated. Annexin V/PI staining was performed after 24, 48 or 72h of treatment using FITC Annexin V Apoptosis Detection Kit (BD biosciences, San Jose, CA). Cells were collected and washed two time with PBS. 100000 cells were resuspended in 100µl of 1X Annexin V binding buffer. Then, 5µl of Annexin V and 5µl of PI solutions were added to cell suspension. Cells were incubated for 15 minutes and then 400µl of 1x Annexin V binding buffer were added. At least 10000 cells were analyzed by flow cytometer.

## 2.8 Western blot

Western blot analysis was performed to assess modifications in protein expression or phosphorylation level.
RPMI 8226 cells were seeded in 6-well plates at 250000 cell/well density. After 24h cells were treated. At different time points cells were collected, washed with PBS and subjected to protein extraction which was performed both with a chemical and a mechanical lysis. Cells were suspended in a lysis buffer (composition in Table 5) and then vortexed for 30 seconds. Afterwards, cell lysate was clarified with a centrifuge at 13500 RPM for 15 minutes and at 4°C.

| | *Final concentration* |
|---|---|
| *Hepes pH7.5* | 5mM |
| *NaCl* | 150mM |
| *Glycerol* | 10% |
| *Triton X100* | 1% |
| *MgCl2* | 1,5mM |
| *EGTA* | 5mM |
| *H2O* | |
| *Phenylmethylsulphonyl fluoride (PMSF)* | 4nM |
| *Aprotinine* | 1% |
| *Sodium pyrophosphate* | 20nM |
| *Sodium orthovanadate* | 92mg/ml |

**Table 5 – Lysis buffer composition**

Protein content was quantified using Bradford method and a Coomassie protein assay reagent kit (Euroclone, Pero, Italy).
Cells lysate suspension with 10µg of proteins was then mixed with Laemmli buffer (5% β-mercaptoethanol, 10% SDS, 50% glycerol, 400 mM Tris HCl (pH 6.8) and 0.5% bromophenol blue, all

from Sigma-Aldrich, S.Louis, MO), denatured at 95°C for 5 min and separated in a 13% acrylamide SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose filters and western blot analysis was performed.
Membranes blocking, washing and antibody incubation were performed according to manufacturer's instructions. Antibodies against LC3B (1:1000, Cell Signaling, Temecula, CA), Beclin-1 (1:1000 Cell Signaling, Temecula, CA), Pro-Caspase-3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), Cleaved-Caspase3 (1:1000, Cell Signaling, Temecula, CA), P-ERK1/2 (1:1000, Cell Signaling, Temecula, CA), pan-ERK (1:5000, BD Transduction Laboratories), P-p38 (1:1000, Cell Signaling, Temecula, CA), p38 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) and beta actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) were used. After incubation with primary antibodies, membrane was washed and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000) (anti-mouse, Chemicon, Temecula, CA, USA; anti-rabbit, PerkinElmer, Boston, MA, USA). Immunoreactive proteins were visualized using an ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA).

## 2.9 Acridine orange staining: flow cytometry analysis and fluorescence microscope imaging

Autophagy was assessed with Acridine Orange (AO) staining. AO is a fluorescent dye that, in normal conditions, emits in green wavelength. In acidic conditions, for example inside autolysosome, the molecule became protonated and change its emission wavelength from green to orange-red. RPMI 8226 cells were seeded in 6-well plates at 250000 cell/well density. After 24h cells were treated. AO staining was performed after further 24h. A solution of AO (Sigma-Aldrich, S.Louis, MO) was added directly to culture medium to reach a final concentration of 1ng/ml. After 15 minutes of incubation at 37°C, cells were collected and washed two times with PBS by centrifugation. For flow cytometry analysis cells were transferred to a 5ml vial and analyzed by flow cytometer.
For fluorescence microscope imaging cells were transferred to a glass slide and micrographs were taken under a fluorescence microscope.

## 2.10 Proteasome activity

Proteasome activity was assessed by N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin fluorogenic substrate for chymotrypsin-like enzymes (Sigma-Aldrich, S.Louis, MO). In the presence of proteasome, the fluorophore, 7-Amido-4-Methylcoumarin, is released and emits fluorescence. The fluorescence obtained is a measure of proteasome activity.
Cell lysate was obtained and quantified as described in Western blot paragraph, but lysis buffer was prepared without proteases and phosphatases inhibitors (Phenylmethylsulphonyl fluoride, Aprotinine, Sodium pyrophosphate, Sodium orthovanadate). Cell lysate containing 40µg of proteins were loaded in dark 96-well plate. Water, 10µl of 10X proteasome buffer and 10µl of proteasome substrate (N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin) were added to protein solution. Plate were then incubated at 37°C and after 2h fluorescence was measured in a microplate reader (Biorad, Hercules, CA).

## 2.11 Boyden chamber

Cell migration and invasion was assessed by Boyden chamber assay. Boyden chamber is a plexiglass plate with 48 wells (Biomap, Agrate, Italy). Each well is divided in one upper and one lower compartment. Compartments were separated by a gelatin coated polycarbonate membrane with 8µm pores (Biomap, Agrate, Italy) to simulate extracellular matrix.

Membrane was washed with a 0.5M acetic acid solution and then two times with PBS. Afterwards, membrane was placed in a 0.2mg/ml gelatin solution at 4°C for 7 days.

The chamber was then prepared (Fig. 15):

1. In lower compartment a complete culture medium was placed. Only in negative control a complete medium without FBS was added.
2. Gelatin coated polycarbonate membrane was placed between compartments.
3. In upper compartment a cell suspension in complete medium with treatments and without FBS were added. 10000 cells for each well were seeded.

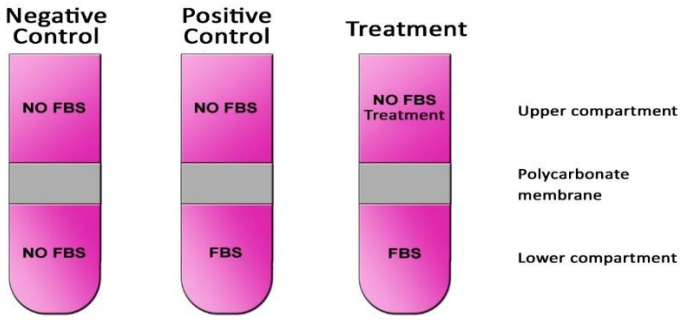



**Fig. 15 – Boyden chamber set up.**

Boyden chamber was incubated at 37°C in a humified incubator. After 20h polycarbonate membrane was removed. The upper side was scraped to remove not-migrated cells. The cells on the lower side were fixed in methanol and stained with Hematoxylin G and Thiazine Blue (DiffQuick staining kit, Biomap, Agrate, Italy). Micrographs of each well were taken under a light microscope (Nikon Eclipse TS100) and the number of migrated cells were counted using ImageJ software.

## 2.12 Statistical analysis

Data are reported as mean ± standard deviation from at least 3 independent experiments. Statistical analysis was performed using GraphPad Prism3 software. The differences between control and treated cells were evaluated using t-student test or One Way ANOVA analysis of variance followed by Dunnet's multiple comparison test. Statistical significance was set at $P<0.05$ or $P<0.01$.

# Chapter 3: **Results**

## 3.1 Phase I: Evaluation of HS antitumoral effect

### 3.1.1. Evaluation of RPMI 8226 cell viability after HSE treatment

Human multiple myeloma RPMI 8226 cells were exposed to different concentrations of HSE (1-50mg/ml). To assess cell viability, MTT assays were performed after 24-48-72h of treatment. Untreated RPMI 8226 cells were used as controls.

HSE impaired RPMI 8226 cell viability in a dose and time dependent manner (Fig. 16) with an $IC_{50}$ at 24h of 20mg/ml.

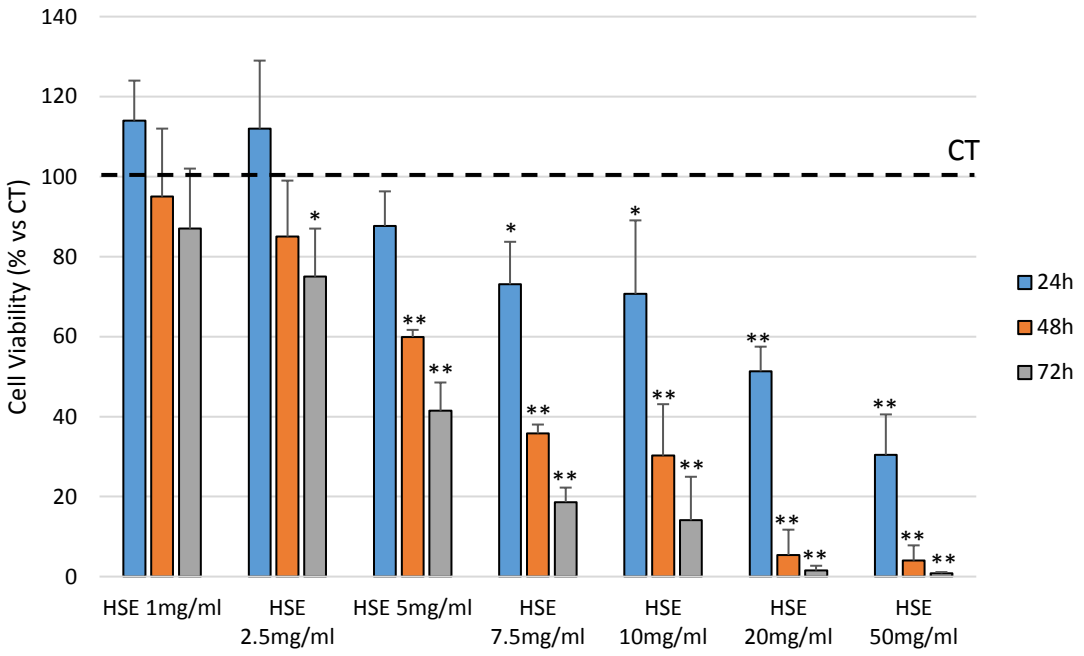



**Fig. 16 – RPMI 8226 cell viability after HSE treatment –** Graph represents the percentage of RPMI 8226 cell viability after exposure to different HSE concentrations. Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.2. Evaluation of RPMI 8226 cell viability after treatment with HSE fractions

Three different HSE fractions were obtained by liquid-liquid extraction using different solvent: water (HSEA), butanol (HSEB) and ethyl acetate (HSEC). All fractions were tested by MTT assay to evaluate RPMI 8226 cell viability. Cells were exposed to different concentration of HSEA (1-10mg/ml), HSEB (1-10mg/ml) or HSEC (1-10mg/ml) and MTT test was performed after 24, 48 and 72h of treatment. Untreated RPMI 8226 cells were used as controls.

HSEA and HSEB were not able to impair cell viability of RPMI 8226 cells. Only 10mg/ml, the highest concentration evaluated, reduced cell viability at 58 and 51% respectively, after 72h of treatment (Fig. 17). On the contrary, HSEC reduced RPMI 8226 cell viability in a dose and time dependent manner. Its effect was higher than HSE with an $IC_{50}$ of 2.5mg/ml after 24h of treatment (Fig. 17).

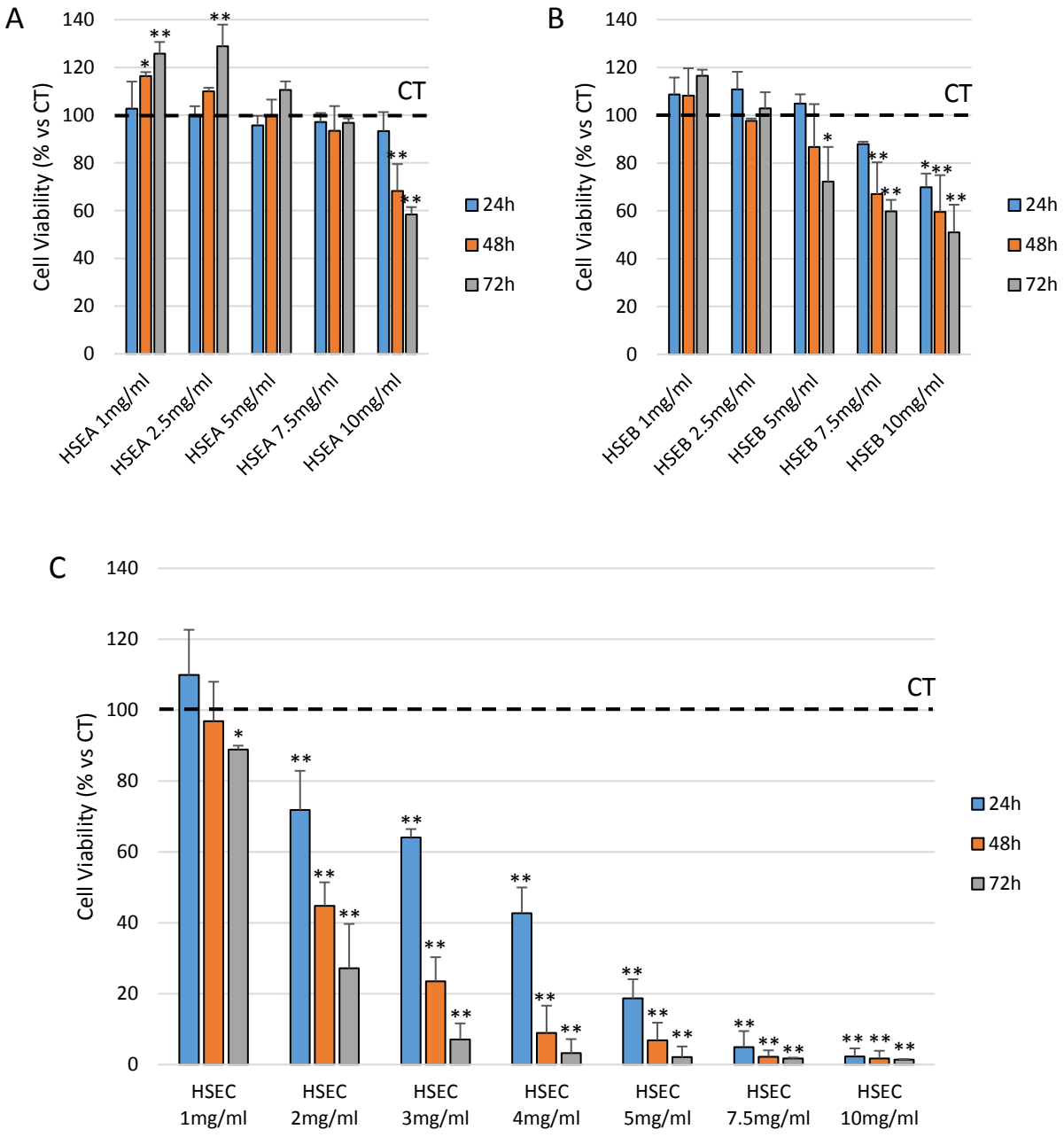
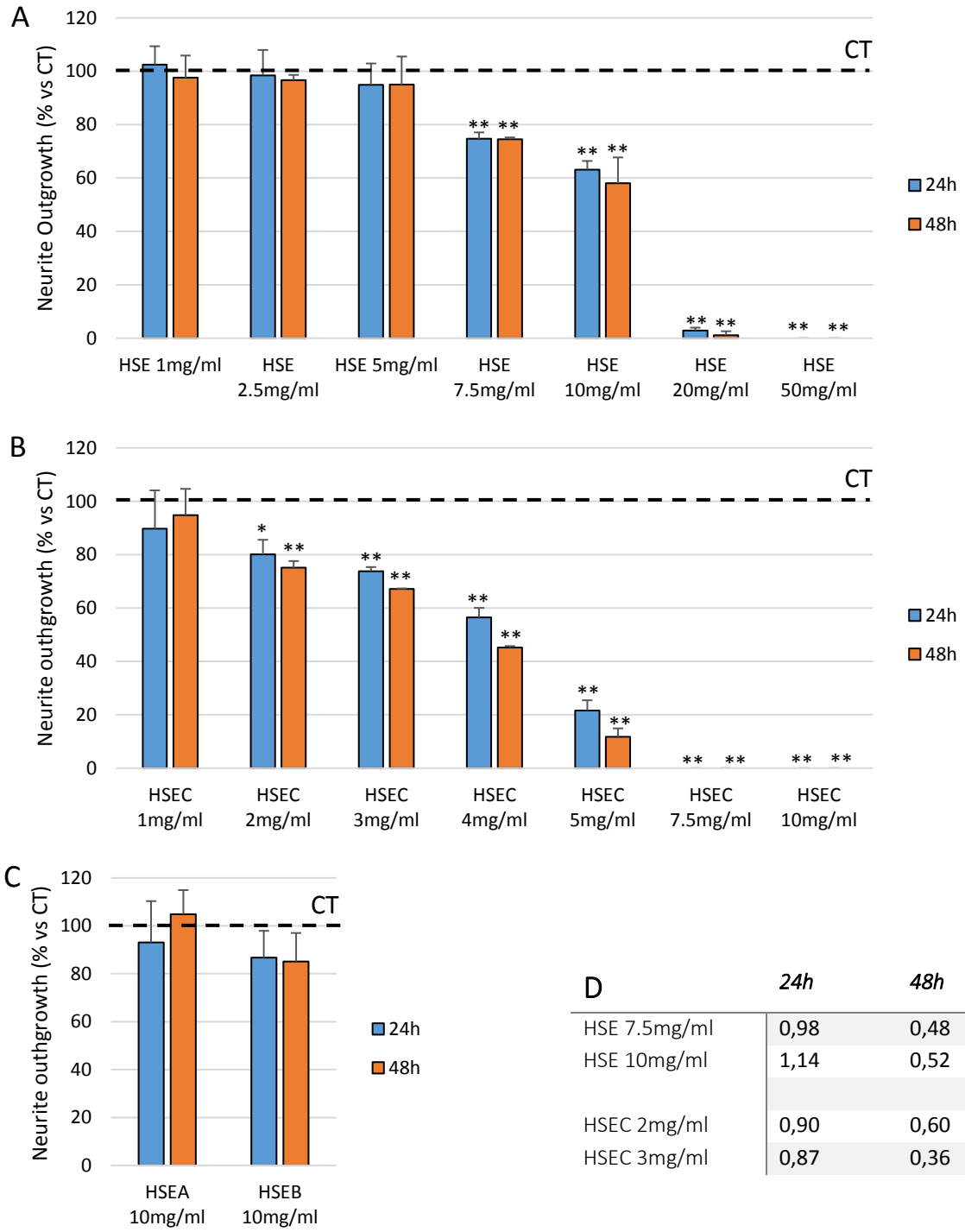



**Fig. 17 – RPMI 8226 cell viability after HSE fractions treatment –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to different concentrations of HSEA (A), HSEB (B) and HSEC (C). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.2. Evaluation of HSE, HSEA, HSEB and HSEC neurotoxicity *in vitro*

The most frequent side effect of several multiple myeloma drugs is peripheral neuropathy. For this reason, we assessed *in vitro* the neurotoxicity of HSE and its fractions using the embryo rat DRG model. In this model a compound is considered not neurotoxic when it allows neurite outgrowth of at least 50% of untreated controls.




D

| | *24h* | *48h* |
|---|---|---|
| HSE 7.5mg/ml | 0,98 | 0,48 |
| HSE 10mg/ml | 1,14 | 0,52 |
| | | |
| HSEC 2mg/ml | 0,90 | 0,60 |
| HSEC 3mg/ml | 0,87 | 0,36 |

**Fig. 18 – HSE, HSEA, HSEB and HSEC DRG neurotoxicity *in vitro* –** Graphs represent percentage of DRGs neurite outgrowth after treatment (24-48 h) with different concentrations of HSE (A), HSEC (B), HSEA and HSEB (C) compared to untreated DRGs (CT, 100%). (D) Table of Cell viability/Neurotoxicity ratio. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

HSE and HSEC resulted not neurotoxic *in vitro* until 10mg/ml and 3mg/ml concentration (Fig. 18). HSEA and HSEB were not neurotoxic at the highest concentration evaluated (Fig. 18).
In order to choose the HSE and HSEC concentrations to be used for subsequent experiments, we calculated the ratio between effect on cell viability and neurotoxicity (Fig. 18). HSE 7.5mg/ml and HSEC 3mg/ml had the best ratio and so they were chosen to perform further experiments.

### 3.1.3. Evaluation of cell viability by Trypan blue vital count assay after HSE and HSEC treatment

Trypan Blue vital count was performed to assess cell viability and mortality of RPMI 8226 cells after 24, 48 and 72h treatment with HSE 7.5mg/ml or HSEC 3mg/ml. Growth curves and cells doubling time were calculated on the basis of Trypan Blue results.
For RPMI 8226 cells treated with HSE 7.5mg/ml a time dependent reduction of cell viability was observed when compared to untreated control cells, confirming the results obtained by MTT assay (Fig. 19). In addition, HSE effect was mostly cytostatic, as demonstrated by growth curve, cell death percentage and the increase of cell doubling time (Fig. 20).
Cell viability data obtained after HSEC 3mg/ml treatment confirmed the MTT assay results. However, HSEC was more cytotoxic compared to HSE causing an increase in cell death (Fig. 19-20).

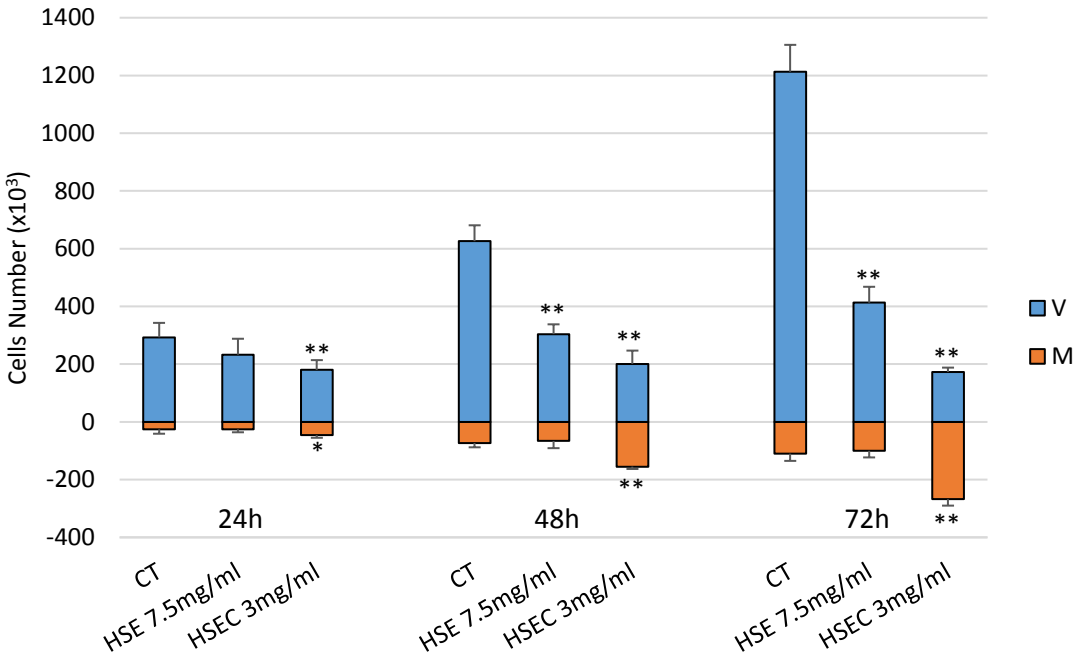
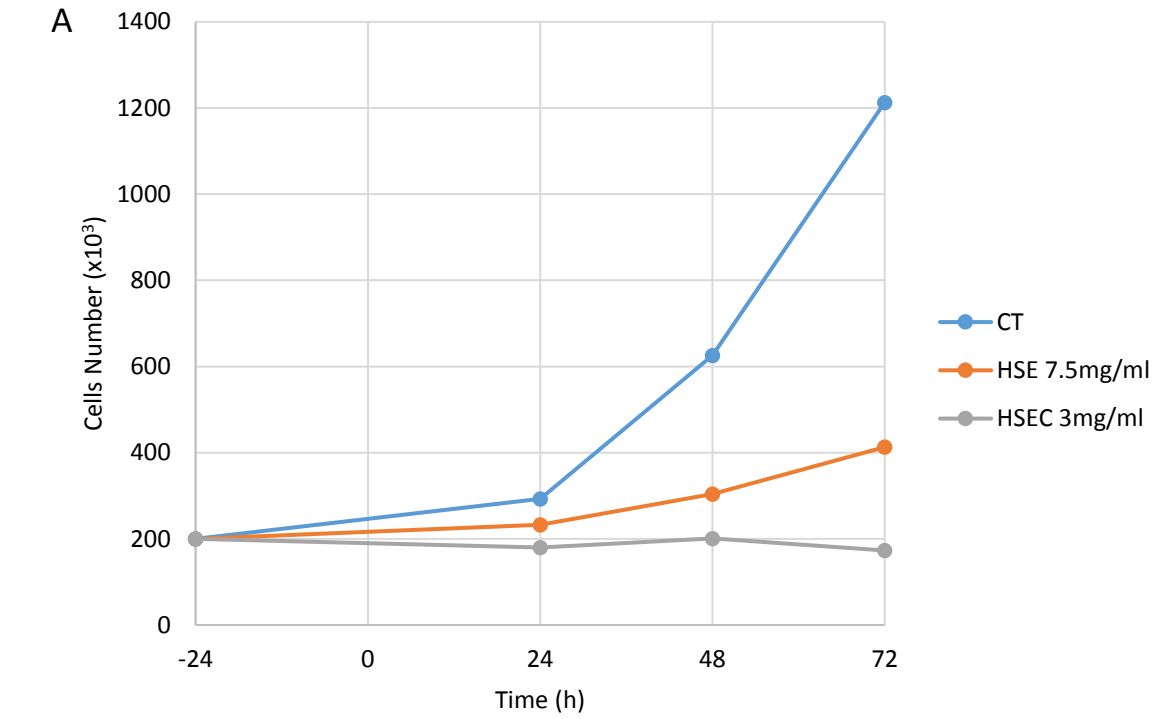
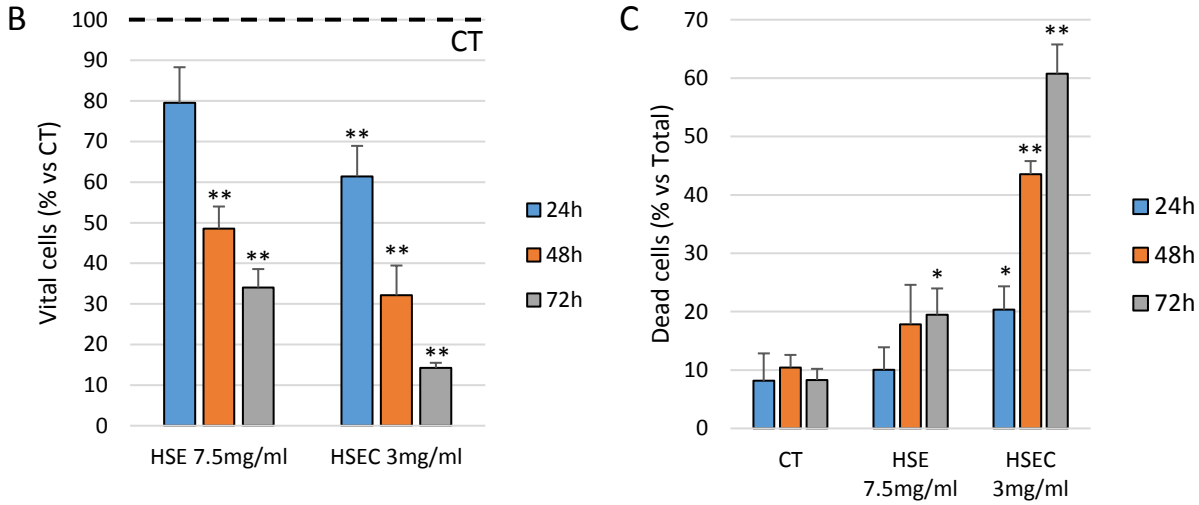



**Fig. 19 – RPMI 8226 trypan blue vital count –** (A) Graph represents the number of viable and death RPMI 8226 cells, untreated (CT) or treated with HSE 7.5mg/ml or HSEC 3mg/ml after 24, 48 and 72h. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.




D

| | *Doubling time (h)* |
|---|---|
| CT | **36.9** |
| HSE 7.5mg/ml | **91.8** |
| HSEC 3mg/ml | **n.c.** |

**Fig. 20 – RPMI 8226 trypan blue vital count –** (A) Growth curve of untreated (CT), HSE 7.5mg/ml or HSEC 3mg/ml treated RPMI 8226 cells. (B) Percentage of viable cells compared to control cells. (C) Percentage of death cells compared to total counted cells. (D) Doubling time of RPMI 8226 cells without treatment or with HSE 7.5mg/ml or HSEC 3mg/ml treatment. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.4. Evaluation of reversibility of HSE and HSEC

To assess the reversibility of HSE and HSEC effect on cell viability, RPMI 8226 cells were treated for 24h with HSE 7.5mg/ml or HSEC 3mg/ml (Fig. 21). Afterwards, treatment medium was replaced with fresh culture medium without HSE or HSEC. Untreated cells were used as controls and were subjected to the same medium change after 24h.

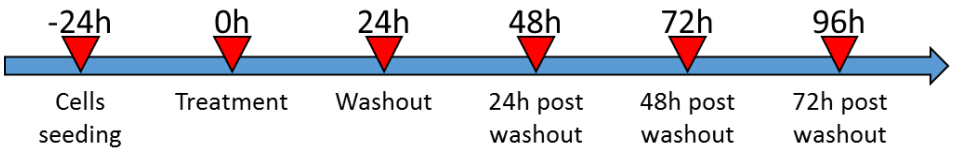



**Fig. 21 – Time schedule of Treatment and Washout**

HSE effect was reversible, because after washout RPMI 8226 cells restarted to grow in comparable manner to controls cells (Fig. 22-23-24). On the contrary, HSEC effect was irreversible: also after washout, cells continued to die (as observed in HSEC continuous treatment) (Fig. 22-23-24).

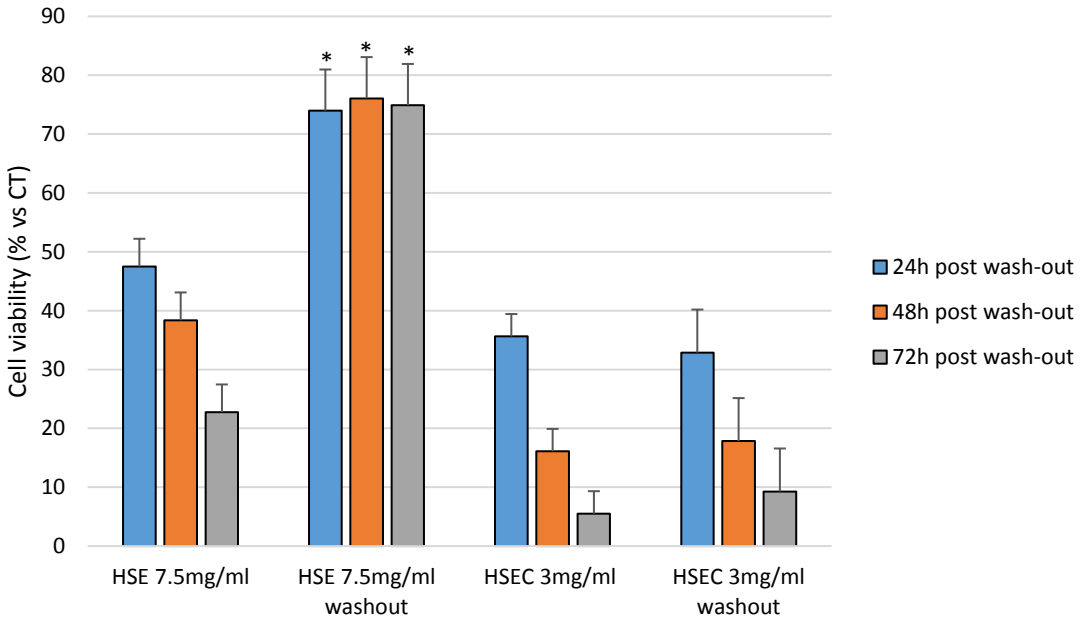



**Fig. 22 – HSE and HSEC reversibility on RPMI 8226 cell viability –** (E) Graph represents the percentage of viability of RPMI 8226 cells after washout of HSE and HSEC, compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSE 7.5mg/ml.

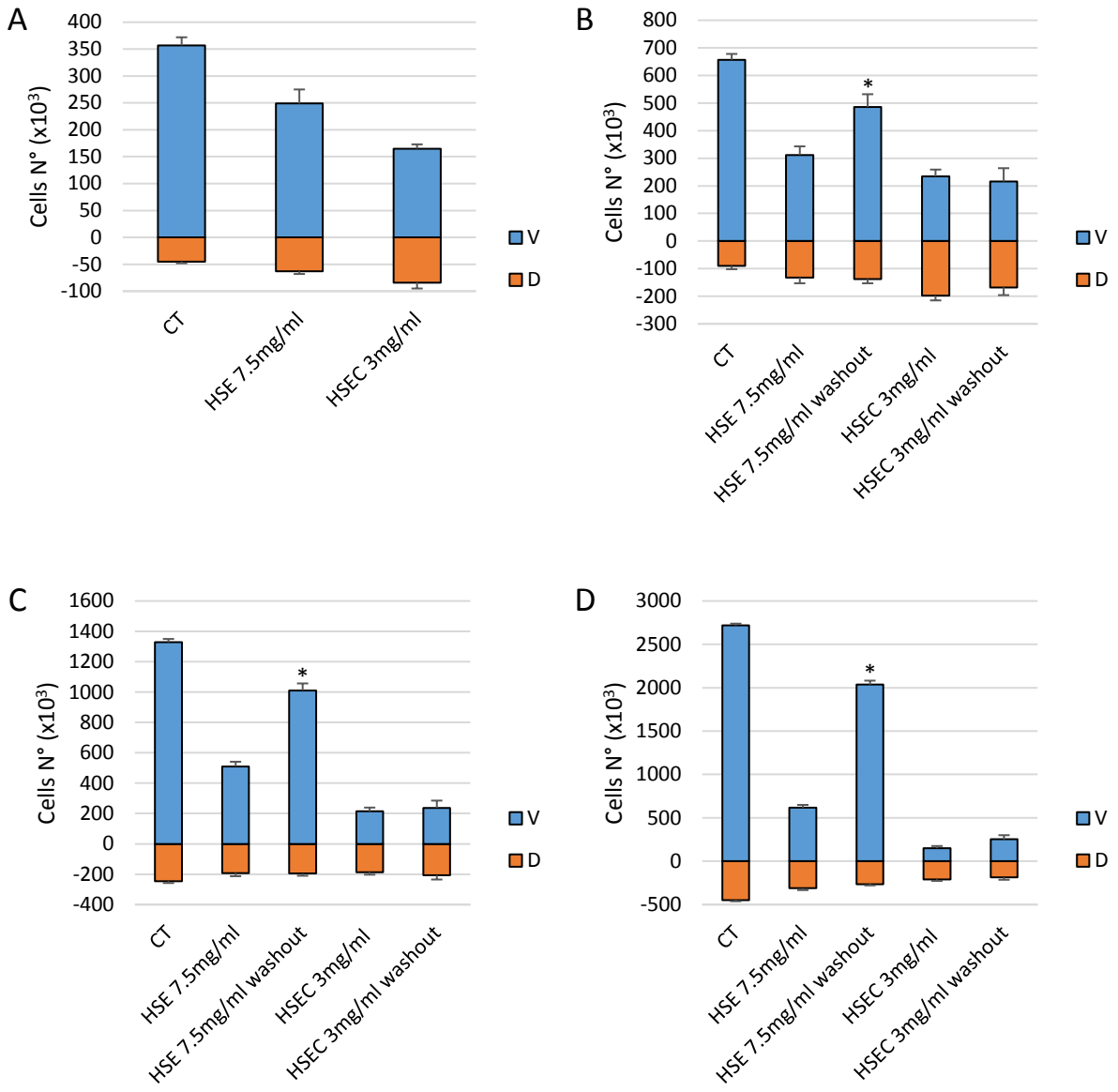



**Fig. 23 – HSE and HSEC reversibility on RPMI 8226 cell viability –** Graphs represent the number of viable and death cells counted after trypan blue staining at different time points: (A) after 24h of HSE or HSEC treatment, just before medium changing; (B) 24h after washout, (C) 48h after washout, (D) 72h after washout. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSE 7.5mg/ml.

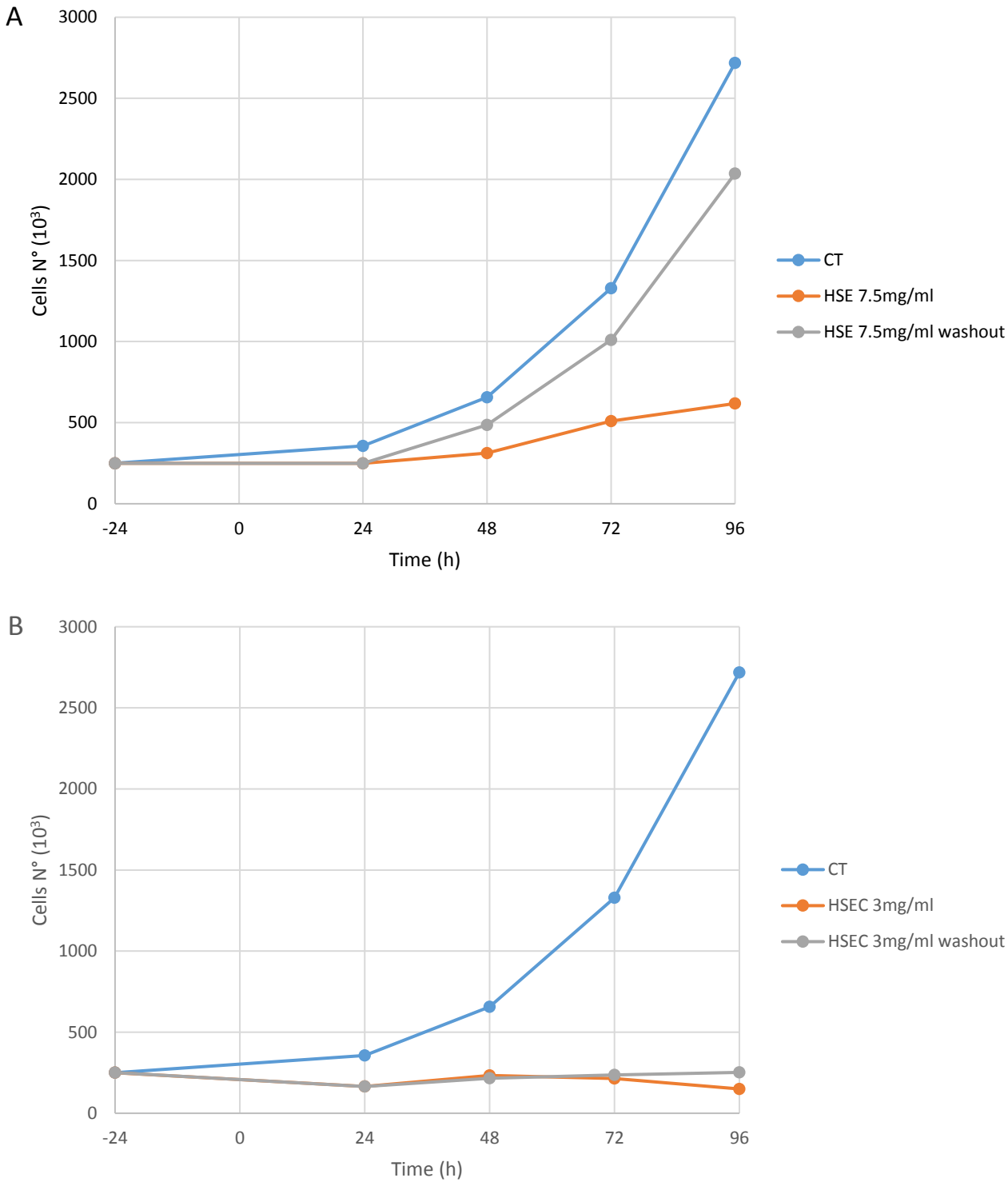



**Fig. 24 – HSE and HSEC reversibility on RPMI 8226 cell viability –** Growth curves of RPMI 8226 cells after (G) HSE and (H) HSEC treatment. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.5. Evaluation of apoptosis after HSE or HSEC treatment

Annexin V/PI double staining assay was performed to evaluate the number of RPMI 8226 cells undergoing apoptosis after HSE or HSEC treatment. RPMI 8226 cells, treated for 24, 48 and 72h with HSE 7.5mg/ml or HSEC 3mg/ml, were labeled with Annexin V/PI and analyzed by flow cytometry. Cells distribution in four groups were done based on Annexin V and PI staining: live (both Annexin V and PI negative), dead (Annexin V negative and PI positive), early apoptosis (Annexin V positive and PI negative) and late apoptosis (both Annexin V and PI positive) (Fig. 25). Apoptotic rate was calculated with the sum of Annexin V positive cells (early and late).

For all the analyzed times, few Annexin V positive cells were found in untreated (CT) and in HSE treated RPMI 8226 cells. On the contrary, in HSEC treated cells, it was observed a progressive increase of Annexin V positive cells, reaching an apoptosis rate of 59% after 72h of treatment (Fig. 25-26).

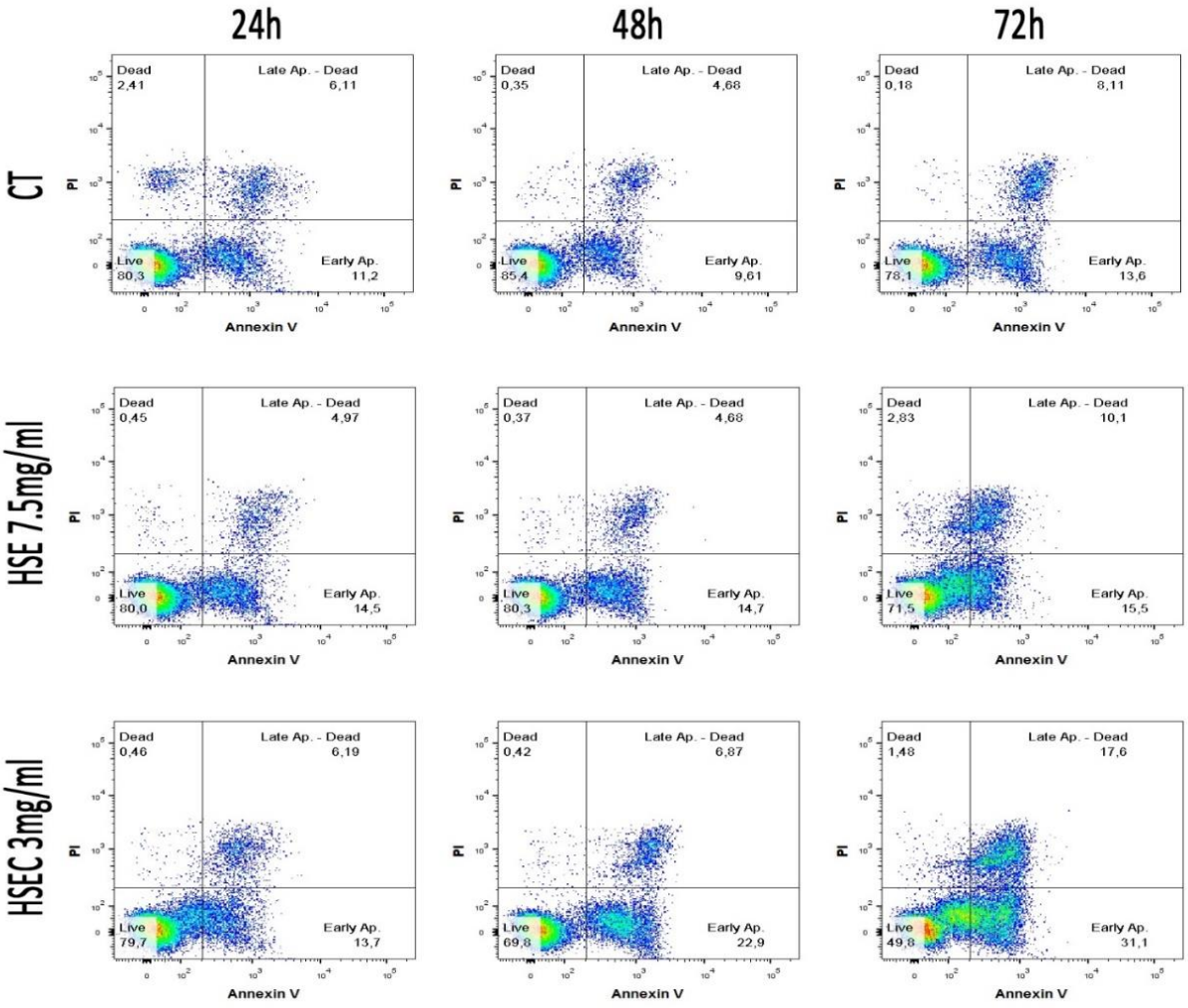



**Fig. 25 – RPMI 8226 Annexin V/IP staining –** Images represent the distribution of RPMI 8226 cells in four groups (live, dead, early apoptotic and late apoptotic) on the basis of Annexin V and PI staining;

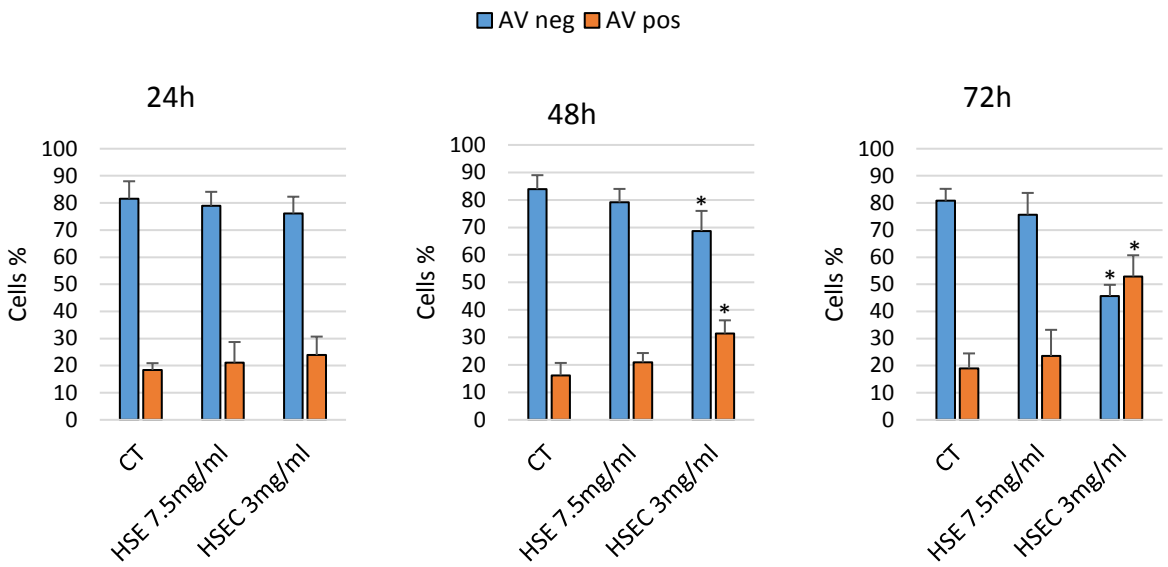



**Fig. 26 – RPMI 8226 Annexin V/IP staining –** Graphs represent the apoptotic rate of RPMI 8226 cells, untreated or treated with HSE 7.5mg/ml or HSEC 3mg/ml, showing negative and positive Annexin V cells. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs CT.

### 3.1.6. Evaluation of Caspase 3 activation after HSE or HSEC treatment

Sequential activation of Caspases (by proteolytic cleavage) plays a central role in the execution-phase of apoptosis. Western blot analysis and quantification of Caspase 3 activation were performed to evaluate of apoptosis. RPMI 8226 cells were treated for 24, 48 and 72h with HSE 7.5mg/ml or HSEC 3mg/ml. Untreated RPMI 8226 cells were used as control (CT).

Untreated RPMI 8226 cells and HSE treated cells had a comparable, percentage of activated Caspase 3 for all the examined times. On the contrary, HSEC induced a significant activation of Caspase 3 since 24h of treatment and was maintained for all the observed times (Fig. 27).

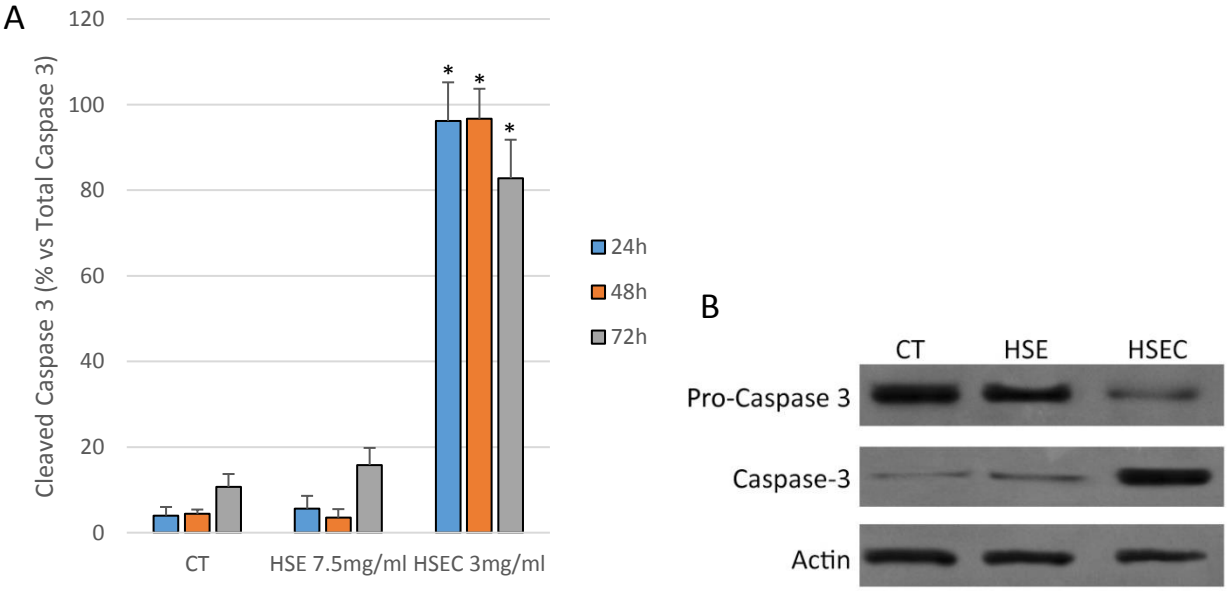



**Fig. 27 – RPMI 8226 Caspase 3 western blot –** (A) Graph represents the percentage of cleaved caspase 3 at 72h compared to total caspase 3 obtained by quantification of western blot. (B) Western blot (image representative) of total and cleaved Caspase 3 in untreated or treated with HSE 7.5mg/ml or HSEC 3mg/ml RPMI 8226 cells. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.7. Evaluation of proteasome activity after HSE or HSEC treatment

Proteasome activity was assessed in RPMI 8226 cells after different time points (30min, 1,2,4,6,16,24,48,72h). RPMI 8226 cells were treated with HSE 7.5mg/ml or HSEC 3mg/ml and untreated cells were used as controls (CT).

Both HSE and HSEC were able to significantly impair proteasome activity of RPMI 8226 cells in a time dependent manner and HSEC was more effective than HSE. Maximum effect was obtained after 24h of treatment and was maintained up to 72h. Residual proteasome activity was 38 and 25% for HSE or HSEC respectively (Fig. 28).

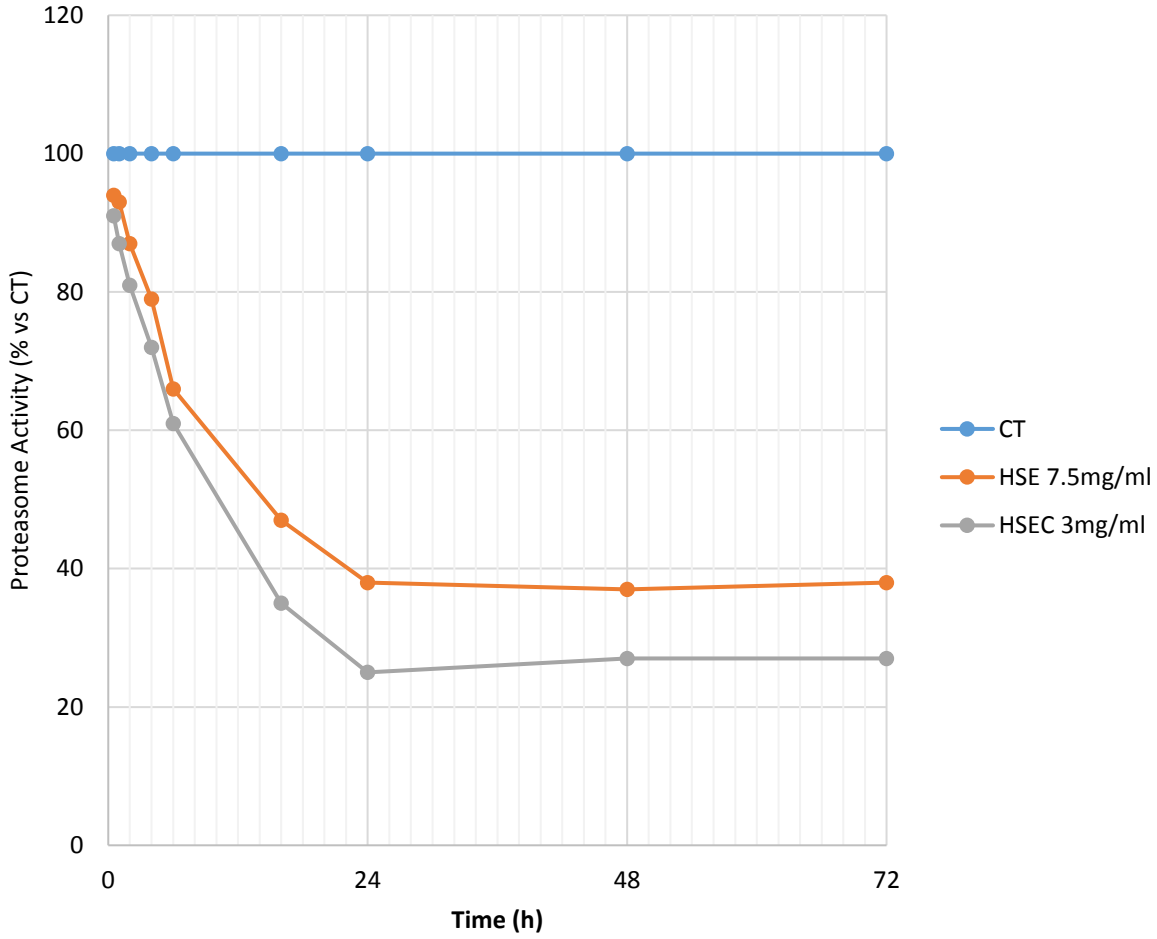



**Fig. 28 – RPMI 8226 Proteasome activity –** Graph represents the percentage of proteasome activity in RPMI 8226 cells after 30min, 1, 2, 4, 6, 16, 24, 48 and 72h of HSE 7.5mg/ml or HSEC 3mg/ml treatment, compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments.

### 3.1.8. Evaluation of autophagy after HSE or HSEC treatment

Autophagy process alterations induced by HSE or HSEC in RPMI 8226 cells were assessed by acridine orange staining and western blot analysis of Beclin1 and LC3.

RPMI 8226 cells were treated with HSE 7.5mg/ml or HSEC 3mg/ml. After 24h of treatment, cells were collected, AO stained and analyzed by FACS or fluorescence microscopy. Untreated cells were used as controls (CT).

As shown in Fig. 28, both HSE and HSEC induced a significant reduction in acidic vesicular organelles (AVOs) positive cells, indicating an impairment of autophagy process. RPMI 8226 control cells had a basal autophagy activity and 60% of cells resulted positive to AVOs. HSE and HSEC treatment reduced AVOs positive cells to 4 and 3% respectively (Fig. 29).

Autophagy impairment was also confirmed by western blot analysis of Beclin-1 and LC3. Beclin-1 has a central role in autophagy because it regulates the localization of autophagic proteins in the early phases of autophagosomes formation. LC3 is a transmembrane protein localized in the membrane of autophagosomes. Its conversion from LC3A to LC3B isoform indicated the progress of autophagic process. Both HSE and HSEC reduced the expression of Beclin-1 and both isoforms of LC3 (A and B) in RPMI 8226 cells after 24h of treatment (Fig. 30). Simultaneous impairment of LC3 A and B indicates a defection in autophagy regulation pathways (Mizushima and Yoshimori, 2007).

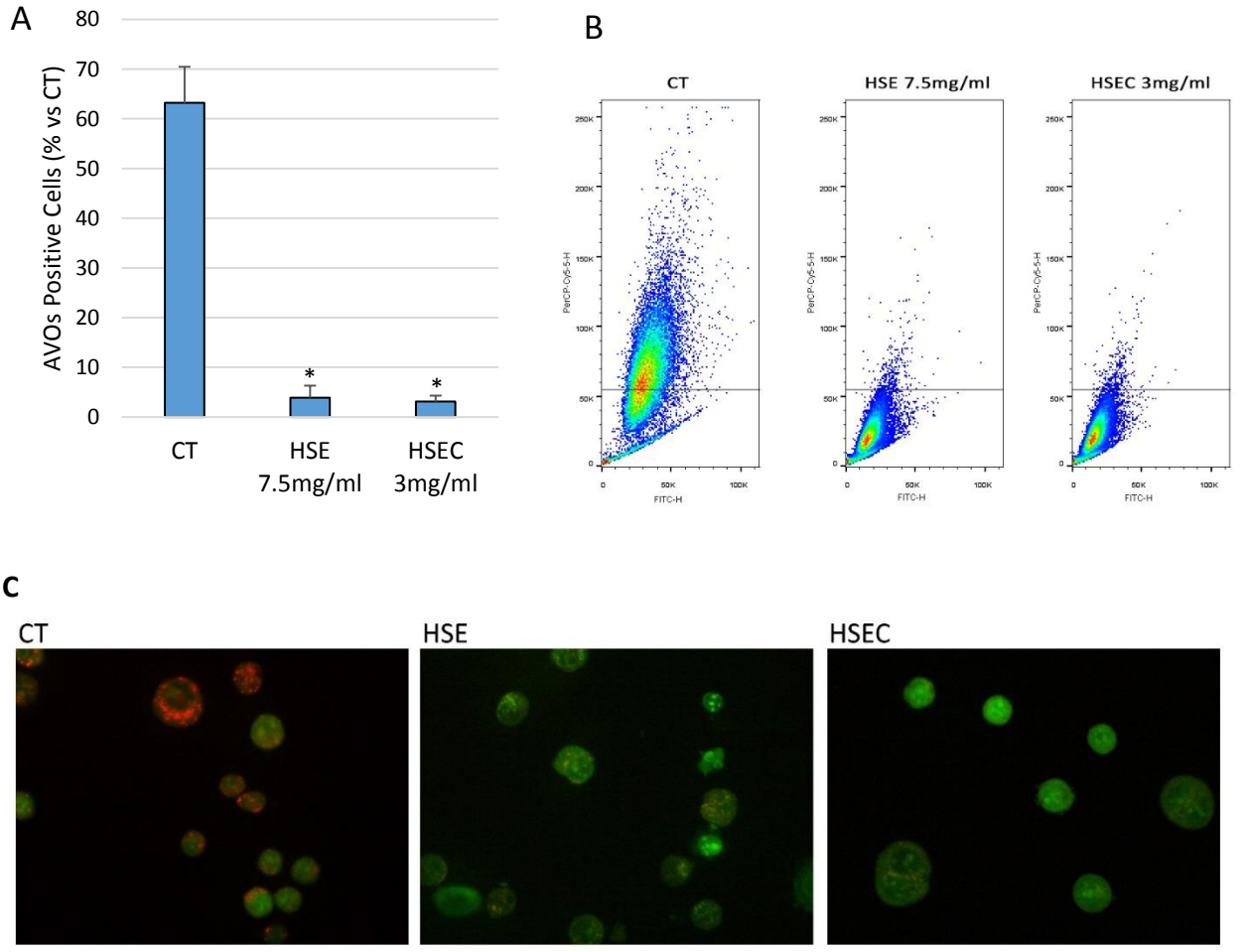



**Fig. 29 – AO staining of RPMI 8226 cells after HSE or HSEC treatment -** (A) Graph represent the percentage of AVOs positive cells, untreated (CT) or treated for 24h with HSE 7.5mg/ml or HSEC 3mg/ml. (B) Image representative of FACS analysis of RPMI 8226 cells after AO staining. (C) Fluorescence microscope images obtained after 24h of treatment with HSE 7.5mh/ml or HSEC 3mg/ml.

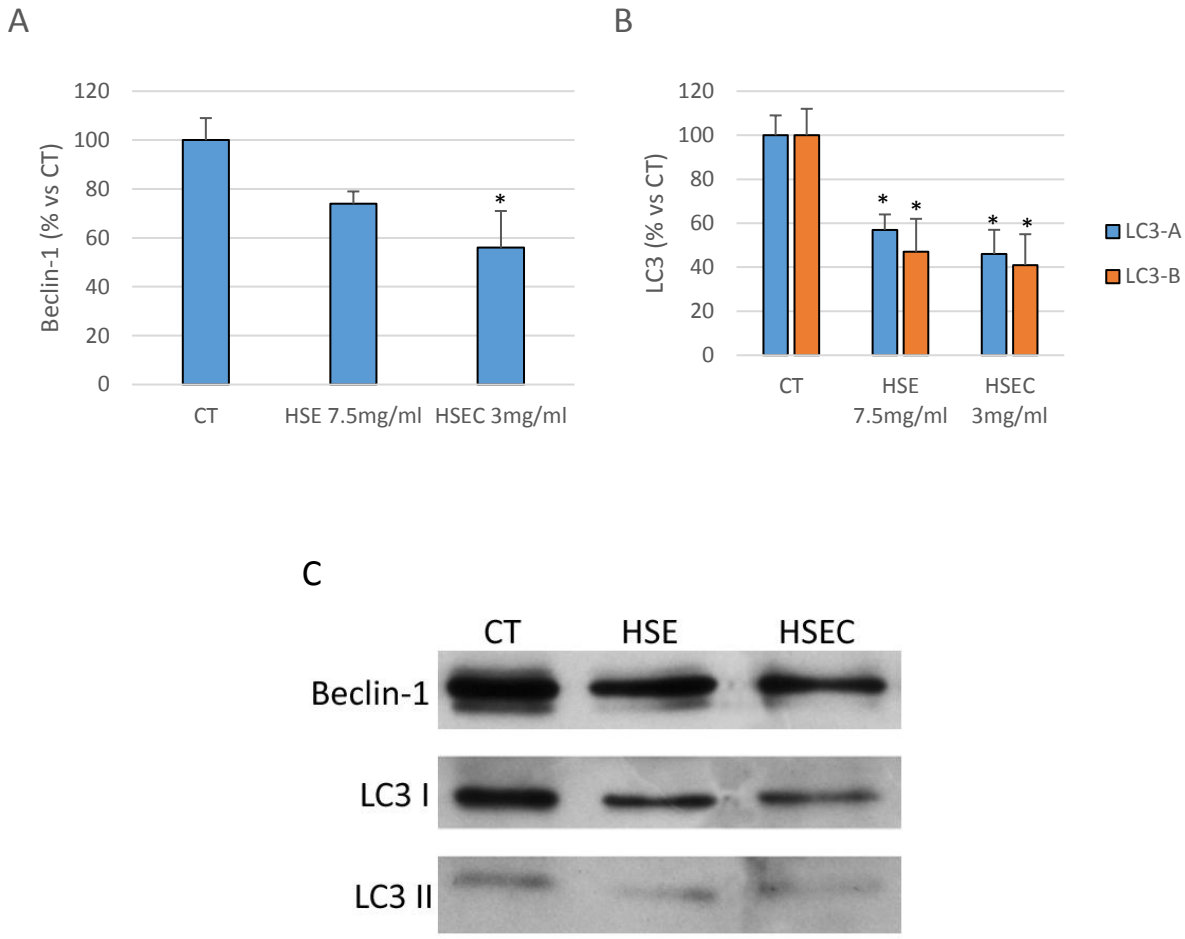



**Fig. 30 – Western blot analysis of Beclin-1 and LC3 expression in RPMI 8226 cells treated with HSE or HSEC –** Images representative of Beclin1 (C) and LC3 (D) WB in RPMI 8226 cells treated with HSE 7.5mg/ml and HSEC 3mg/ml. Graphs represents the percentage of Beclin1 (A) and LC3 (B) levels in RPMI 8226 cells treated with HSE and HSEC. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.9. Evaluation of cell migration and invasion after HSE and HSEC treatment

The ability of HSE and HSEC to affect migration/invasion of RPMI 8226 cells was assessed by Boyden chamber assay. RPMI 8226 cells were treated with HSE 7.5mg/ml or HSEC 3mg/ml and after 24h, cells that have passed through a gelatin coated membrane were counted. FBS was used as cell chemoattractant in all experimental conditions, except in negative controls where FBS were not used.

As shown in Fig. 31, untreated RPMI 8226 cells, used as positive controls, passed through the gelatin coated membrane attracted by FBS, while only few cells of negative control crossed the membrane without FBS stimulus. Both HSE and HSEC reduced the migration/invasion capacity of RPMI 8226 cells. Cell migration/invasion was reduced by 71% and 75% respectively, compared to positive controls.

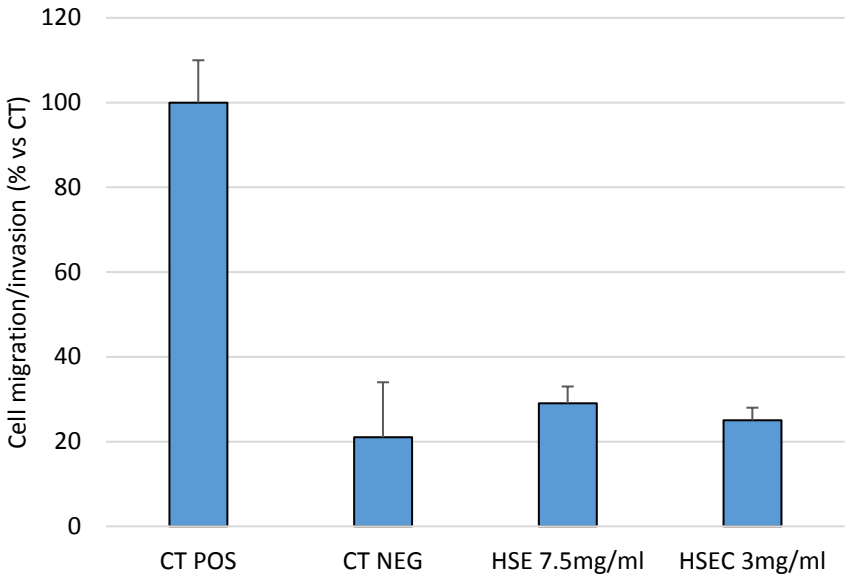



**Fig. 31 – RPMI 8226 Boyden chamber assay –** Graph represents the percentage of RPMI 8226 cells that crossed the gelatin coated membrane after 24h treatment with HSE 7.5mg/ml or HSEC 3mg/ml. Positive control (CT POS) is represented by untreated cells and the number of cells hat crossed the membrane is set to 100%. While negative (CT NEG) control refers to cells that crossed the membrane even in absence of a chemotactic stimulus. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### 3.1.10 Evaluation of MAPKs phosphorylation after HSE and HSEC treatment

WB analysis was performed to evaluate modulation of MAPKs ERK1, ERK2 and p38 phosphorylation after RPMI 8226 HSE or HSEC treatment. MAPKs are kinases involved in many cancer cell process, such as proliferation, gene expression, cell survival and apoptosis (Zhang and Liu, 2002). Moreover, their involvement in natural compound effect on cancer cells has been already demonstrated (Lin *et al.*, 2005).

We analyzed both MAPK activation (phosphorylation) and expression (total amount). RPMI 8226 cells were treated with HSE 7.5mg/ml or HSEC 3mg/ml for 30m, 1, 2, 4, 6, 16, 24 and 48h. At each time point total proteins were extracted, analyzed by WB and compared to phosphorylation levels of untreated cells after quantification.

In RPMI 8226 cells, after HSE or HSEC treatment, increase of ERK1 and ERK2 phosphorylation occurred in late time (6h) and was persistent until 48 h after HSE or HSEC treatment. The effect of both compounds was more effective in enhancing ERK 1 phosphorylation (Fig. 32).

HSE and HSEC induced early increase of p38 phosphorylation (30 m) but after 4 and 6h respectively, phosphorylation decreased at control cell level (Fig. 33).

Total amount of ERK 1, ERK 2 and p38 did not change at all examined times, in presence or not of HSE and HSEC (data not shown).

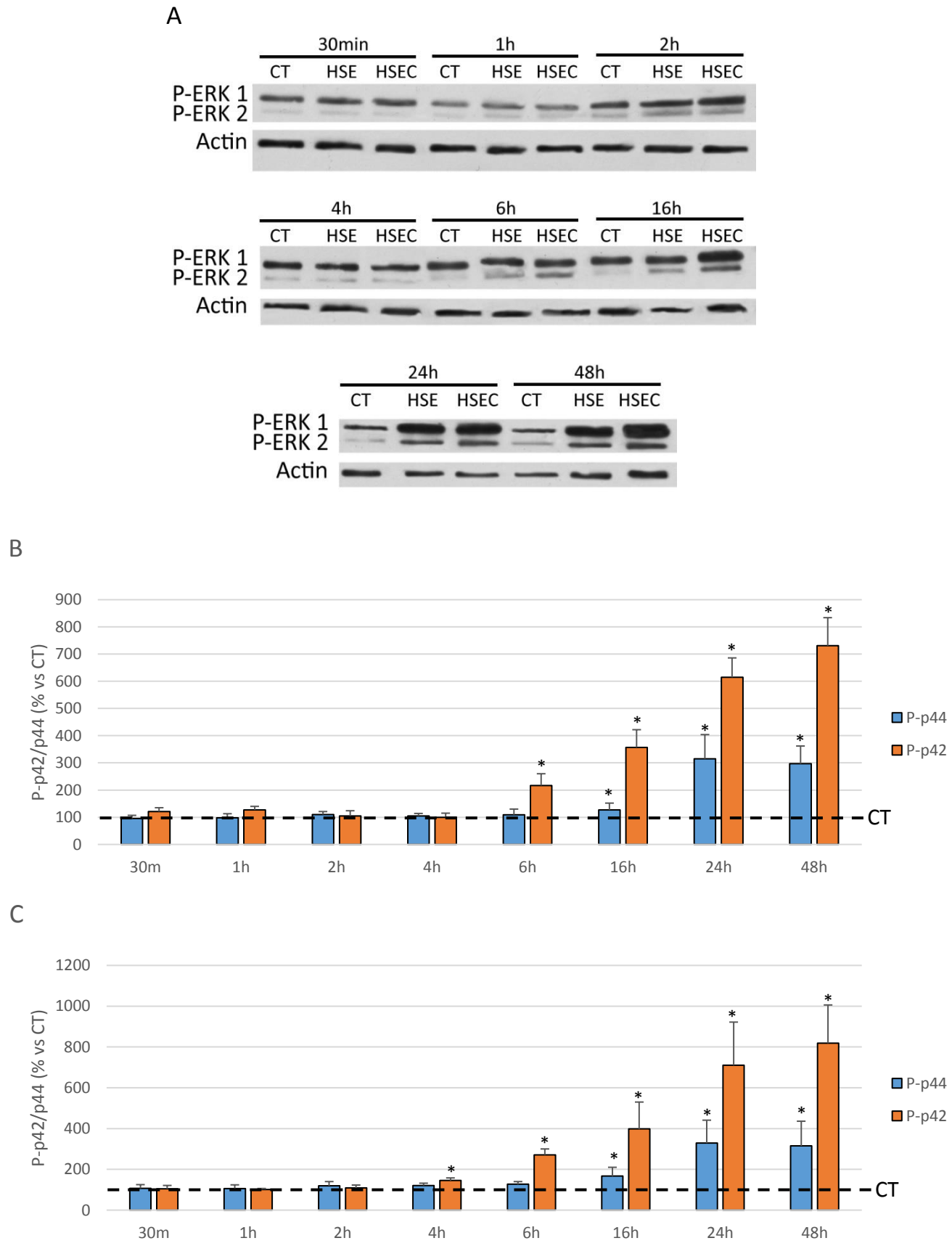



**Fig. 32 – ERK 1/2 phosphorylation levels after HSE and HSEC treatment –** Images representative of P-ERK 1/2 WB in RPMI 8226 cells treated with HSE 7.5mg/ml and HSEC 3mg/ml (A). Graphs represents the percentage of P-ERK 1/2 levels in RPMI 8226 cells treated with HSE (B) and HSEC (C). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

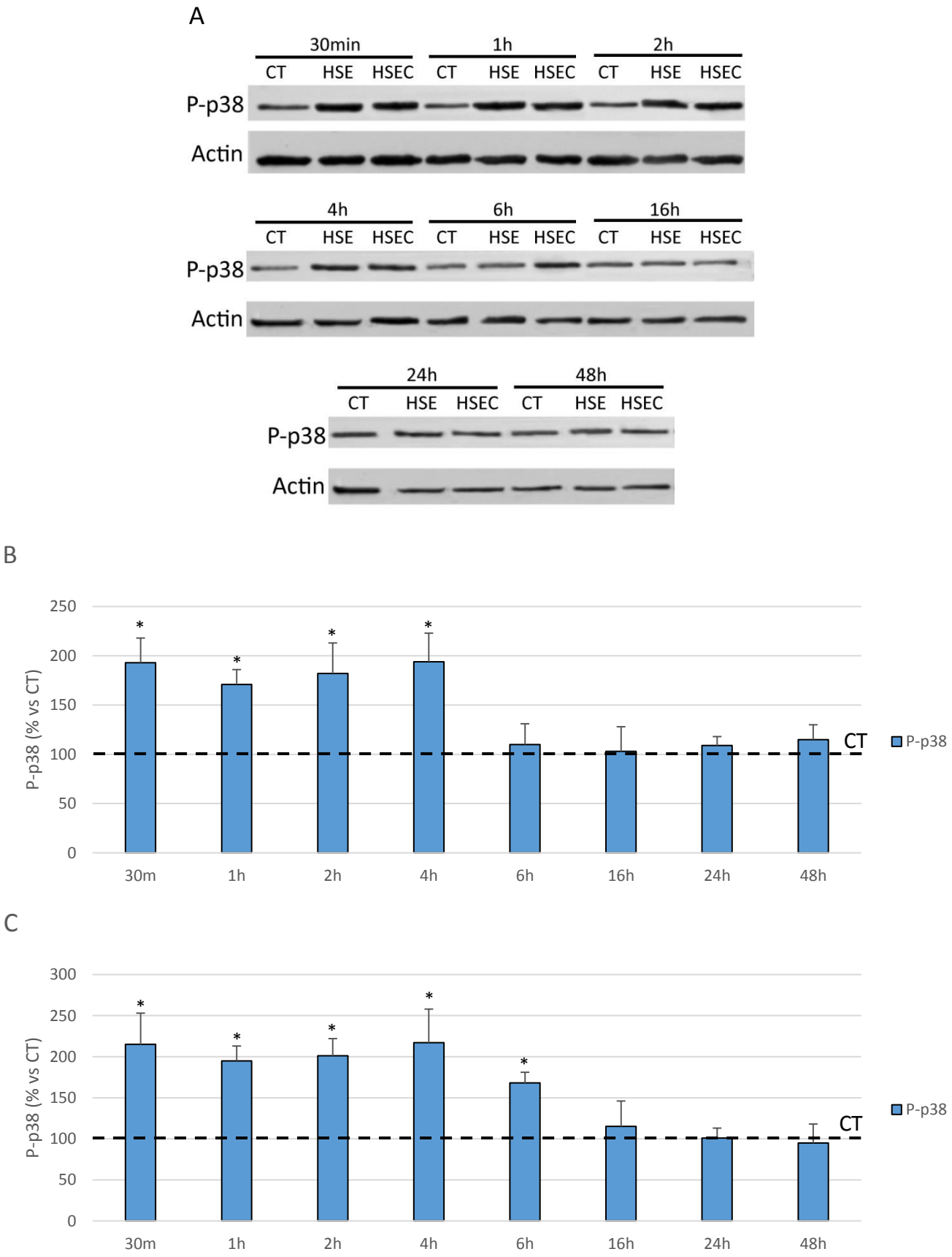



**Fig. 33 – p38 phosphorylation levels after HSE and HSEC treatment –** Images representative of P-p38 WB in RPMI 8226 cells treated with HSE 7.5mg/ml and HSEC 3mg/ml (A). Graphs represents the percentage of P-p38 levels in RPMI 8226 cells treated with HSE (B) and HSEC (C). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

## 3.2. Phase II: Evaluation of BTZ and HS combination

In the second phase of my PhD thesis I have evaluated in RPMI 8226 cells the effect of combination of BTZ with HSE or HSEC by using different concentrations and treatment times. BTZ is the most used and probably the most effective drug for multiple myeloma treatment. Despite this, BTZ has several important side effects, among which peripheral neuropathy is the most dose limiting. For these reason, it is important to research new therapeutic approaches, with new molecules or with combinations of already available drugs.

### 3.2.1. Simultaneous treatment

RPMI 8226 cells were treated simultaneously with HSE 7.5mg/ml or HSEC 3mg/ml and different concentrations of BTZ (1, 5 and 10nM) (Fig. 34). Untreated cells were used as controls and set to 100%, while cells treated with single compounds were used to assess the increased efficacy of simultaneous combination treatment. After 24, 48 and 72h an MTT assay was performed to evaluate RPMI 8226 cell viability.

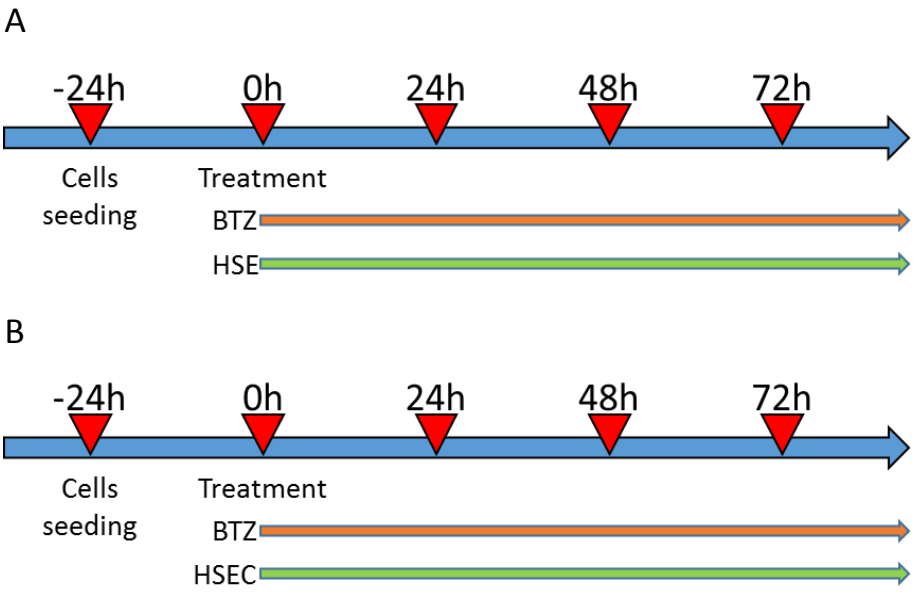



**Fig. 34 – Time schedule of RPMI 8226 simultaneous treatment –** Figures represent the simultaneous combination of BTZ with HSE (A) or HSEC (B) on RPMI 8226 cells.

As shown in the graphs of Fig. 35, simultaneous combination of BTZ with HSE or HSEC had not a synergistic or increased effect against RPMI 8226 cells.
BTZ 1nM was not effective in reducing cell viability at any considered time. Its simultaneous combination with HSE 7.5mg/ml or HSEC 3mg/ml reduced cell viability at a percentage at most comparable with the one obtained by HSE or HSEC single treatment.

BTZ 5nM and 10nM were significantly effective on RPMI 8226 cell viability. Both BTZ concentrations had a higher effect than HSE 7.5mg/ml and HSEC 3mg/ml. Their simultaneous combinations reduced the effectiveness of BTZ treatment and results were, as observed for BTZ 1nM, at most comparable with HSE or HSEC single treatment.

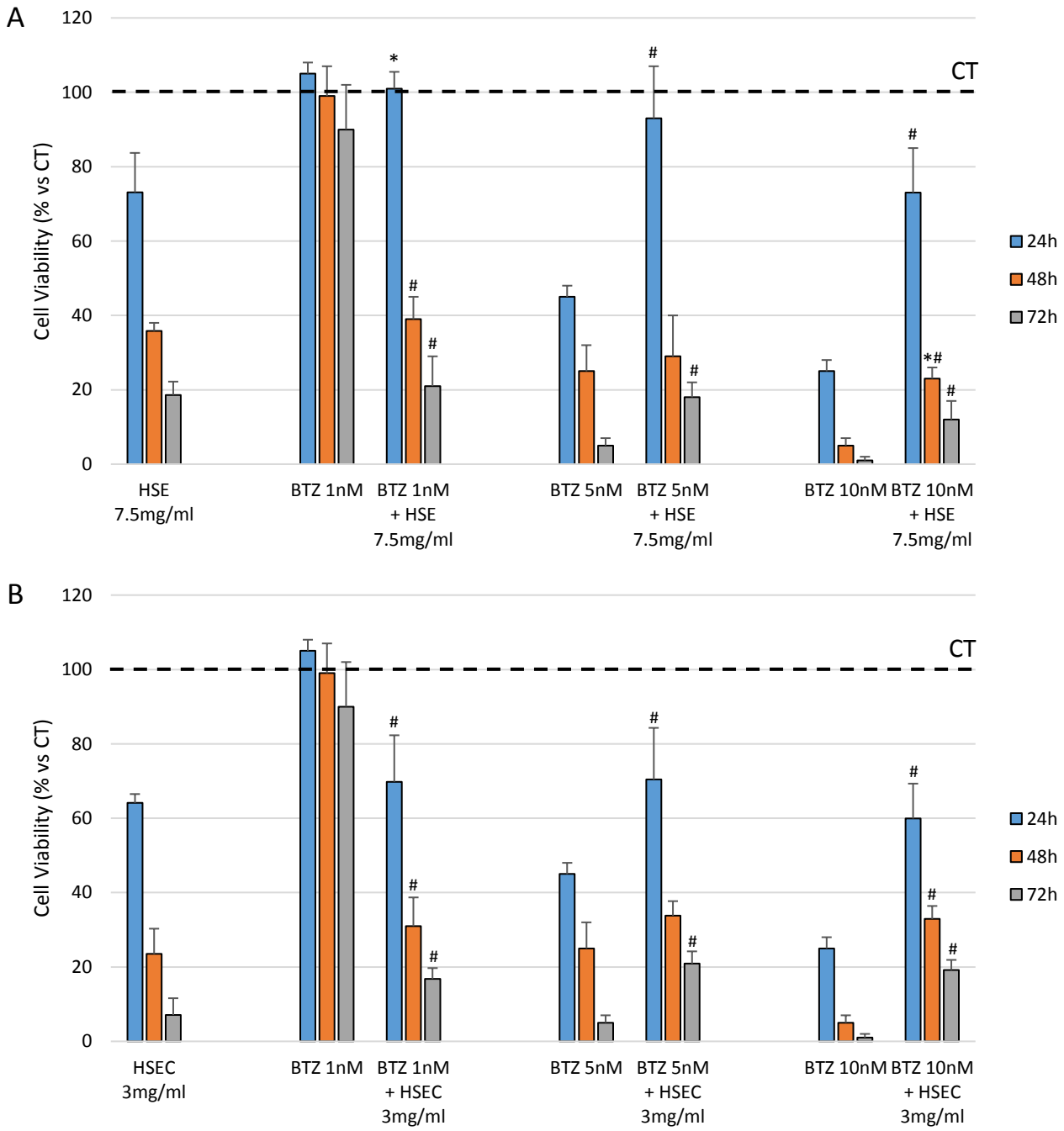



**Fig. 35 - RPMI 8226 cell viability after simultaneous treatment with BTZ and HSE or HSEC –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to simultaneous combinations between different concentrations of BTZ (1, 5 and 10nM) and HSE 7.5mg/ml (A) or HSEC 3mg/ml (B). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSE, # P<0.05 vs respective BTZ concentration.

In previous results, a significant reduction in the effectiveness of BTZ was observed when it was used in combination with HSE or HSEC. To determine whether this reduction was due to simultaneous presence of the two compounds, RPMI 8226 cells were treated with BTZ 10nM and increasing concentration of HSE (1-10mg/ml) or HSEC (1-5mg/ml). After 24, 48 and 72h cells were subjected to MTT assay. The effect of BTZ against RPMI 8226 cells was reduced in a dose dependent manner both from HSE and HSEC (Fig. 36).

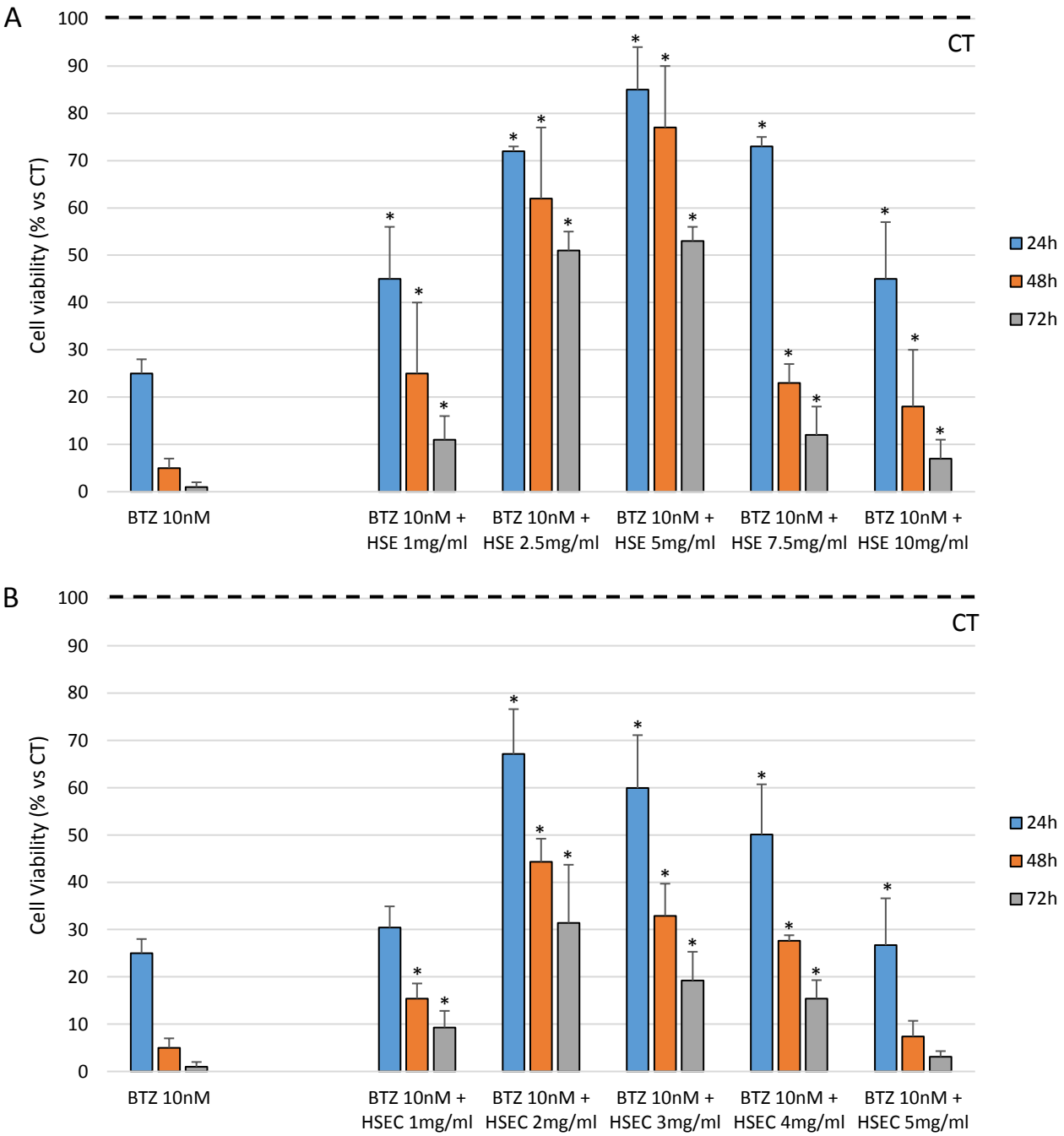



**Fig. 36 – RPMI 8226 cell viability after simultaneous treatment with BTZ 10nM and HSE or HSEC increasing concentrations –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to simultaneous combinations between BTZ 10nM and increasing concentrations of HSE (1-10mg/ml) (A) or HSEC (1-5mg/ml) (B). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs BTZ 10nM.

### 3.2.2. Not-Simultaneous treatment

RPMI 8226 cells were treated for 24h with HSE 7.5mg/ml or HSEC 3mg/ml and subsequently with different concentrations of BTZ (1, 5 and 10nM) for further 24 and 48h (not-simultaneous treatment) (Fig. 37). Alternatively, cells were treated first with BTZ and after 24h with HSE or HSEC (Fig. 37). In both types of combination, the first compound was kept in culture medium until the end of the treatment. At each time point a MTT assay was performed to assess RPMI 8226 cell viability. Untreated RPMI 8226 cells were used as control and were set to 100%, while cells treated with single compounds were used to evaluate the effect of combination treatments.

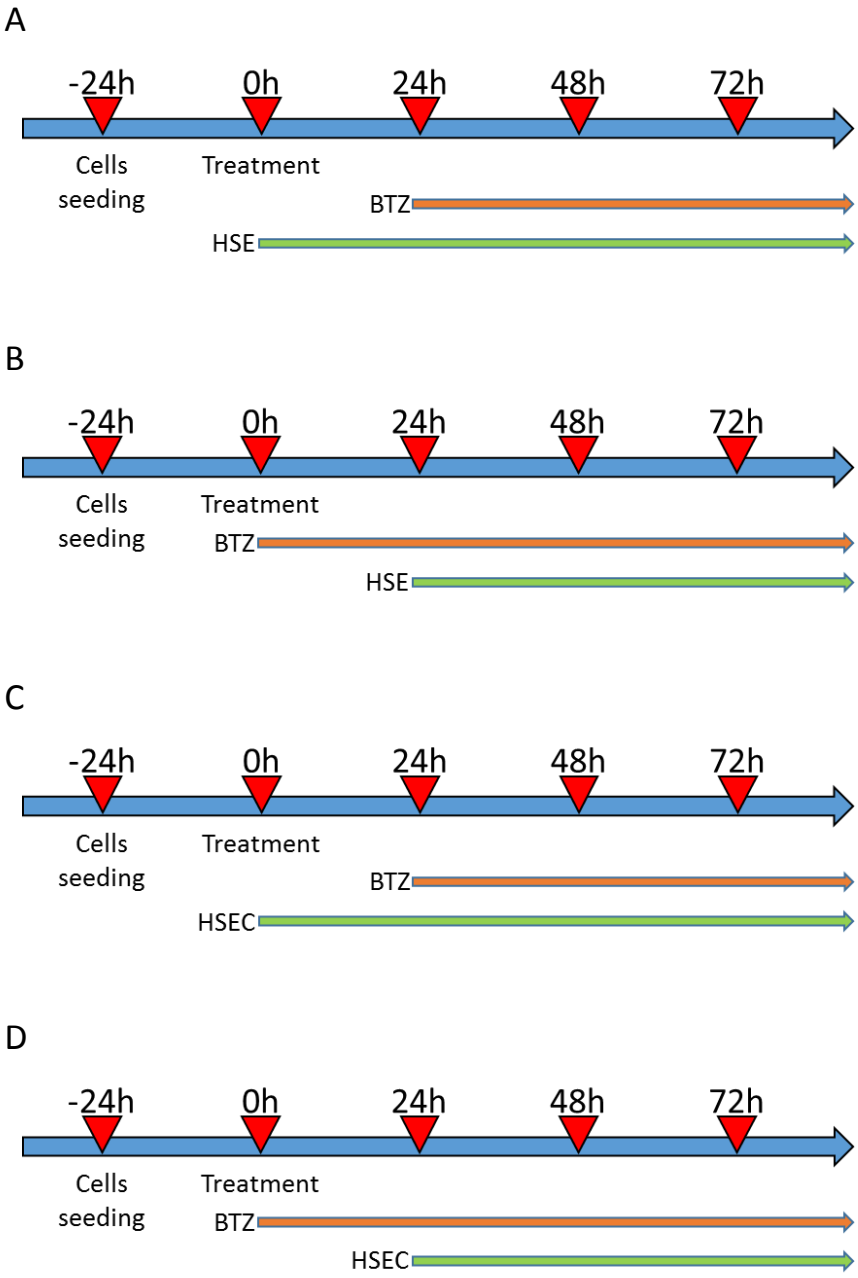



**Fig. 37 – Time schedule of RPMI 8226 not-simultaneous treatment -** Figures represent the not-simultaneous treatment of RPMI 8226 cells with BTZ and HSE or HSEC. Cells were treated with HSE 7.5mg/ml (A) or HSEC 3mg/ml (C) and after 24h with different concentrations of BTZ (1, 5 and 10nM). Alternatively, cells were treated with BTZ (1, 5 and 10nM) and after 24h with HSE 7.5mg/ml (B) or HSEC 3mg/ml (D).

In this type of combination, we did not observe a synergic effect on RPMI 8226 cell viability between BTZ and HSE or HSEC, compared to single compound treatments.
RPMI 8226 cells, treated for 24h with BTZ 1nM and then with HSE or HSEC, had a cell viability lower than BTZ 1nM only treatment, but it was higher than HSE or HSEC only treatment.
Considering RPMI 8226 cells treated for 24h with BTZ 5nM or 10nM and then with HSE or HSEC, cell viability was at most comparable with that obtained after BTZ only respective treatments.
On the contrary, when cells were treated for 24h with HSE or HSEC and then with any concentration of BTZ, results were completely comparable to HSE or HSEC only treatments (Fig 38-39).

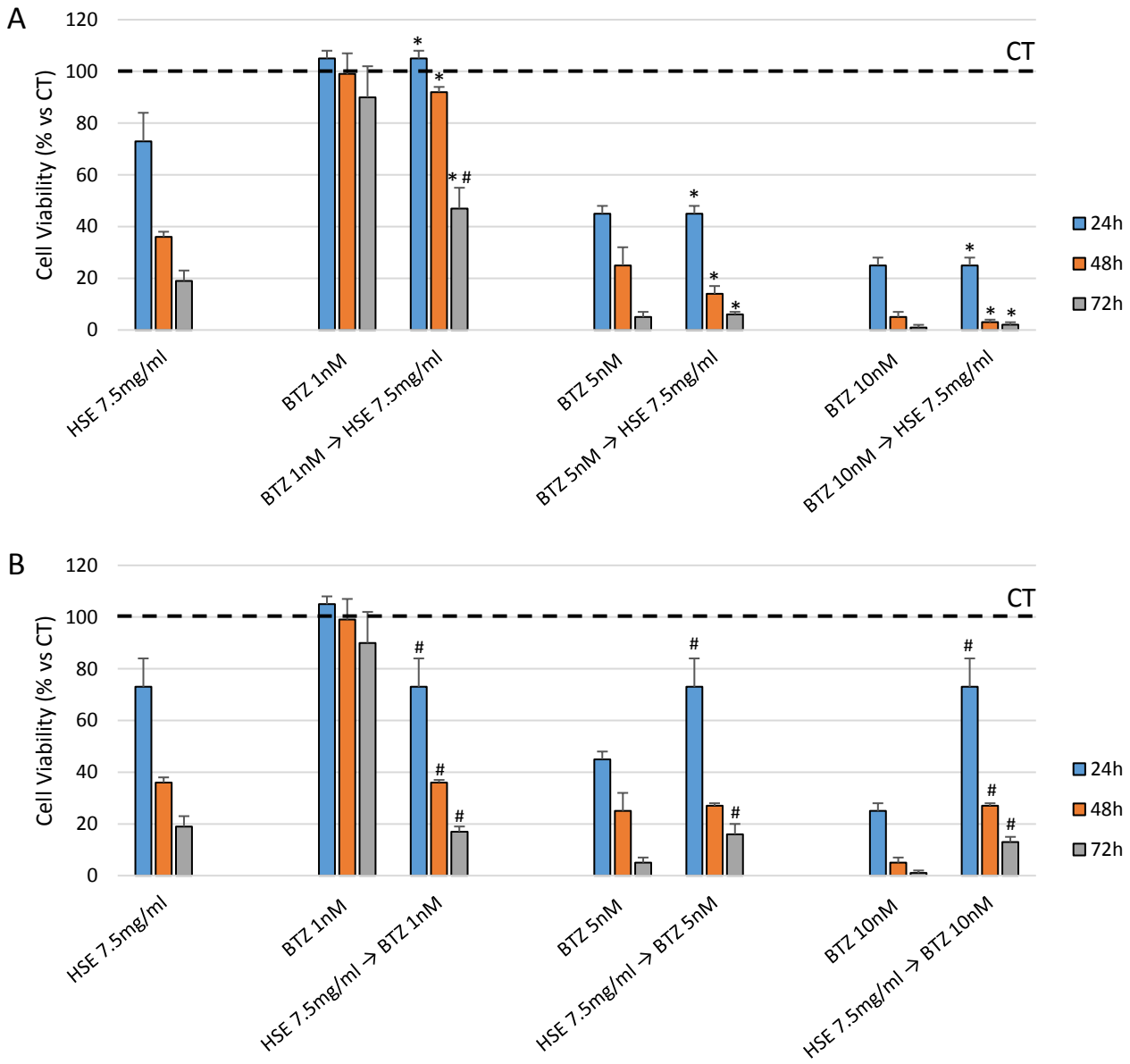



**Fig. 38 - RPMI 8226 cell viability after not-simultaneous treatment with BTZ and HSE –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to not-simultaneous combinations of different concentrations of BTZ (1, 5 and 10nM) and HSE 7.5mg/ml. Cells were treated for 24h with BTZ and then with HSE for further 24, 48 and 72h (A). Alternatively, cells were treated with HSE and after 24h with BTZ for further 24,48 and 72h (B). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSE, # P<0.05 vs respective BTZ concentration.

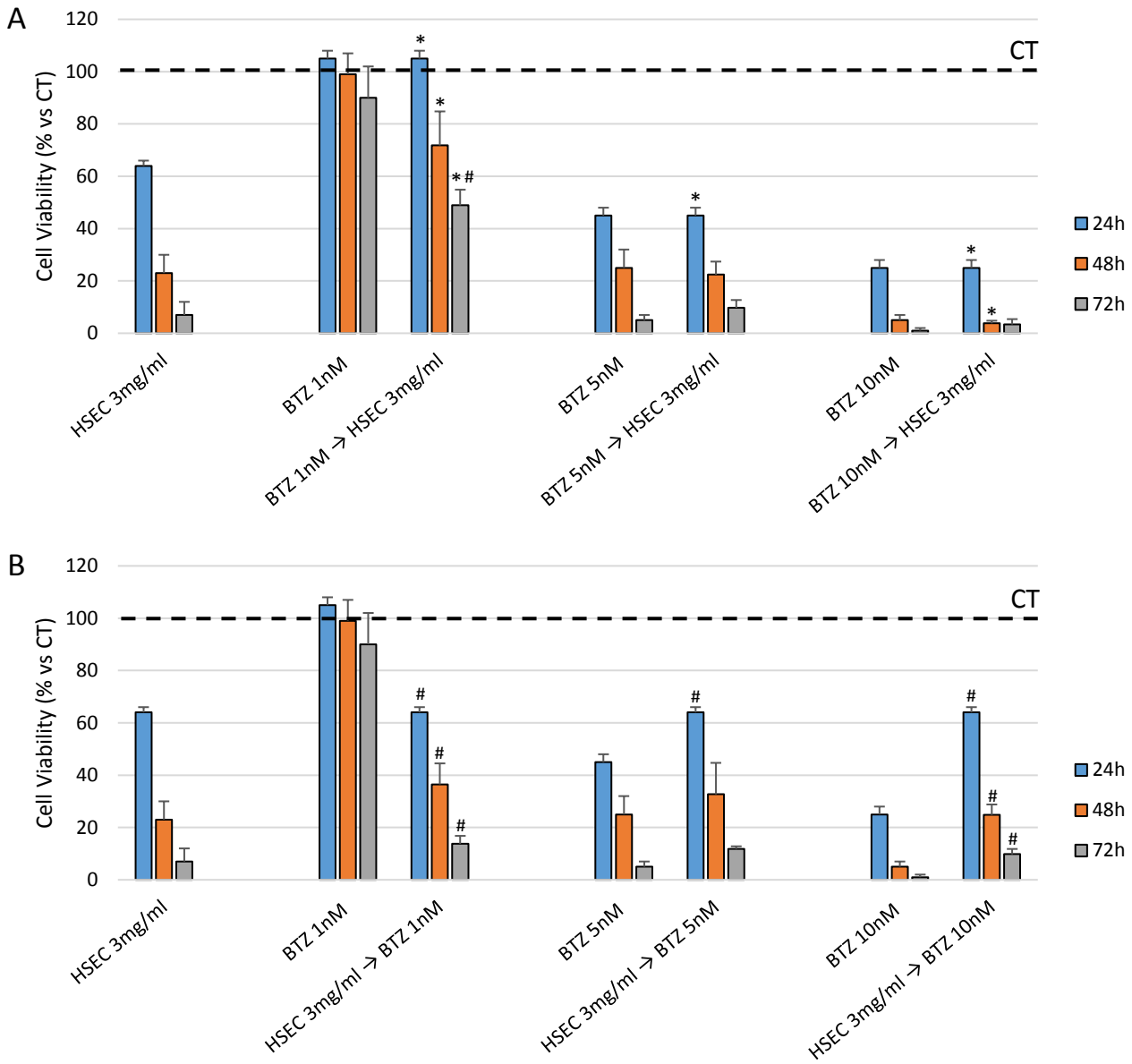



**Fig. 39 - RPMI 8226 cell viability after not-simultaneous treatment with BTZ and HSEC –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to not-simultaneous combinations of different concentrations of BTZ (1, 5 and 10nM) and HSEC 3mg/ml. Cells were treated for 24h with BTZ and then with HSEC for further 24, 48 and 72h (A). Alternatively, cells were treated with HSEC and after 24h with BTZ for further 24,48 and 72h (B). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSEC, # P<0.05 vs respective BTZ concentration.

### 3.2.3. Pretreatment

RPMI 8226 cells were pretreated with BTZ 1nM for 24 h. BTZ was then removed from culture medium and replaced with HSE 7.5mg/ml or HSEC 3mg/ml for further 24-48h (Fig. 40). Alternatively, cells were pre-treated with HSE 7.5mg/ml or HSEC 3mg/ml for 24 h. HSE or HSEC were then removed from culture medium and replaced with BTZ 1 nM for further 24-48 h (Fig. 40). Untreated RPMI 8226 cells were used as controls, while cells treated only with BTZ or HSE or HSEC, without pretreatment, were used to evaluate the effectiveness of combinations. At the end of each time point MTT assay was performed to evaluate cell viability

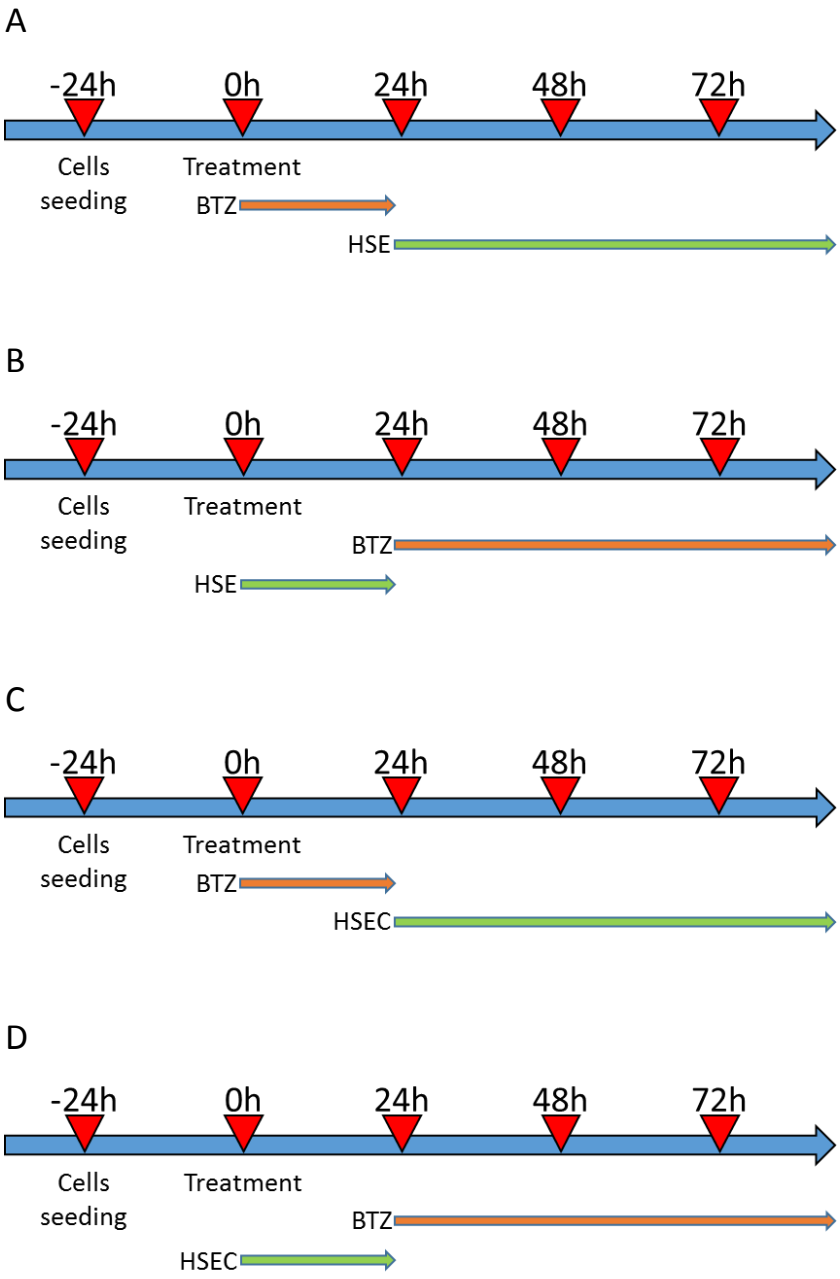



**Fig. 40 – Time schedule of RPMI 8226 pretreatment -** Figures represent the pre-treatment of RPMI 8226 cells with BTZ, HSE or HSEC. Cells were pretreated with BTZ 1nM for 24h and subsequently with HSE 7.5mg/ml (A) or HSEC 3mg/ml (C). Otherwise, cells were pretreated with HSE 7.5mg/ml (B) or HSEC 3mg/ml (D) for 24h and then with BTZ 1nM.

In a MTT assay, RPMI 8226 cells, pretreated with BTZ 1nM and subsequently treated with HSE 7.5mg/ml or HSEC 3mg/ml, underwent a significant reduction of cell viability, compared to cells treated only with single compounds without pretreatments (Fig. 41). BTZ pretreatment followed by HSEC treatment was more effective than BTZ pretreatment followed by HSE treatment. On the contrary, cells pretreated with HSE or HSEC didn't undergo a higher reduction of cell viability and results were comparable with those obtained with single compounds treatment. The effectiveness of BTZ pretreatment followed by HSEC treatment was also confirmed by Trypan blue vital count assay (Fig. 42).

Between all assessed types of combinations, only BTZ 1nM pretreatment, followed by HSE or HSEC treatment, was the only more effective than single compounds. For this reason, we decided to perform further studies to better describe its effect on RPMI 8226 cells.

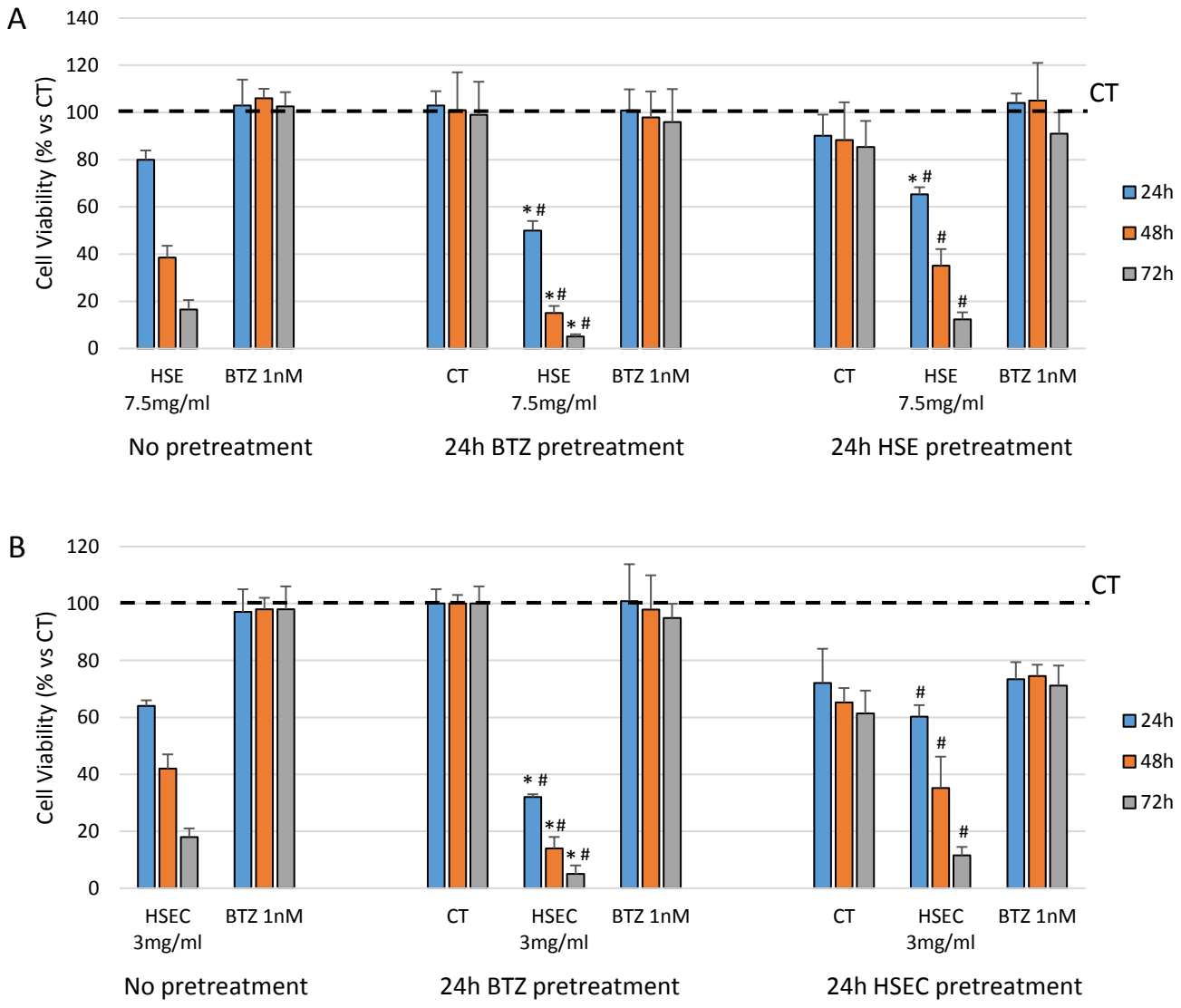



**Fig. 41 - RPMI 8226 cell viability after pretreatment with BTZ, HSE or HSEC –** Graphs represent the percentage of RPMI 8226 cell viability after exposure to BTZ 1nM, HSE 7.5mg/ml (A) or HSEC 3mg/ml (B) treatment, without pretreatment or with 24h BTZ 1nM pretreatment or 24h HSE or HSEC pretreatment. Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05 vs HSE/HSEC no pretreatment, # P<0.05 vs BTZ no pretreatment.

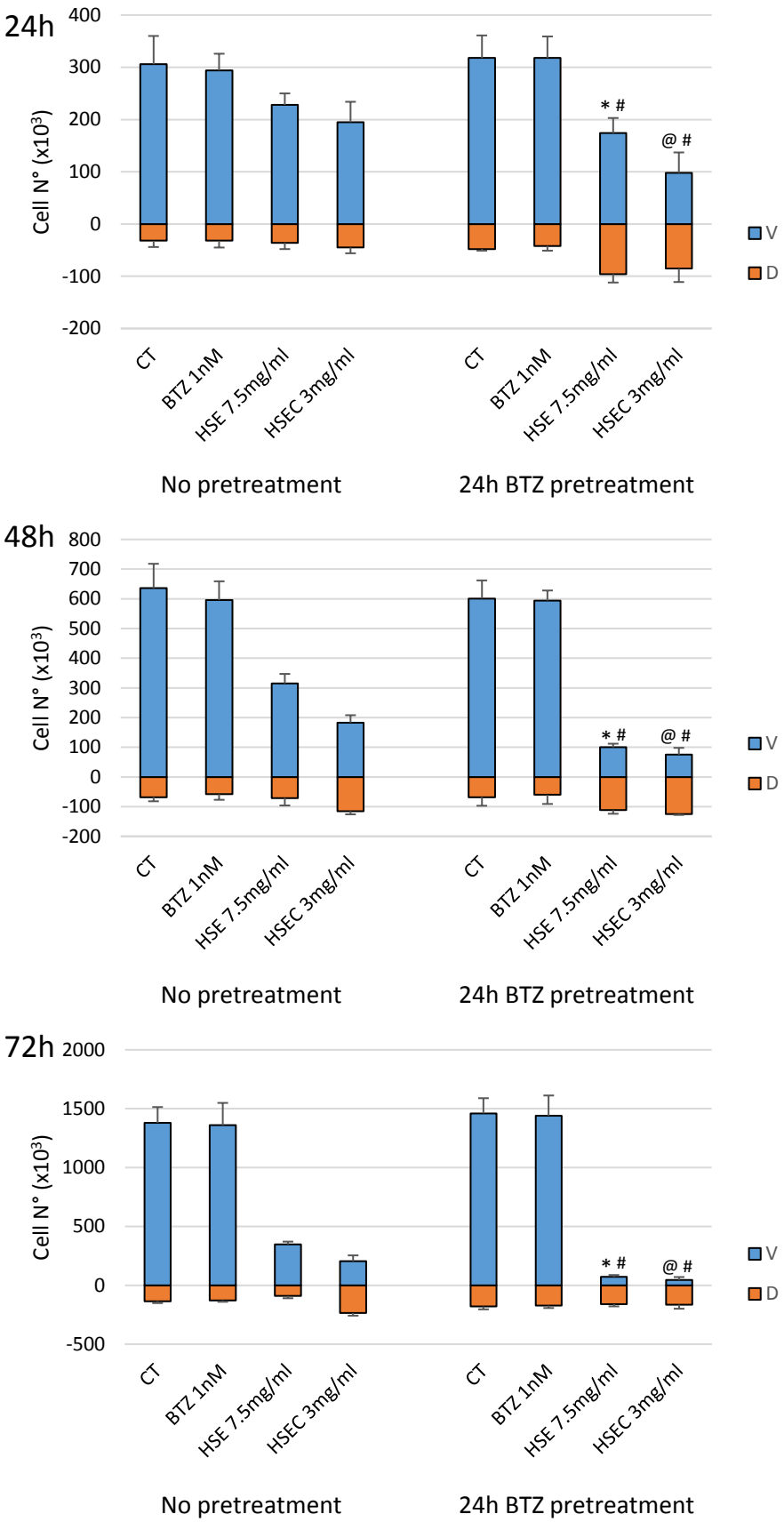



**Fig. 42 – RPMI 8226 trypan blue vital count –** Graph represent the number of counted viable and death RPMI 8226 cells, untreated (CT) or treated with BTZ 1nM, HSE 7.5mg/ml or HSEC 3mg/ml, with or without 24h BTZ 1nM pre-treatment. * P<0.05 vs HSE no pretreatment, @ P<0.05 vs HSEC no pretreatment; # P<0.05 vs BTZ no pretreatment.

### Evaluation of proteasome activity

RPMI 8226 cells, pretreated for 24h with BTZ 1nM and then treated with HSE 7.5mg/ml or HSEC 3mg/ml (in absence of BTZ) for additional 24h, were analyzed for proteasome activity. Proteasome inhibition induced by HSE or HSEC was comparable both after BTZ 1nM pretreatment or not (Fig. 43). Noteworthy BTZ 1nM alone didn't significantly reduce proteasome activity when compared to untreated control cells.

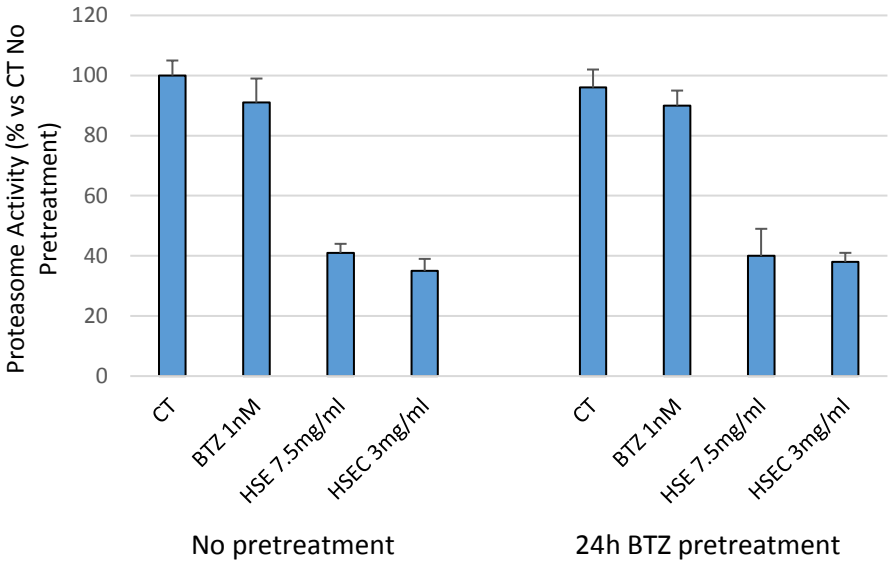



**Fig. 43 – RPMI 8226 Proteasome activity after 24h BTZ pretreatment –** Graphs represent the percentage of proteasome activity in RPMI 8226 cells after 24h BTZ 1nM pretreatment followed by HSE 7.5mg/ml or HSEC 3mg/ml treatment, compared to untreated cells (CT no pre, 100%) and single HSE or HSEC treatment without pretreatment.

### Evaluation of Autophagy

As described in paragraph 3.1.8, untreated RPMI 8226 cells had a basal autophagic activity and approximatively 60% of cells were AVOs positive in AO staining assay. Treatment for 24h with BTZ 1nM (with or without 24h BTZ pretreatment) induced a significant increase of AVOs positive cells due to higher autophagic activity. In RPMI 8226 cells a reduction of AVOs positive cells percentage below to 10% was observed after HSE or HSEC treatment both in presence or in absence of BTZ pre-treatment. These results demonstrated that BTZ pre-treatment was not able to affect the autophagy inhibition induced by HSE or HSEC (Fig. 44).

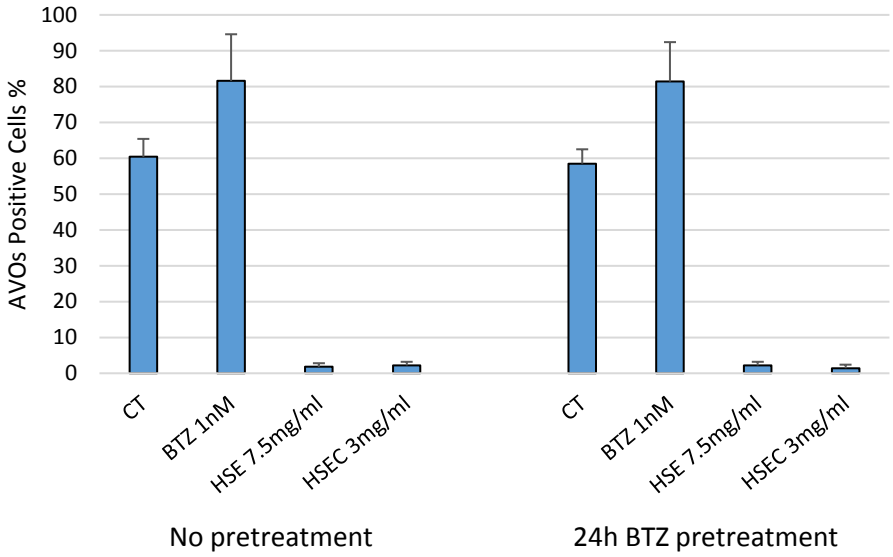



**Fig. 44 – AO staining of RPMI 8226 cells after HSE or HSEC treatment -** (A) Graph represent the percentage of AVOs positive cells of untreated RPMI 8226 cells (CT) or treated for 24h with BTZ 1nM, HSE 7.5mg/ml or HSEC 3mg/ml, with or without 24h BTZ 1nM pretreatment.

## Evaluation of cell migration/invasion

24h BTZ 1nM pretreatment did not alter the inhibition of RPMI 8226 cell migration/invasion induced by HSE 7.5mg/ml or HSEC 3mg/ml. In effect with or without BTZ pre-treatment HSE and HSEC reduced the number of cells that crossed the membrane of Boyden chamber to approximatively 30 an 20% respectively compared to untreated control cells. In contrast, BTZ 1nM alone did not inhibit migration/invasion of RPMI 8226 cells and it was comparable to untreated cells (Fig. 45).

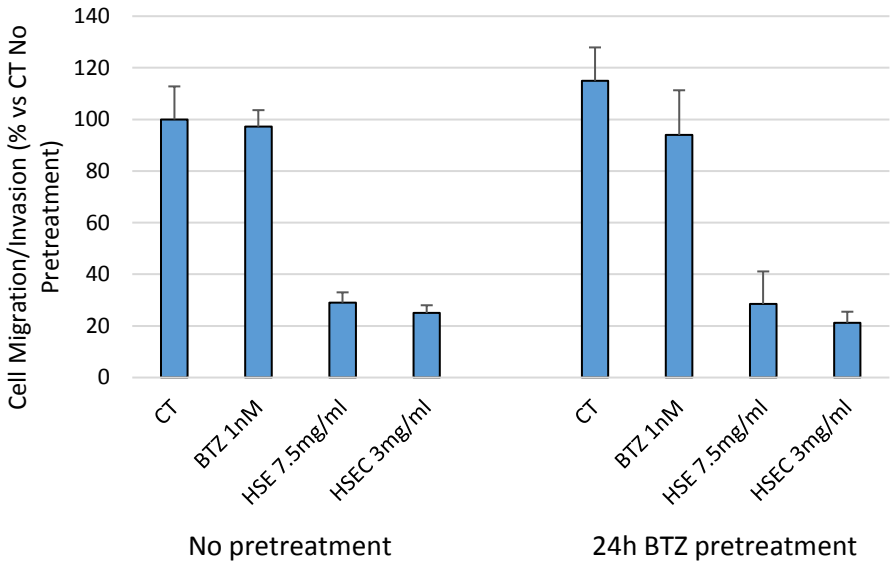



**Fig. 45 – RPMI 8226 Boyden chamber assay after 24h BTZ pretreatment –** Graph represent the percentage of RPMI 8226 cells that crossed the gelatin coated membrane after 24h pretreatment with BTZ 1nM followed by HSE 7.5mg/ml or HSEC 3mg/ml treatment. Control is represented by untreated cells without pretreatment and the number of cells hat crossed the membrane is set to 100%.

### 3.2.3.6 Evaluation of neurotoxicity

Neurotoxicity of BTZ 1 nM pre-treatment followed by HSE 7.5mg/ml or HSEC 3mg/ml induction was evaluated in embryo rat DRG model. This combination resulted not neurotoxic and neurite outgrowth observed was comparable to those obtained after HSE or HSEC treatment without BTZ pre-treatment. BTZ 1nm alone had a neurite outgrowth similar to untreated DRG (Fig. 46)

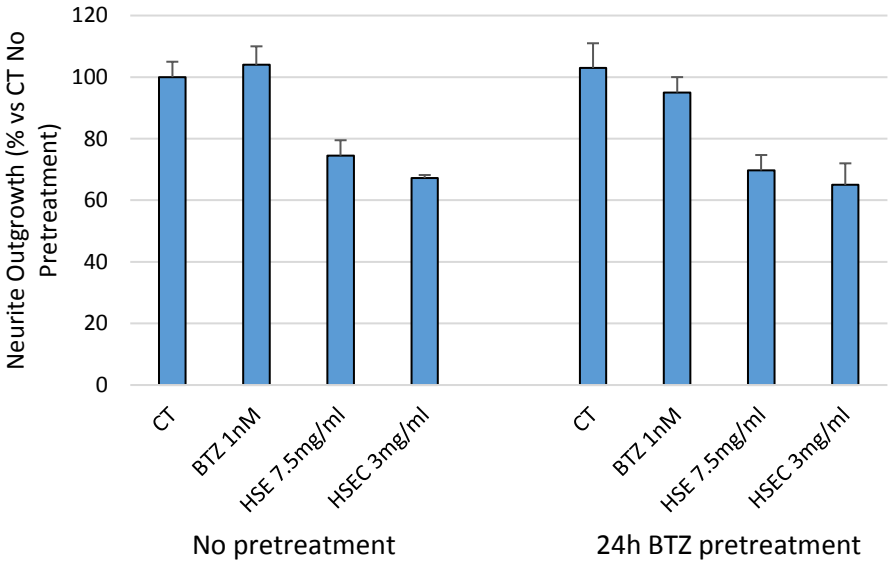



**Fig. 46 – 24h BTZ pretreatment neurotoxicity *in vitro* –** Graphs represent the percentage of DRGs neurite outgrowth after 24h pretreatment with BTZ 1nM followed by HSE 7.5mg/ml or HSEC 3mg/ml. Results are compared to untreated DRGs without pretreatment (CT no pre, 100%) or single HSE or HSEC treatment without pretreatment. Data are represented as the mean ± SD of at least 3 independent experiments.

## 3.3 Phase III: Isolation and characterization of HSEC molecules

HSEC was the most effective HS fraction against RPMI 8226 multiple myeloma cells. In order to identify and isolate the molecules responsible of its antitumoral effect, we performed several bioassay-guided isolations. Obtained compounds were subsequently molecularly and a biologically characterized. This part of the project was performed in collaboration with the laboratory of Professor Simona Collina of Pavia University.

### 3.3.1 Characterization of HSE components

HSEC was subjected to analytical analysis by Gas Chromatography-Mass spectrometry (GC-Mass), with 1-eptanol used as standard, to assess the presence of volatile compounds. From a literature research, identified molecules did not seem to have biological interest (Table 6). Further analysis also identified the presence of flavonoids and polyphenols in HSEC (Table 6).

A

| | *Retention time (min)* | *Concentration (µg/L)* | | *Retention time (min)* | *Concentration (µg/L)* |
|---|---|---|---|---|---|
| *Ethyl valerate* | 6.24 | 12 | *Isovaleric acid* | 29.28 | 14 |
| *3-penten-2-ol* | 6.72 | 56 | *Diethyl succinate* | 29.6 | 7097 |
| *Ethyl isopropyl ether* | 7.35 | 15 | *Verbenone* | 30 | 352 |
| *Ethyl hexanoate* | 8.82 | 123 | *Borneol I* | 30.4 | 59 |
| *4-ethoxy-2-pentanone* | 8.93 | 123 | *3-ethyl-4-nonanol* | 30.6 | 39 |
| *Ethyl 2-oxopropanoate* | 10 | 29 | *d-carvone* | 31.72 | 87 |
| *2-hexanol* | 11.28 | 62 | *Ethyl phenylacetate* | 35.02 | 357 |
| *Ethyl lactate* | 12.76 | 61 | *Hexanoic acid* | 38.36 | 384 |
| *Hexanol* | 13.17 | 28 | *Benzyl alcohol* | 39.56 | 20 |
| *2-butoxyethanol* | 15.29 | 42 | *Ethyl hdroxyoctanoate* | 40.38 | 31 |
| *Ethyl 3-hydroxy-3-methyl butanoate* | 15.73 | 35 | *Phenyl ethyl alcohol* | 41.16 | 77 |
| *Heptanol* | 18.03 | 547 | *2,3-dimethyl-5-propyl thiophene* | 42.3 | 1634 |
| *Furfural* | 18.44 | 657 | *Maltol* | 43.53 | 197 |
| *Camphor* | 20.37 | 44 | *2-furancarboxyaldehyde* | 44.65 | 128 |
| *Benzaldehyde* | 21.11 | 64 | *Diethyl pimelate* | 45.89 | 140 |
| *Ethyl 3,3-diethoxypropionate* | 21.52 | 77 | *p-anisaldehyde* | 46.21 | 322 |
| *Ethyl 4-oxobutyrate* | 22.87 | 39 | *Diethyl malate* | 47.85 | 8476 |
| *Pinocarvone* | 23.07 | 31 | *Diethyl octanoate* | 50.81 | 162 |
| *Nopinone* | 23.5 | 37 | *Vanillin* | 51.54 | 49 |
| *Hydroxymethylfurfural* | 23.94 | 216 | *Ethyl undecanoate* | 56.4 | 195 |
| *Diethyl malonate* | 24.63 | 52 | *Benzoic acid* | 62.68 | 1344 |
| *Ethyl levulinate* | 26 | 4061 | *Triethyl citrate* | 64.1 | 640 |
| *Myrtenol* | 26.17 | 38 | *Vanillin* | 66.52 | 1710 |
| *Ethyl 2-furancarboxylate* | 26.7 | 56 | *Adipic acid ethyl ester* | 67.17 | 597 |
| *Diethyl fumarate* | 28 | 64 | *Glutamic acid* | 67.88 | 1076 |
| *Ethyl benzoate* | 28.6 | 53 | *Homovanillic acid* | 77.6 | 1005 |

B

| | *Concentration (mg/L)* |
|---|---|
| *Procyanidins* | 0 |
| *Anthocyanins* | 0 |
| *Flavonoids* | 3104 |
| *Polyphenols* | 546 |

**Table 6 –** (A) GC-mass analysis of volatile compounds of HSEC. (B) Content of procyanidins, anthocyanins, flavonoids and polyphenols in HSEC.

The molecular fingerprint of HSEC was obtained by TLC (Fig. 51) and by HPLC-UV analysis. The best HPLC method, in terms of peak resolution and analysis time, has been identified as result of various attempt with different eluent mixtures and different columns (Chromolith RP-18, C18 Discovery and XBridge Phenyl). The most suitable method and its experimental conditions are reported in Table 7. The analysis of HSEC with this HPLC method allowed the identification of 5 major peaks at different retention times (Fig. 47).

| | |
|---|---|
| *Column* | XBridge Phenyl (4.6x150mm, 5µm) |
| *Solvent A* | H2O |
| *Solvent B* | ACN |
| *Flux* | 1ml/min |

| *Time* | *% A* | *% B* |
|---|---|---|
| *0.1* | 50 | 50 |
| *10* | 0 | 100 |
| *11* | 50 | 50 |
| *16* | 50 | 50 |

**Table 7 –** HPLC method and experimental conditions for HSEC analysis.

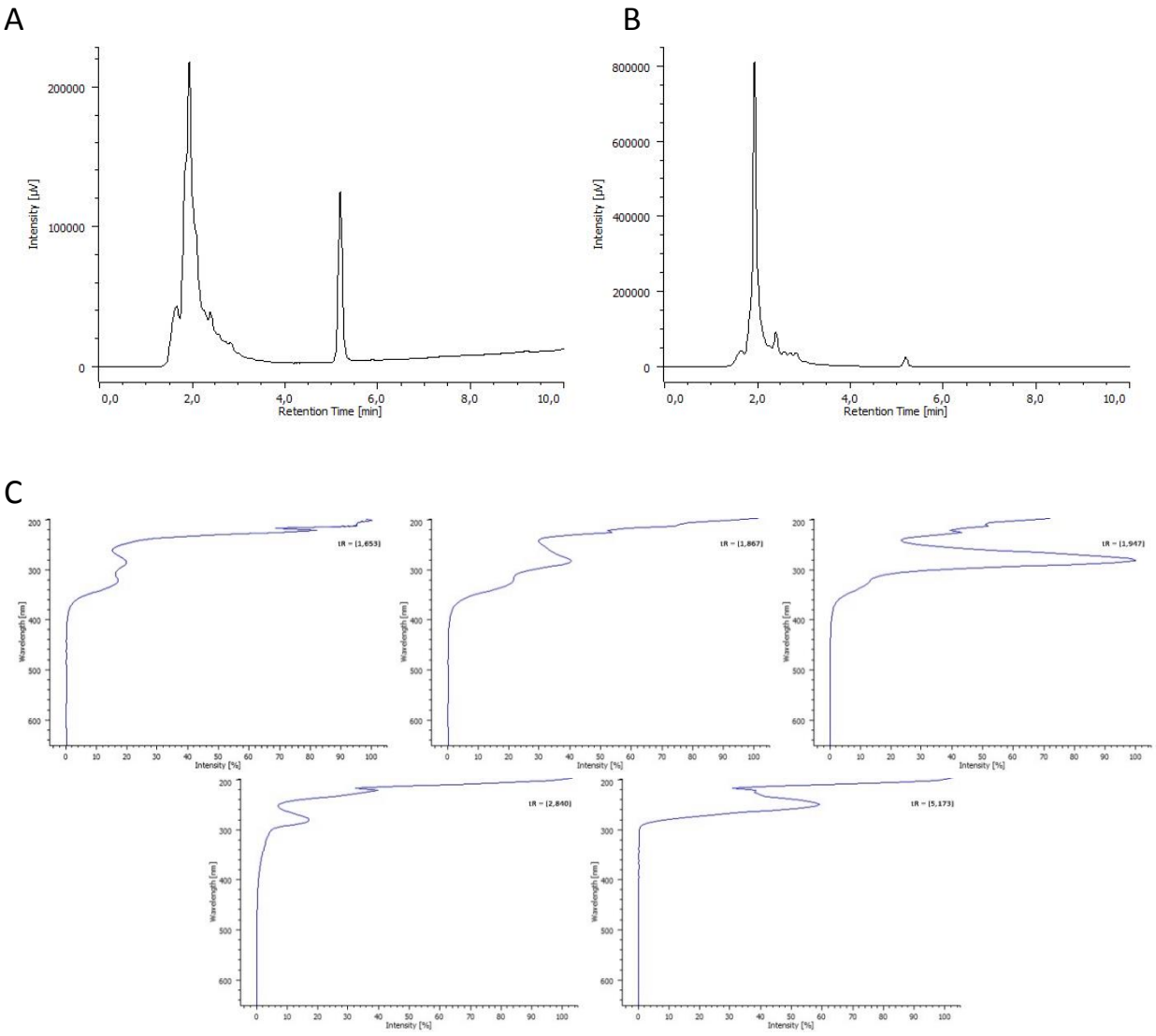



**Fig. 47 – HPLC analysis of HSEC –** HPLC-UV chromatograms of HSEC at 250nm (A) and 279nm (B) with UV spectra of each principal peak (C).

### 3.3.2 First HSEC fractions isolation

The first separation of HSEC components was accomplished by flash column chromatography. The most suitable mobile phase (Ethyl acetate : Toluene : Methanol, 5:3:1) was selected on the basis of several TLC profiles obtained with different mobile phases (data not shown).
HSEC was loaded in the column and the eluate was collected in four different fractions on the basis of TLC profiles (F1, F2, F3 and F4) (Fig. 48). Fraction 4 was further fractionated with flash column chromatography and a second most suitable mobile phase (Ethyl acetate : Toluene : Methanol, 5:1:1). Two new fractions were obtained (F4-F1 and F4-F2).

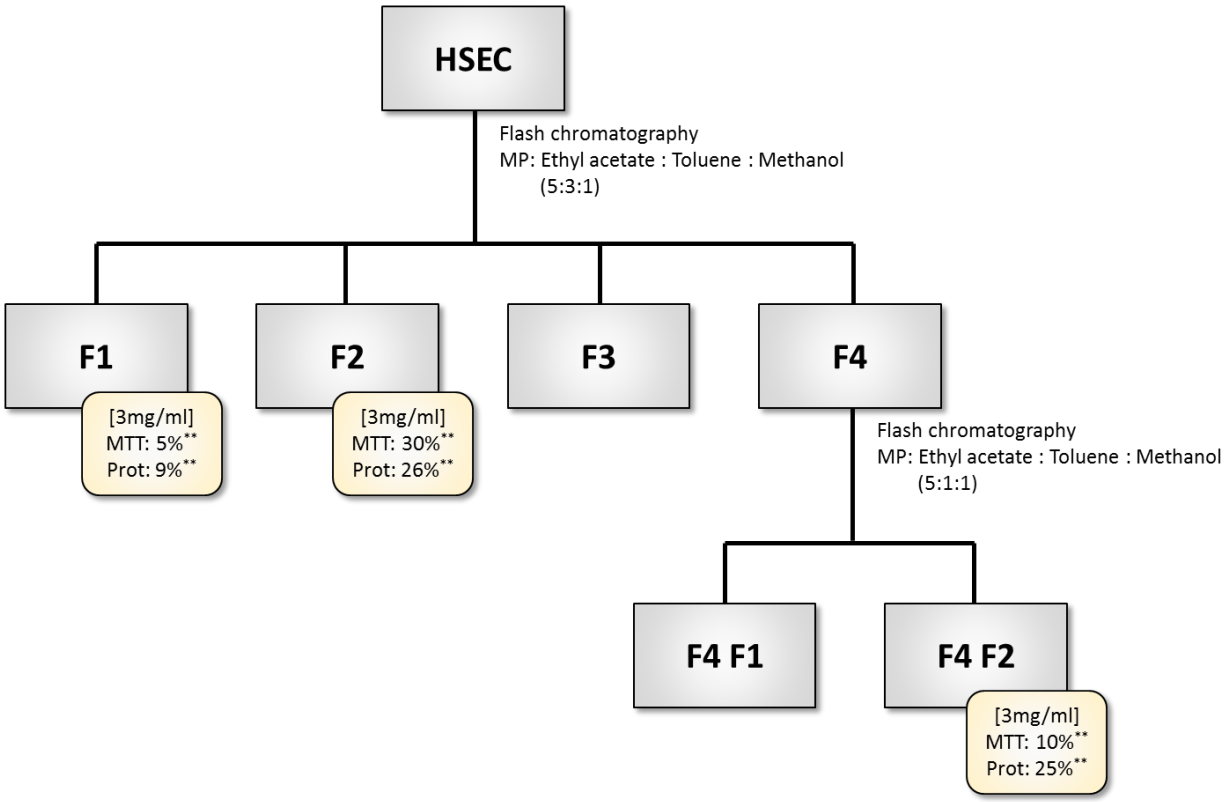



**Fig. 48 – Schematic representation of HSEC fractions obtained by Flash column chromatography and their activity on RPMI 8226 cells.** RPMI 8226 cells were treated for 24h with F1, F2 and F4-F2 fractions at 3mg/ml concentration, then MTT and proteasome activity (prot) assays were performed. Data are represented as the mean ± SD of at least 3 independent experiments. ** P<0.01 vs CT.

F3 and F4-F1 fractions resulted not completely pure at a first TLC analysis (data not shown) so they were not assessed in further chemical and biological experiments.
F1, F2 and F4-F2 fractions were analyzed by HPLC-UV using the method previously described in Table 7 (Fig. 49). Chromatograms of all fractions resulted on specific isolated peaks, but probably they were not completely clean fractions. Further analysis and separations are necessary to obtain single molecules.

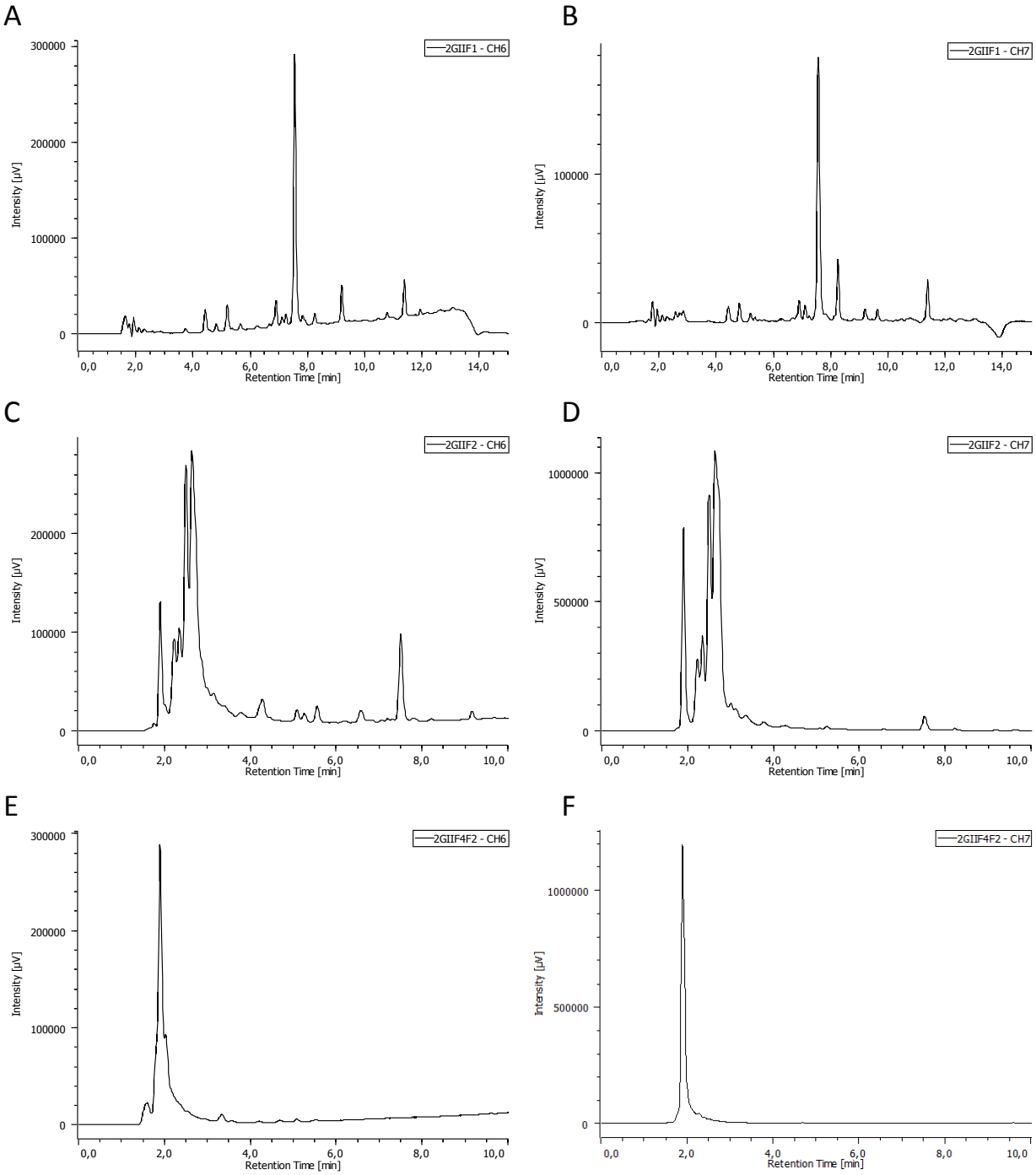



**Fig. 49 – HPLC analysis of HSEC fractions –** HPLC-UV chromatograms of: F1 at 250nm (A) and 279nm (B); F2 at 250nm (C) and 279nm (D); F4-F2 at 250nm (E) and 279nm (F).

Nevertheless, the biological effect of the three compounds against multiple myeloma cells was also assessed *in vitro*. RPMI 8226 cells were treated for 24h with F1 or F2 or F4-F2 fractions at 3mg/ml concentration. Subsequently viability (by MTT assay) and proteasome activity experiments were performed.

All three compounds were able to impair both cell viability and proteasome activity when compared to untreated control cells, but only F1 was the most effective on both biological aspects

(Fig. 48). This fraction impaired cell viability to 5% and residual proteasome activity was 9%, when compared to untreated control cells.

### 3.3.3 Second HSEC fractions isolation

A second procedure to fractionate HSEC components was performed using a carbonate resin and following the steps illustrated in Fig. 50.

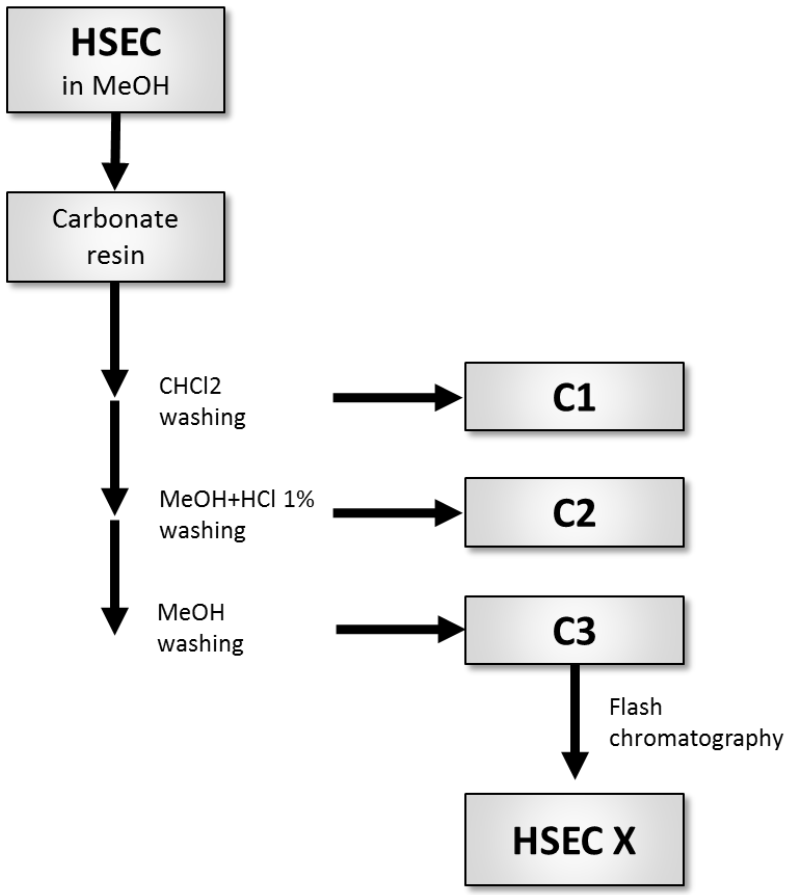



Fig. 50 - **Schematic representation of HSEC fractions obtained using a carbonate resin.** HSEC was solubilized in Methanol (MeOH) and added to a carbonate resin. After three different washing with dichloromethane (CHCl2), MeOH + Chloridric Acid 1% (HCl) and MeOH, three compounds were obtained (C1, C2 and C3). C3 were than subjected to Column flash chromatography and the compound of interest, HSEC X, was obtained.

At the end of the process a 99.99% pure compound from HSEC (HSEC X) was obtained and its purification yield was about 2%. HSEC X had a specific TLC profile (Fig. 51), but it was not detectable under UV light. For this reason, it was not possible to run a HPLC-UV analysis. However, the substance was unequivocally identified by NMR and HPLC-mass analysis (data not shown).
Afterwards, HSEC X was evaluated from a biological point of view. RPMI 8226 cells were treated with different concentration of HSEC X (0.3, 1 and 3mg/ml). After 24h, cells were subjected to biological assays.

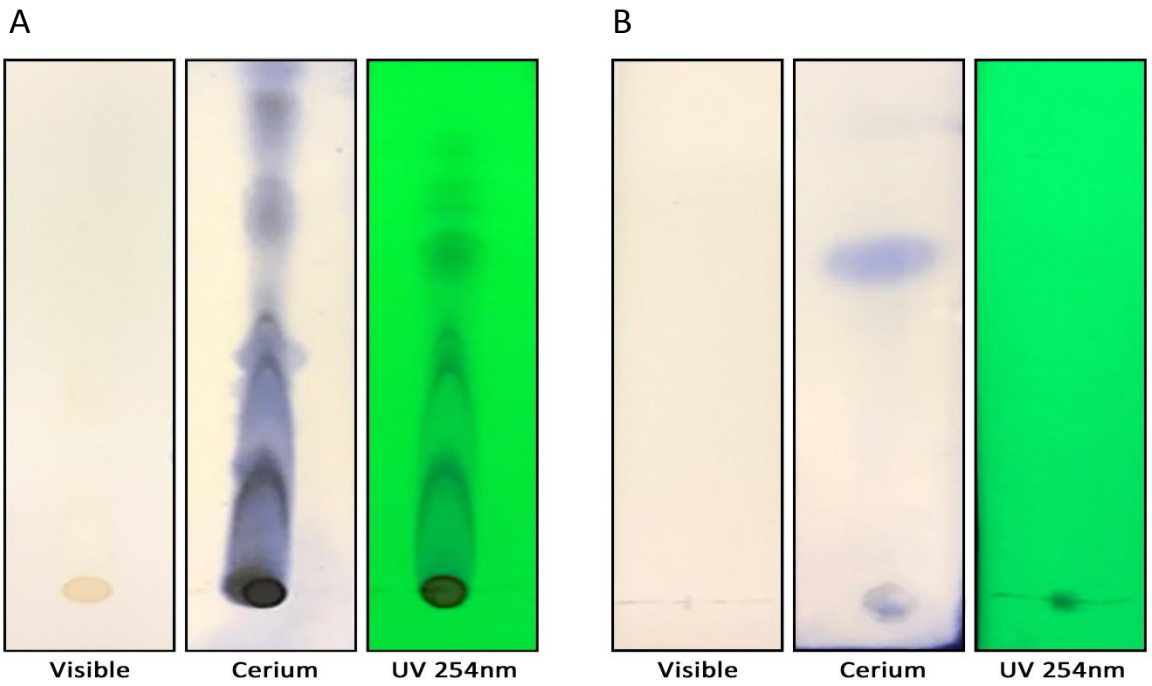



**Fig. 51 – TLC analysis of HSEC and HSEC X –** Photographs of HSE (A) and HSEC (B) TLCs under visible light, with cerium staining or under a 254nm UV lamp. Mobile phase used to get the TLC profile was Toluene : Ethyl Acetate : Methanol; 5:1:1.

### Evaluation of RPMI 8226 cell viability after HSEC X treatment

A dose dependent reduction of RPMI 8226 cell viability was observed after HSEC X treatment, when compared to untreated control cells. This reduction was demonstrated both in MTT (Fig. 52) and Trypan blue assay (Fig. 53), although showing a difference in HSEC X efficacy. In MTT assay, less than 3% of RPMI 8226 cells survived to HSEC X 3mg/ml treatment, while, in Trypan blue assay, cell viability was approximatively 40%. This difference was probably due to a cell death type that did not lead to membrane disruption and Trypan blue could be not trustworthy to detect cell viability of RPMI 8226 cells after HSEC X treatment.

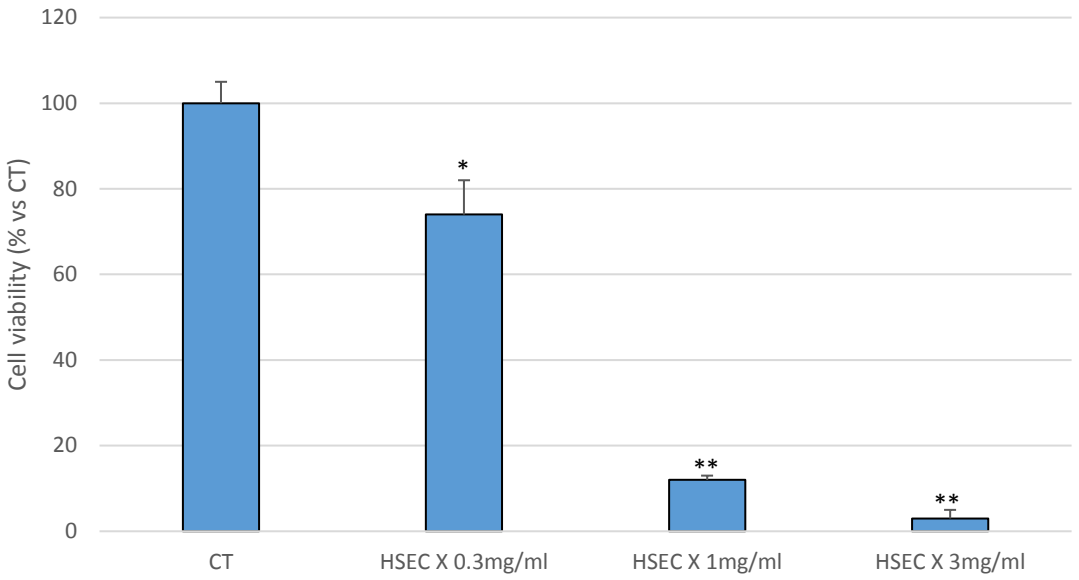



**Fig. 52 - RPMI 8226 cell viability after HSEC X treatment –** Graphs represent the percentage of RPMI 8226 cell viability in MTT assay and after exposure to different concentrations of HSEC X (0.3, 1 and 3mg/ml). Results are compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

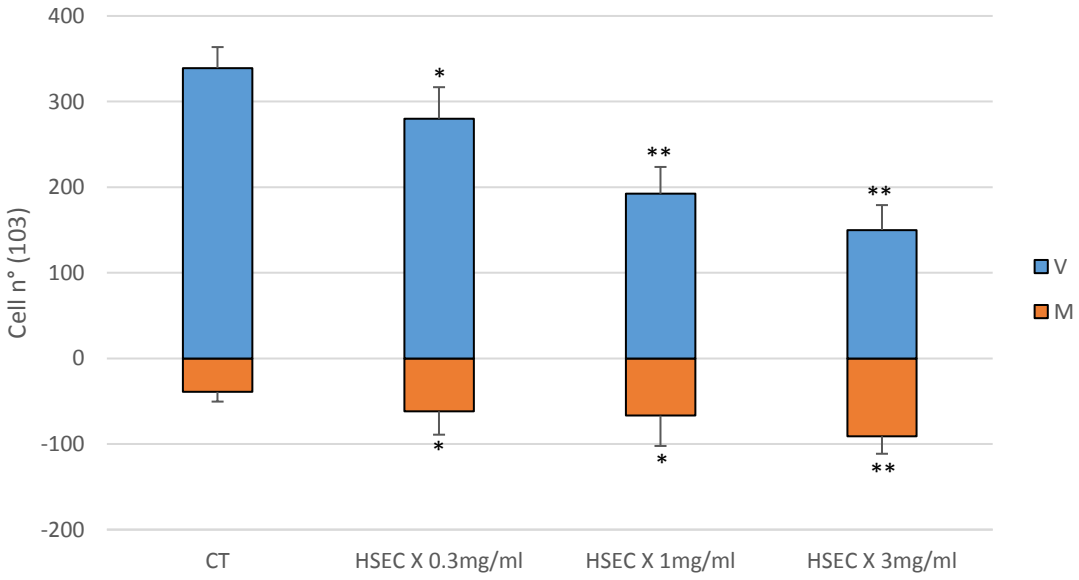



**Fig. 53 – RPMI 8226 trypan blue vital count after HSEC X treatment –** Graph represent the number of viable and death RPMI 8226 cells, untreated (CT) or treated with HSEC X at different concentrations (0.3, 1 and 3mg/ml), counted in trypan blue vital count assay after 24h of treatment. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### Evaluation of RPMI 8226 cell migration and invasion after HSEC X treatment.

RPMI 8226 cell migration and invasion was significantly reduced by HSEC X in a Boyden chamber assay. Both concentrations evaluated, 0.3 and 1mg/ml, reduced cell migration and invasion to 25 and 12% respectively, when compared to positive controls, set to 100%. 3mg/ml concentration was not evaluated because it was too toxic in MTT assay and could not return reliable results (Fig. 54).

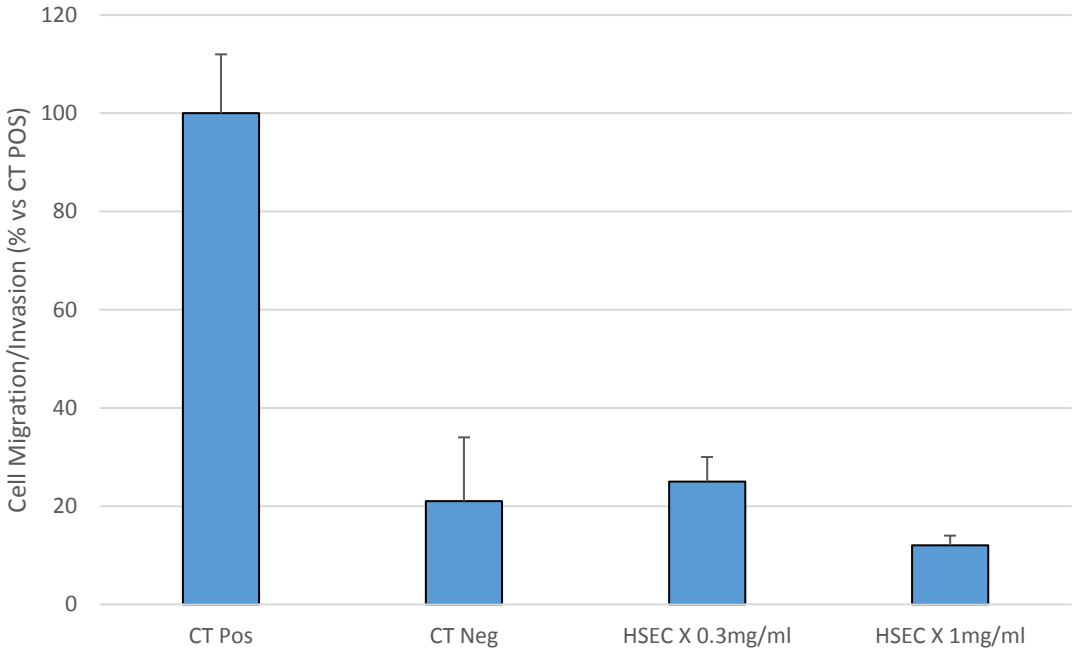



**Fig. 54 – RPMI 8226 Boyden chamber assay after HSEC X treatment –** Graph represent the percentage of RPMI 8226 cells that crossed the gelatin coated membrane, attracted by FBS stimulus, after 24h treatment with different concentrations of HSEC X (0.3 and 1mg/ml). Positive control (CT POS) is represented by untreated cells attracted by the same stimulus. The number of CT Pos cells that crossed the membrane is set to 100%. While negative control (CT Neg) refers to cells that crossed the membrane even in absence of a chemotactic stimulus. Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

### Proteasome activity and autophagy of RPMI 8226 cells after HSEC X treatment

Proteasome activity and AVOs are both impaired by HSEC X when compared to untreated control cells. Proteasome inhibition was dose dependent, while AVOs reached the maximum reduction already with the lower dose of HSEC X assessed (Fig. 55-56)

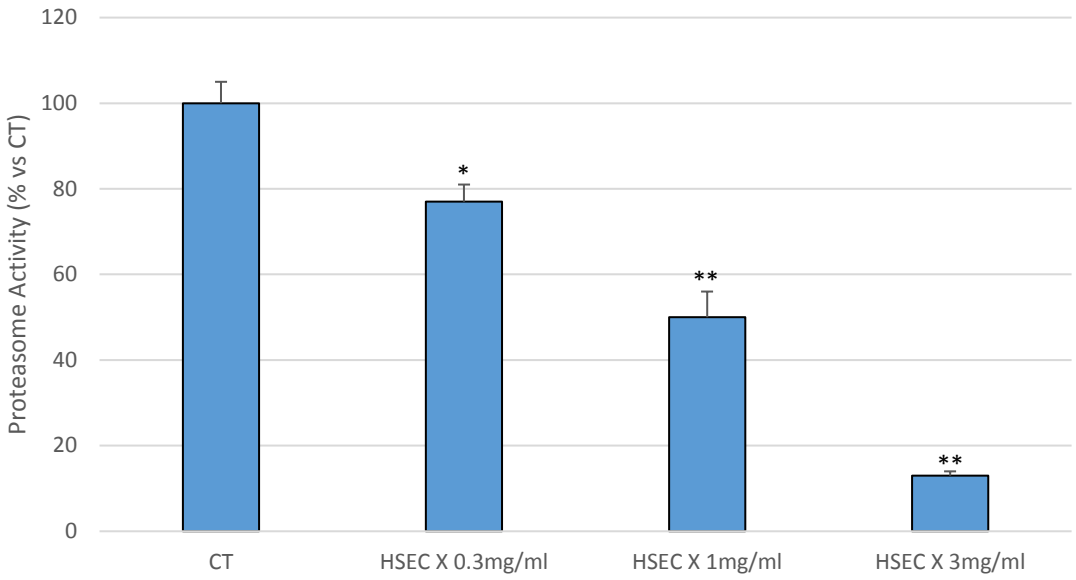



**Fig. 55 – RPMI 8226 Proteasome activity after HSEC X treatment –** Graphs represent the percentage of proteasome activity in RPMI 8226 cells after 24h of treatment with different concentrations of HSEC X (0.3, 1 and 3mg/ml), compared to untreated cells (CT, 100%). Data are represented as the mean ± SD of at least 3 independent experiments. * P<0.05, ** P<0.01 vs CT.

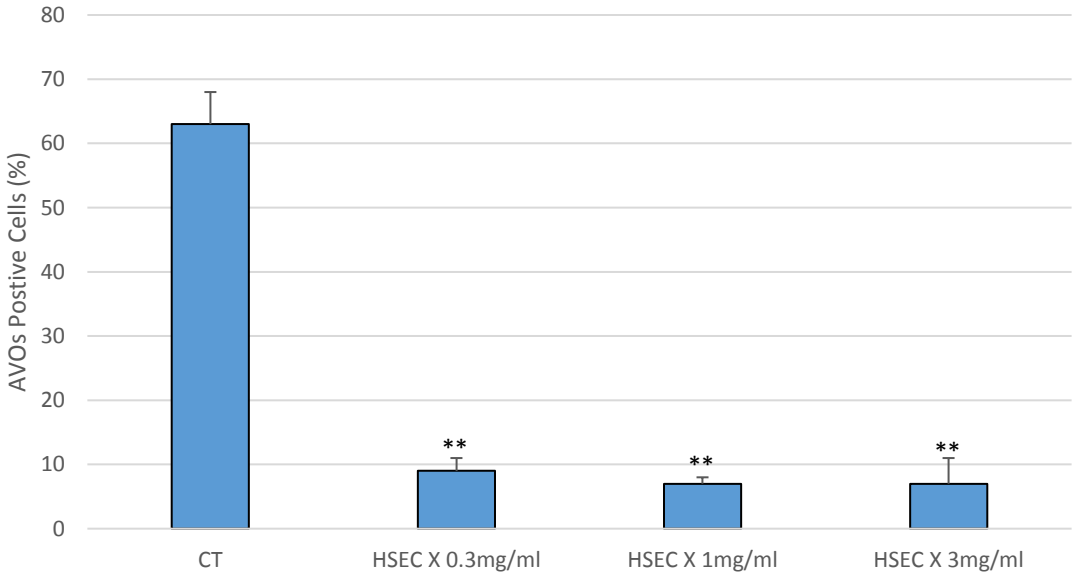



**Fig. 56 – AO staining of RPMI 8226 cells after HSEC X treatment -** Graph represent the percentage of AVOs positive cells, untreated (CT) or treated for 24h with different concentrations of HSEC X (0.3, 1 and 3mg/ml). Data are represented as the mean ± SD of at least 3 independent experiments. ** P<0.01 vs CT.

# Chapter 4: **Discussion**

Multiple myeloma is the most common hematologic malignancy and represent about 1% of all cancers. Although in recent years new therapeutic techniques and new drugs have improved life expectancy of patients to 4-5 years, the research of novel therapeutic approaches is required. Indeed, drugs commonly used for multiple myeloma treatment have several side effects, some of which are dose limiting and often require the complete cessation of the treatment.

In the last decade, compounds from natural sources are gaining a lot of interest, especially because of their high bioavailability, low costs and minimal adverse effects. From this perspective, *Hibiscus sabdariffa* (HS) could be considered a promising alternative to common drugs. It is a plant cultured in tropical and subtropical countries and it has already been used for a long time in folk medicine for its antioxidant and anti-inflammatory effects (Ross, 2003).

In our previous work (Malacrida *et al.*, 2016), we demonstrated the antitumoral effects of an HS extract against multiple myeloma cells and oral squamous carcinoma cells in vitro. On the basis of our results, in this PhD project we decided to further investigate the effects of HS on multiple myeloma cells.

In dose finding experiments, we evaluated the effect of different concentrations of a total HS extract (HSE) on cell viability of RPMI 8226 human multiple myeloma cells. Concentrations were chosen on the basis of a literature research, in which concentrations between 0.05 and 20m/ml are used, and our experience gained in previous work (Tseng *et al.*, 1998; Lin *et al.*, 2005, 2007; Chiu *et al.*, 2015; Malacrida *et al.*, 2016).

Subsequently, we also evaluated the effectiveness of three fractions obtained by liquid-liquid extraction from HSE. Among obtained fractions, aqueous and butanol fractions showed no particular activity against RPMI 8226 cells and their characterization did not continue. Otherwise, HSE and ethyl acetate fraction HSEC impaired cell viability of RPMI 8226 cells in a dose and time dependent manner. An antitumoral effects of a fraction of HS total extract (aqueous fraction) has been already observed on gastric carcinoma AGS cells and leukemia HL-60 cells *in vitro* (Chang *et al.*, 2005; Lin *et al.*, 2005). In these works, viability impairment was due to apoptosis induction and consequent cell death. Otherwise, in our study, apoptosis was observed after ethyl acetate fraction (HSEC) treatment. Moreover, we observed that HSE had a cytostatic effect, while HSEC was more cytotoxic. Differences between HSE and HSEC were also observed in the reversibility of the effect on cell viability. HSE was reversible after 24h of treatment and this feature is common for many antitumoral drugs of natural origin (Akita and Sliwkowski, 2003; Moucadel *et al.*, 2011; Teicher and Tomaszewski, 2015). On the contrary, HSEC was not reversible and after washout, we observed a reduction of cell viability comparable to HSEC continuous treatment.

The differences observed between HSE and HSEC in cytotoxicity and reversibility are probably due to liquid-liquid isolation method. The process divides the molecules of HSE on the basis of their affinity with solvents, in this case water and ethyl acetate. It is possible that, during the process, pro-apoptotic molecules became more concentrated in HSEC, while anti-apoptotic or pro-proliferative molecules were removed by water solvent.

One of the most problematic aspects of multiple myeloma is the capability to establish bone metastasis and bone marrow infiltrates. These areas are a favorable microenvironment for multiple myeloma development and proliferation. Therefore, it is important to identify not only drugs effective on cell viability, but also on migration and invasion. In our work, we demonstrated for the first time that both HSE and HSEC impaired this feature in multiple myeloma cells. Nowadays, there is no information on the effect of HS on cell migration and invasion, except some molecules isolated from other natural sources that could be also contained in HS extracts. For example, delphinidin, quercetin and EGCC were able to impair cell migration and invasion of breast cancer and oral squamous carcinoma cells in vitro (Chiang *et al.*, 2006; Ho *et al.*, 2007; Syed *et al.*, 2008; Lai *et al.*, 2013). Therefore, in our experiments, HS demonstrated to be effective against two important cancer features, cell viability and cell migration and invasion.

The molecular mechanisms responsible of HS antitumoral effects have not yet been studied in depth. In our work, we focused our attention in particular on proteasome, autophagy and MAPKs.

Proteasome and autophagy are the main degradation tools of cells. Proteasome is a multiprotein complex that degrades damaged or unfolded proteins, while during autophagy, whole cell areas are included in autophagosomes to be degraded by lysosomes. Both processes were significantly reduced by both HSE and HSEC. There are no works in literature that report the use of HS (or one of its molecules) as an inhibitor of proteasome or autophagy. Furthermore, it is also difficult to find a drug or compound that is able to inhibit both processes. Indeed, in most cases, the inhibition of one process induces the cell to compensate increasing the activity of the second one (Wojcik, 2013; Niewerth *et al.*, 2015). Moreover, proteasome and autophagy were found to communicate in a much broader system-wide context and their activities are tightly coordinated. For example, they share many processes and proteins, such as the ubiquitin system for cargo recognition, chaperones and stress sensing molecules. The relevance of both proteasome and autophagy inhibition remains largely unknown. Understand the crosstalk between them is highly relevant for translational research. It is however evident that simultaneous inhibition of both degradative processes could lead to an important stress condition for the cell. A direct consequence could be the accumulation of misfolded proteins and damaged organelles, that could induce cell death (by apoptosis, necrosis, mitotic catastrophe or other types cell death) (Wong and Cuervo, 2010; Lilienbaum, 2013; Schreiber and Peter, 2014).

Next, we evaluated phosphorylation levels of MAPKs after HSE and HSEC treatment. MAPKs are proteins involved in many processes of cancer cells, such as proliferation, gene expression, cell survival and apoptosis (Zhang and Liu, 2002; Koul *et al.*, 2013). Moreover, some researchers demonstrated the involvement of MAPKs in the antitumoral effects of drugs derived from natural sources (Lin *et al.*, 2007; Peng *et al.*, 2014). Several works reported that ERK 1 and ERK 2 can be pro-apoptotic or anti-apoptotic regulators, and this depends upon cell type, nature of the stimuli, localization of the kinases and interactions with substrates (Lu and Xu, 2006). In our previous work (Malacrida *et al.*, 2016), we demonstrated that ERK 1 and ERK 2 inhibition by U0126 enhanced the effect of HS on cell viability supporting the hypothesis that these kinases are involved in anti-apoptotic process. In this study, after HSE and HSEC treatment, we performed a time point study of ERK1 and ERK2 activation and we observed an increase in ERK 1 and ERK 2 phosphory-

lation after 16, 24 and 48h of treatment. Further studies are needed to understand if the inhibition of ERK activation at different times could have different effects on cell viability and could therefore act as a pro apoptotic stimulus.

p38 phosphorylation was also increased both by HSE and HSEC, but the activation induced by HSEC lasts for a slightly longer time: HSE induced an increase until 4h of treatment, while HSEC until 6h. This difference could be important to explain the difference between the two compounds. We already demonstrated the importance of p38 phosphorylation in our previous work (Malacrida *et al.*, 2016), where we demonstrated that p38 phosphorylation inhibition by SB203580 reduced the effectiveness of HSE treatment. Furthermore, the importance of p38 was also described by Lin and collaborators that observed the link between p38 and the induction of apoptosis in gastric cancer cells after HS treatment. In fact HS induced the activation of p38 in the first hours of treatment and consequently it induced apoptosis through the FasL cascade (Lin *et al.*, 2005). Also in our experiments, we observed apoptosis induction and early p38 activation, but more studies are needed to link the alterations observed in RPMI 8226 cells after HS treatment.

Peripheral neuropathy is a common adverse effect of many antitumoral drugs. This debilitating condition is dose limiting and often leads to total cessation of the treatment. Thanks to DRGs neurotoxicity model in vitro, widely used in our laboratory (Meregalli *et al.*, 2014), we were able to select HSE and HSEC concentrations that could be not neurotoxic in vivo. Even if the concentrations used in most of the experiments were not neurotoxic, we observed an increase of neurotoxicity in response to higher concentrations of both HSE and HSEC. To date, there are no studies in literature that discuss the neurotoxicity of HS both in vitro and in vivo. There are also not information about concentrations that should be used in vivo or pharmacokinetic studies of HS. We could not affirm that our tested concentrations are biologically achievable. Lo and collaborators hypothesized that 0.05-4mg/ml in vitro concentrations should correspond to 0.2% of an animal standard diet or to a human consumption of 1.5g/day as diet supplement (Lo *et al.*, 2007). Therefore, the concentrations we used in our experiments could be valid, but more studies are needed to confirm this important topic.

In the second phase of the project, we evaluated different combinations between Bortezomib (BTZ) and HSE or HSEC. BTZ was chosen because it is one of the most used and probably one of the most effective drug for multiple myeloma, reaching a complete recovery in 60-70% of patients. However, use of BTZ presents different problems, not only of clinical kind. BTZ costs are really high and in Italy they are estimated around 45000€ for complete treatment cycle. Moreover, from a clinical point of view, BTZ has several important side effects. Some of them, such as hematological and gastrointestinal, can be pharmacologically treated. Instead, peripheral neuropathy is not curable, is a real dose-limiting factor and sometimes requires total therapy suspension. For these reasons, it is important to research new therapeutic approaches, with new molecules or with combinations of already available drugs.

In clinic, combinations with BTZ and other drugs are already being used to treat the high heterogeneity of multiple myeloma cell populations. In fact, some subpopulations are BTZ resistant and this could lead to relapsed and refractory multiple myeloma (Richardson *et al.*, 2007). Combinations between BTZ and Lenalidomide, Thalidomide, Melphalan are only some of the common

examples of combination therapy (Schwartz and Davidson, 2004; Palumbo and Cerrato, 2013; Wojcik, 2013; Genadieva-stavric *et al.*, 2014).

In the second part of this PhD project we evaluated several combinations between BTZ and HSE or HSEC with the aim to maintain or increase the effectiveness of the treatment, while reducing the doses and consequently the side effects of BTZ.

Among all combinations evaluated, simultaneous and not-simultaneous treatment were not more effective than single compounds treatment and we also observed a reduction in BTZ effectiveness. This could be due to a direct interaction between BTZ and one or more molecules of HS. This results are in agreement with a previous work of Golden and colleagues (Golden *et al.*, 2009). In this research, BTZ was inhibited by the presence of molecule, probably polyphenols, contained in green tea extracts in vitro. The situation was different in *in vivo* experiments, where, probably because of different body distribution or metabolism of the molecules, BTZ antitumoral effect was not impaired (Jia and Liu, 2013).

On the contrary, BTZ pretreatment, followed by HSE or HSEC treatment, was two times more effective than single compounds. Interesting is the fact that BTZ dose (1nM), used in the pretreatment, has no effect on cell viability of multiple myeloma cells when used alone throughout treatment time. Probably at the concentration 1nM, BTZ induces the modulation of pathways, or specific molecules, that make cells more sensible to subsequent HSE or HSEC treatment. A possible candidate to explain what was observed is autophagy. Indeed, as mentioned previously, BTZ induces autophagy process as a consequence of proteasome inhibition (Driscoll and De Chowdhury, 2012). On the contrary, both HSE and HSEC inhibits autophagy and this could enhance the cytotoxic effect of HS. This is not the first time that the simultaneous combination with proteasome and autophagy inhibitors are considered to enhance the effect on multiple myeloma cells or other types of cancer (Jagannathan *et al.*, 2015; Baranowska *et al.*, 2016). For example, some researchers reported that the use of Chloroquine (a known autophagy inhibitor), in combination with Carfilzomib (a novel proteasome inhibitor), potentiates the induction of apoptosis in multiple myeloma cells both *in vitro* and *in vivo*.

In the third phase of the project, the aim was the isolation and characterization of one or more molecule responsible of HS effect. We used a bioguided isolation method: molecules were obtained from HSEC with chemical techniques and were analyzed by HPLC, NMR and mass spectrometry. In parallel, compounds were assessed by a biological point of view, using cell viability and proteasome activity assay to screen active molecules.

Today there are no such detailed work on isolation and characterization of molecules from HS, especially about its antitumoral effect. Several research focused their attention on total HS extract composition, but without studying the effect of single molecules. Some exceptions are the studies on most known compounds that have been already isolated from other natural sources. For example, anthocyanins and protocatechuic acid.

We performed two bioguided fraction isolation processes. In the first one, we isolated three different fractions from HSEC that keep the antitumoral effects of the starting extract. Despite their promising activity, fractions were not completely pure and a further characterization was not possible both from a molecular and biological point of view. In the second process, we obtained a high purity (99.99%) fraction. It was significantly effective against multiple myeloma cells and

so we studied its effects on other biological aspects (autophagy, migration and invasion). Moreover, we completely characterized the compound from a molecular point of view. Its method of isolation and its antitumoral effects will be considered as a possible subject of a patent. It is the first time that someone describes an isolation method from HS of this molecule and its effects on multiple myeloma cell have never been observed.

In conclusion, in this PhD work it has been demonstrated the antitumoral effects of HS against multiple myeloma cells in vitro. The total extract and the ethyl acetate fraction are effective on cell viability, cell migration and invasion and the concentrations used in our experiments are not neurotoxic in vitro. Proteasome and autophagy inhibition, p38 and ERK1 and 2 activation are probably the pathways involved in the action of HS but more studies are need to better understand its molecular mechanisms.
Moreover, we demonstrate the inhibition of BTZ antitumoral effect against multiple myeloma cells during a simultaneous treatment with HS. We also figure out a possible combination between BTZ and HS, a 24h BTZ pretreatment followed by HS treatment, that could enhance their effectiveness against multiple myeloma cells and reduce adverse effects.
In the last phase, we identify a novel molecule isolated for the first time from HS with interesting antitumoral properties and probably partly responsible for the observed HS effect on human multiple myeloma cells.
Our results suggest that HS is a potential alternative for multiple myeloma treatment, but further studies will be needed to deepen the molecular mechanisms involved in its action and to test its efficacy in *in vivo* experiments.

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NUTRITION AND CANCER
2016, VOL. 68, NO. 7, 1161–1170
http://dx.doi.org/10.1080/01635581.2016.1208830

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# Antitumoral Effect of *Hibiscus sabdariffa* on Human Squamous Cell Carcinoma and Multiple Myeloma Cells

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**ABSTRACT**

Cancer is a leading cause of death worldwide. Despite therapeutic improvements, some cancers are still untreatable. Recently there has been an increasing interest in the use of natural substances for cancer prevention and treatment. *Hibiscus sabdariffa* (HS) is a plant, belonging to Malvaceae family, widespread in South Asia and Central Africa. HS extract (HSE) used in folk medicine, gained researchers' interest thanks to its antioxidant, anti-inflammatory, and chemopreventive properties. In the present study, we initially assessed HSE effect on a panel of human tumor cell lines. Then we focused our study on the following that are most sensitive to HSE action cell lines: Multiple Myeloma (MM) cells (RPMI 8226) and Oral Squamous Cell Carcinoma (OSCC) cells (SCC-25). In both RPMI 8226 and SCC-25 cells, HSE impaired cell growth, exerted a reversible cytostatic effect, and reduced cell motility and invasiveness. We evaluated the involvement of MAPKs ERK1/2 and p38 in HSE effects by using specific inhibitors, U0126 and SB203580, respectively. For both SCC-25 and RPMI 8226, HSE cytostatic effect depends on p38 activation, whereas ERK1/2 modulation is crucial for cell motility and invasiveness. Our results suggest that HSE may be a potential therapeutic agent against MM and OSCC.

**ARTICLE HISTORY**
Submitted 25 August 2015
Accepted 9 May 2016

## Introduction

Cancer is a global health and social problem and, despite improvements in therapeutic options, it remains a leading cause of death worldwide. Therefore, further research for more effective treatments is highly needed and several innovative approaches are proposed.

Based on epidemiological data reporting reduced cancer risk associated to highly fruit and vegetable consumption, in the last decades the attention has been focused on chemoprevention using natural substances, with the aim of preventing or at least delaying cancer progression (1). In particular the chemopreventive properties of many constituents of plant-derived food and beverages have been investigated (2).

*Hibiscus sabdariffa* (HS) is a plant, belonging to Malvaceae family, widely diffused in South Asia and Central Africa (3). Its flowers are consumed worldwide in a cold or hot beverage (Karkadè). Moreover, its extract, characterized by a high content of polyphenol, flavonoids, and anthocyanins, has been used for a long time in folk medicine against liver disease, fever, and hypertension (3). Due to its antioxidant properties HS extract (HSE) has recently gained attention as chemopreventive compound; indeed some studies have demonstrated its efficacy in counteracting both in vitro and in vivo gastric cancer growth (4). However, few data are available up to now regarding the molecular mechanisms underlying HSE anticancer effect. Some studies have reported HSE ability to modulate signaling transduction pathways, such as Mitogen Activated Protein Kinases (MAPKs), a family of structurally related serine-threonine kinases, including ERK1/2, p38, and JNK/SAPK. MAPKs members are activated by different upstream regulators, differ in their substrate specificity, and they have a pivotal role in transducing different signals, regulating cell viability, apoptosis, and differentiation (5). Both ERK1/2 and p38 deregulation have been reported in several tumors. Upregulation of ERK1/2 is generally associated to cancer cell proliferation (6), while p38 inactivation is usually considered as a mechanism for cancer cells to avoid cell cycle blockade and apoptosis (7).

In the present study, we evaluated the effect of HSE, obtained from the dried flower of HS, on a panel of human tumor cell lines. Then we focused our study on the following that are most sensitive to HSE action cell lines: Multiple Myeloma (MM) cells (RPMI 8226) and Oral Squamous Cell Carcinoma (OSCC) cells (SCC-25).

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HSE impaired cell growth and migration of SCC-25 and RPMI 8226 cells. In addition, we analyzed the involvement of ERK1/2 and p38 in HSE effects both on cell growth and migration. These cell lines were representative of tumors that were different both for ontogenic and progression mechanisms. SCC-25 and RPMI 8226 cells derived, respectively, from human tongue squamous cell carcinoma and human multiple myeloma.

## Materials and methods

### Cell cultures and materials

SCC-25 human squamous cell carcinoma cells (ATTC, USA) were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS) (Euroclone, Pero, Italy), 400 ng/ml hydrocortisone (Sigma-Aldrich, St Louis, MO) and 1% Penicillin and Streptomycin. MM RPMI 8226 cells (ATTC, USA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) medium (Invitrogen, Carlsbad, CA) with 20% FBS, 1% L-glutamine, and 1% Penicillin and Streptomycin. Human immoral Keratinocyte HaCaT (German Cancer Research Center, Heidelberg, Germany), human embryonic kidney derived HEK-293 (kindly provided by Prof D. Barisani, University of Milano-Bicocca, Milano, Italy), and human glioblastoma U87-MG (kindly provided by Dr D. Ballinari, Nerviano Medical Sciences, Nerviano, Italy) cells were cultured in high glucose DMEM (Euroclone, Pero, Italy) supplemented with 10% FBS and 1% Penicillin and Streptomycin. Human pancreatic carcinoma Capan-1, human ovarian cancer Igrov-1, human prostate cancer PC3, and large cell lung cancer H460 cells (kindly provided by Prof. M. D'Incalci, Istituto Mario Negri, Milano, Italy) were maintained in RPMI 1640 medium (Euroclone, Pero, Italy) supplemented with 10% FBS, 1% glutamine, and 1% Penicillin and Streptomycin. Cells were incubated at 37°C and 5% $CO_2$ in a humidified incubator.

HS total extract (HSE), kindly provided by CREA Research Unit for Floriculture and Ornamental Species (Sanremo, Italy), was dissolved in phosphate-buffered saline (PBS) and then diluted directly into culture medium to working concentrations.

U0126 (Merck, Darmstad, Germany) and SB203580 (Promega, Madison, WI) inhibitors were solubilized in DMSO at 10 mM concentration. Further dilutions were done directly into culture medium.

### MTT assay

SCC-25, RPMI 8226, HaCaT, HEK-293, U87-MG, Capan-1, Igrov-1, PC3, and H460 cells were seeded in 96-well plates at 10,000 cells/well density and were treated with different HSE concentrations (0.5–1–3 and 5 mg/ml) for 24, 48, and 72 h at 37°C.

Culture medium was then replaced with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St Louis, MO) 0.5 mg/ml medium solution. After 2 h of incubation at 37°C, formazan crystals were solubilized in 100% ethanol and absorbance was read at 560 nm in a microplate reader (Biorad, Hercules, CA). HSE effect on cell survival was calculated normalizing data to the absorbance of non-treated cells. The reversibility of HSE effect on cell growth was assessed by treating cells for 24 h with HSE. Afterwards HSE was removed and substituted with culture medium (without HSE) for 24 and 72 h. At these times MTT assays were performed.

### Trypan blue vital staining

Cells were cultured in 60-mm diameter dish at initial density of 200,000 cells/dish. After 24 h and 48 h of HSE treatment, SCC-25 adherent and floating cells were harvested after trypsinization and stained with Trypan blue vital dye. RPMI 8226 cells were harvested and stained with Trypan blue vital dye. Viable and death cells were counted in a Burker hemocytometer under a light microscope (Nikon Eclipse TS100).

### Scratch wound healing assay

Scratch wound healing assay was performed to assess SCC-25 cells motility. Cells were cultured in a 6-well plate since they reached 100% confluence. Complete culture medium was replaced with a serum-free medium for 16 h. A scratch with 200 $\mu$l plastic tips was made on the cell monolayer. Medium was removed and after two washes in PBS, cells were treated with HSE for 24 h. Micrographs were taken just after the scratch (t0) and after 24 h of treatment. Control was represented by cells in culture medium. Migration areas of treated and untreated cells were measured using ImageJ software.

### Boyden chamber assay

A Boyden chamber was used to evaluate SCC-25 and RPMI 8226 cells migration and invasion. This chamber consists of two different compartments separated by a gelatin-coated polycarbonate membrane with 8 $\mu$m pores (Biomap, Agrate, Italy). In the lower compartment of the chamber a complete medium was placed and 10% FBS was used as chemo-attractant. In the upper compartment 5,000 cells suspension in serum-free medium was added. HSE was added in the upper chamber medium. After 6 h (for SCC-25 cells) or 16 h (for RPMI

8226 cells) of HSE treatment at 37°C, the membrane was removed. Cells on the upper side were detached mechanically whereas the ones on the lower side were fixed and stained with Diff Quick kit (Biomap, Agrate, Italy). Membranes were mounted on a glass slide and micrographs were taken under a light microscope. ImageJ software was used to count the number of stained migrated cells. Untreated cells were used as positive control. Untreated cells without FBS chemo-attractant were used as negative control.

### Western blotting

Cells were cultured in 60-mm dishes at 200,000 cells/dish density and after different times of HSE treatment were lysed in lysis buffer (Hepes pH 7.5 5 mM, NaCl 150 mM, glycerol 10%, Triton X100 1%, $MgCl_2$ 1.5 mM, EGTA 5 mM) with proteases and phosphatases inhibitors (phenylmethanesulfonyl fluoride 4 nM, aprotinine 1%, sodium pyrophosphate 20 nM, and sodium orthovanadate 92 mg/ml). Lysate was clarified by centrifugation at 4°C, 18,000 *g* for 15 min. Total protein suspension concentration was determined by Bradford assay using a Coomassie protein assay reagent kit.

Cells lysate suspension with 10 $\mu$g of proteins was then mixed with Laemmli buffer (5% $\beta$-mercaptoethanol, 10% SDS, 50% glycerol, 400 mM Tris HCl (pH 6.8) and 0.5% bromophenol blue, all from Sigma-Aldrich, St Louis, MO), denatured at 95°C for 5 min and separated in a 13% acrylamide SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose filters and immunoblotting analysis was performed.

Membranes blocking, washing, and antibody incubation were performed according to manufacturer's instructions. Antibodies against P-ERK1/2 (1:1000, Cell Signaling, Temecula, CA), pan-ERK (1:5000, BD Transduction Laboratories), P-p38 (1:1000, Cell Signaling, Temecula, CA), p38 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and beta actin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) were used. After incubation with primary antibodies, membrane was washed and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000) (anti-mouse, Chemicon, Temecula, CA, USA; anti-rabbit, PerkinElmer, Boston, MA, USA). Immunoreactive proteins were visualized using an ECL chemiluminescence system (Amersham, Arlington Heights, IL, USA).

### Statistical analysis

Data are reported as mean ± standard deviation from at least three independent experiments. Statistical analysis was performed using GraphPad Prism3 software. The differences between control and treated cells were evaluated using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical significance was set at $P < 0.05$.

## Results

### HSE effect on cell viability of different tumor cell lines

We evaluated the effect of HSE on a panel of tumor cell lines by MTT assay (Table 1). Cells were exposed to various HSE concentrations (0.5–1–3 and 5 mg/ml) for 24–48–72 h. In Table 1 we report results by using the highest HSE concentration (5 mg/ml) for 24–48–72 h that are representative of data obtained by using HSE at the different concentrations (0.5–1–3 mg/ml) (data not shown). For each cell line, control (CT) was represented by cells grown in culture medium in absence of HSE stimulation. SCC-25 and RPMI 8226 cells were the most sensitive to HSE effect, which was time and concentration dependent. We have focused our subsequent studies on these two cell lines by using HSE at 5 mg/ml concentration, able to reduce cell viability for both cell lines around 50%.

### HSE effect on SCC-25 and RPMI 8226 cells

Trypan blue exclusion assay demonstrated that HSE induced a significant reduction of cell growth for SCC-25 and RPMI 8226 cells with respect to controls at 48 and 72 h. Furthermore, both for SCC-25 and for RPMI 8226 the number of dead cells was relatively low and did not significantly differ between controls and HSE-treated cells (Fig. 1A–B).

We next aimed at evaluating whether HSE-induced reduction of cell growth was a reversible or irreversible process. SCC-25 and RPMI 8226 cells were treated with 5 mg/ml HSE for 48 h, washed extensively to remove HSE, and incubated in fresh medium for additional 24 and 72 h. The recovery after 24 h was very partial both

**Table 1.** List of cell lines used in MTT experiments.

| Cell line | | MTT cell growth (% of CT) 24 h | 48 h | 72 h |
|---|---|---|---|---|
| HaCaT | Immortal keratinocyte | 106 ± 6 | 84 ± 15 | 81 ± 4 |
| Capan-1 | Pancreatic carcinoma | 95 ± 15 | 79 ± 4 | 73 ± 6 |
| Igrov-1 | Ovarian cancer | 105 ± 7 | 78 ± 5 | 77 ± 7 |
| PC3 | Prostate cancer | 109 ± 2 | 96 ± 3 | 85 ± 5 |
| U87-MG | Glioblastoma | 94 ± 3 | 98 ± 1 | 103 ± 2 |
| H460 | Large cell lung cancer | 105 ± 4 | 88 ± 2 | 71 ± 6 |
| HEK-293 | Human embryonic kidney | 87 ± 8 | 120 ± 6 | 112 ± 9 |
| SCC-25 | Oral squamous cell carcinoma | 87 ± 11 | 58 ± 11 | 53 ± 14 |
| RPMI 8226 | Multiple myeloma | 96 ± 17 | 61 ± 11 | 39 ± 13 |

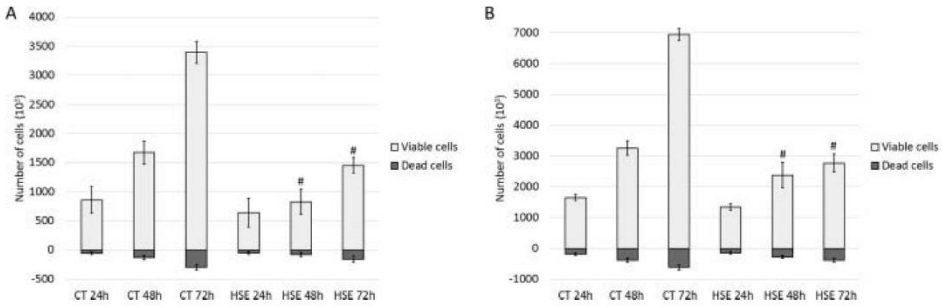


**Figure 1.** SCC-25 and RPMI 8226 Trypan blue vital count. SCC-25 (A) and RPMI 8226 (B) cells, untreated (CT) or treated with HSE 5 mg/ml for 24, 48, and 72 h, were harvested, stained with Trypan blue, and counted in a hemocytometer. The chart represents the mean ± SD of viable and dead cells, counted in three independent experiments. # $P < 0.05$ vs. CT.

for SCC-25 and RPMI 8226 cells (a further decrease of cell growth was even observed for RPMI 8226) but after 72 h the recovery was total and the number of cells became similar to the number of control cells (Fig. 2A–B). After recovery, morphology of SCC-25 and RPMI 8226 cells previously treated with HSE was comparable to control cells (data not shown). Together these results suggested that HSE had a cytostatic rather than a cytotoxic effect on SCC-25 and RPMI 8226 cells, and this effect was reversible.

### HSE effects on cell motility and transmigration

To evaluate HSE effect on cell motility we performed a scratch assay only for SCC-25 and not for RPMI 8226 cells, because the latter grow in suspension. Untreated SCC-25 cells almost completely closed the wound during the 24 h following the scratch. HSE significantly inhibited SCC-25 cell motility on plastic dish because the complete closure of wound was prevented and the migration area was 40% if compared with untreated cells (Fig. 3: HSE vs. CT).

The ability to pass through anatomic barriers and propagate in cell matrix is one of the most important properties of aggressive tumor cells. To assess the transmigration capacity of SCC-25 and RPMI 8226 across an artificial barrier in vitro in the presence or not of HSE, we performed a Boyden chamber assay using a polycarbonate porous membrane coated with gelatin. Untreated RPMI 8226 and SCC-25 cells passed through the membrane, while HSE treatment reduced cell transmigration more than 50% in both SCC-25 and RPMI 8226 cells (Fig. 4A–B: HSE vs. CT).

### HSE effect on the modulation of MAPKs ERK1/2 and p38

To evaluate the modulation of the MAPKs ERK1, ERK2, and p38 after HSE stimulation of SCC-25 and RPMI 8226 cells, we performed immunoblotting experiments analyzing both kinases activation (i.e., phosphorylation) and expression (total amount) at different time points (from 30 min to 48 h). For each time, cells cultured in the absence of HSE represented the control (CT).

At most of the examined times HSE-treated SCC-25 cells presented a level of ERK1/2 phosphorylation lower than CT (every HSE treatment had a corresponding CT) and this evidence resulted more pronounced and significant for ERK1 than ERK2 (Fig. 5A–B–C). The total amount of ERK1 and ERK2 proteins was unchanged at all examined times in the presence or not of HSE (data not shown). Compared with the corresponding CT, p38 phosphorylation showed a significant increase at 30 min, 1 and 4 h after

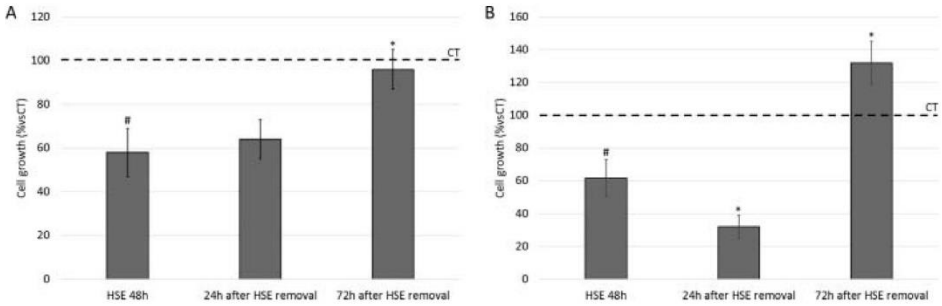


**Figure 2.** Reversibility of HSE effect on SCC-25 and RPMI 8226 cell viability. SCC-25 (A) and RPMI 8226 (B) were treated with HSE 5 mg/ml. After 48 h HSE was removed and replaced with culture medium without HSE for 24 or 72 h. MTT assays were performed to assess cell viability at each time point. Data represent percentage of cell viability in comparison to untreated control cells (CT) that were arbitrarily set to 100% and are reported as mean ± SD of three independent experiments. # $P < 0.05$ vs. CT; * $P < 0.05$ vs. HSE 48 h.

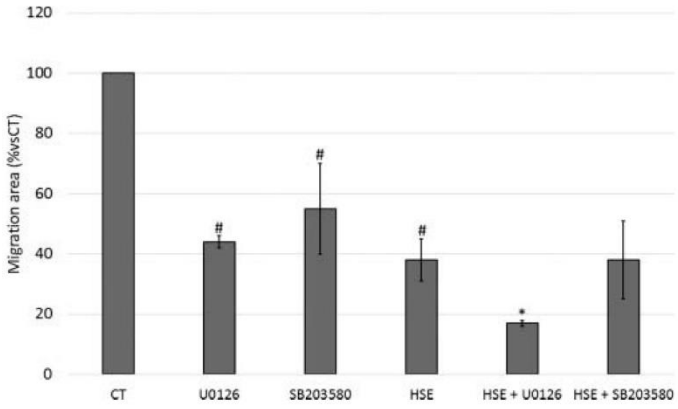


**Figure 3.** Effect of HSE on SCC-25 cell migration. SCC-25 cell migration was assessed with a scratch wound healing assay. 24 h after the scratch, migration areas of untreated (CT) and treated (U0126 10 $\mu$M, SB 203580 1 $\mu$M, HSE 5 mg/ml, HSE 5 mg/ml + U0126 10 $\mu$M and HSE 5 mg/ml + SB 203580 1 $\mu$M) SCC-25 was measured using ImageJ software. Data represent the percentage of wound closure with respect to CT arbitrarily set to 100% and are expressed as mean ± SD of three independent experiments. # $P < 0.05$ vs. CT; * $P < 0.05$ vs. HSE.

HSE treatment. Instead, at 16 h a significant reduction was evident while at 24 and 48 h p38 phosphorylation was quite comparable between treated and CT cells (Fig. 5D–E). At all the examined times the total amount of p38 did not change following treatment with HSE compared with the CT (data not shown).

In HSE-treated RPMI 8226 cells, ERK1 and ERK2 showed a different trend of phosphorylation. ERK1 phosphorylation was similar to CT from 30 min to 16 h then significantly increased at 24 and 48 h. On the other hand, ERK2 phosphorylation was similar to CT from 30 min to 6 h, then for the subsequent times ERK2 phosphorylation increased (Fig. 6A–B–C).

In HSE-treated RPMI 8226 cells, compared with the CT, p38 phosphorylation significantly increase from 30 min to 4 h, then from 6 h to 48 h was similar to CT and no difference in phosphorylation level was evident (Fig. 6D–E). At all the examined times the total amount of ERK1/2 and p38 did not change following treatment with HSE compared with the CT (data not shown).

### *Involvement of ERK1/2 and p38 in SCC-25 and RPMI 8226 cell growth*

To investigate ERK1/2 and p38 involvement in SCC-25 and RPMI 8226 cell growth, we used specific ERK1/2 and p38 inhibitors, U0126 and SB203580, respectively, both in the presence or absence of HSE. MTT assays were performed at 24, 48, and 72 h. For each time, SCC-25 and RPMI 8226 cells cultured in the absence of HSE and U0126 or SB203580 represented the control.

SCC-25 and RPMI 8226 cell growth was reduced by the ERK1/2 inhibitor U0126 (10 $\mu$M) in a time-dependent manner and the decrease became significant after 48 and 72 h of U0126 treatment (Fig. 7A–B: U0126 vs. CT). The reduction of cell growth in presence of U0126 resulted to be higher in RPMI 8226 cells compared with SCC-25 cells. When SCC-25 cells were treated with HSE and U0126, a further and significant decrease of cell growth was observed at 48 and 72 h compared with cells treated only with HSE (Fig. 7A: HSE+U0126 vs. HSE). On the contrary, in RPMI 8226 cells no change was observed in HSE-treated cells either in the presence or absence of U0126 (Fig. 7B: HSE+U0126 vs. HSE).

Cell growth of SCC-25 cells treated with SB203580 (1 $\mu$M) for 24–48–72 h was comparable to untreated SCC-25 cells (Fig. 8A: SB203580 vs. CT). After treatment with SB203580 and HSE, SSC-25 cell growth increased compared with cell treated only with HSE in a time-dependent manner becoming significant at 48 and 72 h (Fig. 8A: HSE+SB203580 vs. HSE).

At all the examined times, RPMI 8226 cells treated with SB203580 (1 $\mu$M) showed an increase in cell growth that was not significant compared with RPMI 8226 cells cultured in absence of the inhibitor (Fig. 8B: SB203580 vs. CT). When RPMI 8226 cells were treated with HSE and SB203580, cell growth increased compared with cells

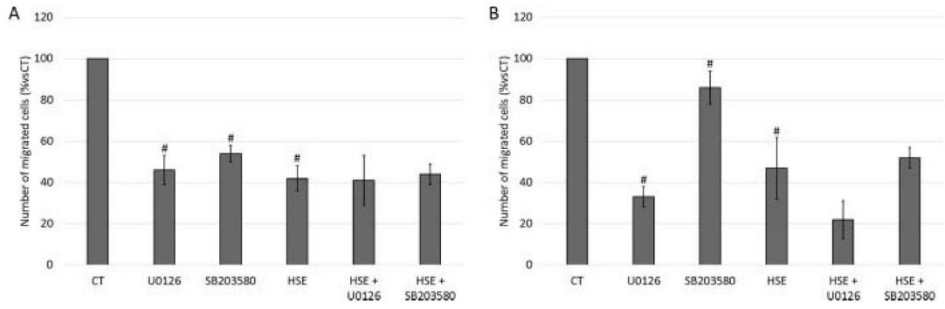


**Figure 4.** Effect of HSE on transmigration. Transmigration of SCC-25 (A) and RPMI 8226 (B) cells was assessed by a Boyden chamber migration assay. Cells were treated with U0126 10 $\mu$M, SB203580 1 $\mu$Mm, HSE 5 mg/ml and their combination (HSE + U0126 and HSE + SB203580. Data represent the percentage of migrating cells with respect to CT arbitrarily set to 100% and are expressed as mean ± SD of three independent experiments. # $P < 0.05$ vs. CT.

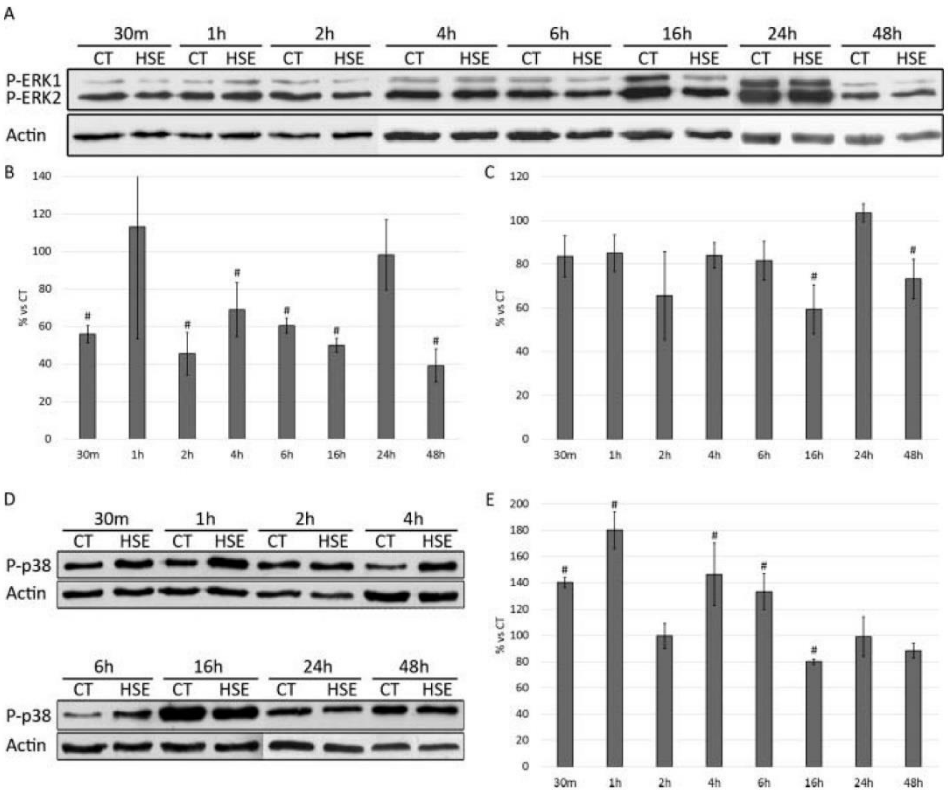


**Figure 5.** HSE effect on ERK 1/2 and p38 phosphorylation in SCC-25 cells. SCC-25 cells were treated with HSE 5 mg/ml at different time points. Total protein extracts were prepared and separated by 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with specific anti P-ERK1/ERK2 and anti P-p38 antibodies. Representative Western blot of P-ERK 1/2 (A) and P-p38 (D). Charts represent the band mean intensity of P-ERK 1 (B), P-ERK 2 (C), and P-P38 (E) normalized to actin. The results are expressed as mean ± SD of two independent experiments, reported as percentage of respective untreated control (CT). # $P < 0.05$ vs. CT.

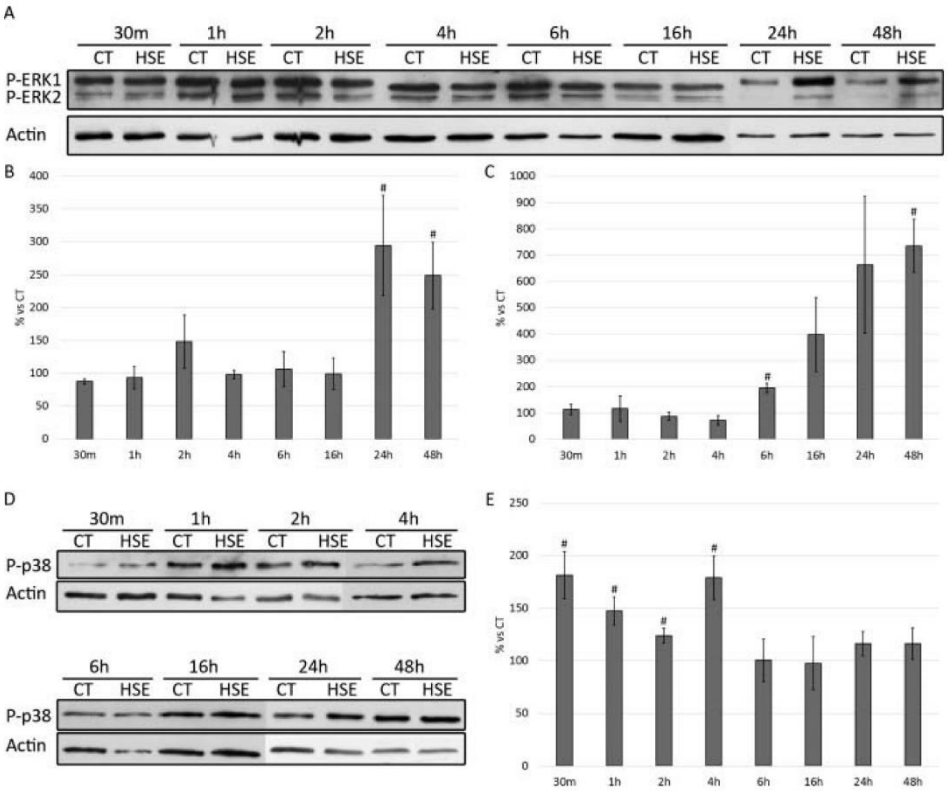


**Figure 6.** HSE effect on ERK 1/2 and p38 phosphorylation in RPMI 8226 cells. RPMI 8226 cells were treated with HSE 5 mg/ml at different time points. Total protein extracts were prepared and separated by 13% SDS-PAGE and transferred to a nitrocellulose membrane that was blotted with specific anti P-ERK1/P-ERK2 and anti P-p38 antibodies. Representative Western blot of P-ERK 1/2 (A) and P-p38 (D). Charts represent the band mean intensity of P-ERK 1 (B), P-ERK 2 (C) and P-p38 (E) normalized to actin. The results are expressed as mean ± SD of two independent experiments, reported as percentage of untreated control (CT). # $P < 0.05$ vs. CT.

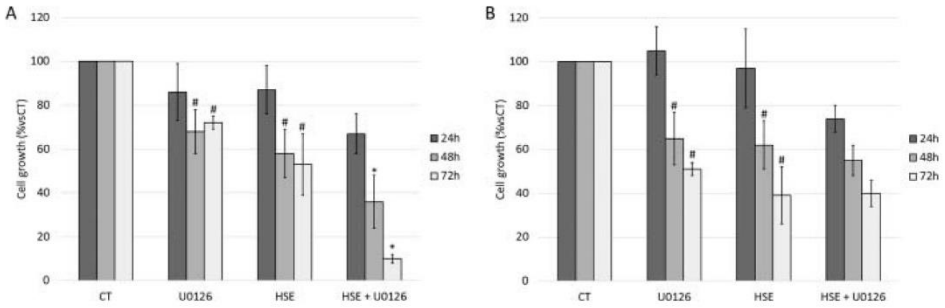


**Figure 7.** Involvement of ERK1/2 in cell viability. SCC-25 (A) and RPMI 8226 (B) cells were treated with HSE 5 mg/ml in the presence or absence of U0126 10 $\mu$M. Cell viability was assessed by MTT assay after 24, 48, and 72 h. Data represent percentage of cell viability in comparison to untreated control cells (CT) that were arbitrarily set to 100% and are reported as mean ± SD of three independent experiments. # $P < 0.05$ vs. CT; * $P < 0.05$ vs. HSE.

treated only with HSE and the increase became significant at 48 and 72 h (Fig. 10B: HSE+SB203580 vs. HSE).

### *Involvement of ERK1/2 and p38 in cell motility and transmigration*

To investigate the involvement of ERK1/2 and p38 in SCC-25 cell motility and transmigration and RPMI 8226 cell transmigration we used U0126 and SB203580 both in the presence or absence of HSE.

Treatment of SCC-25 cells with U0126 (10 $\mu$M) or SB203580 (1 $\mu$M) significantly reduced the closure of wound and the migration area was respectively about 40% and 50% if compared with cells cultured in absence of the inhibitor (Fig 3: U0126 vs. CT; SB203580 vs. CT). In the presence of HSE and U0126, SCC-25 cell migration was further significantly reduced with respect to HSE reduction (see above paragraph) (Fig 3: HSE+U016 vs. HSE). On the contrary the simultaneous HSE and SB203580 treatment did not modify the migration area compared with when cells were treated only with HSE (Fig. 3: HSE+SB203580 vs. HSE).

SCC-25 cells transmigration ability in the presence of U0126 and SB203580 was reduced about 50% compared with untreated cells (Fig. 4A: U0126 vs. CT, SB203580 vs. CT). Nevertheless, when SCC-25 cells were treated simultaneously with HSE and U0126 or SB203580 no difference was evident with respect to HSE-treated SCC-25 (Fig. 4A: HSE + U0126 vs. HSE, HSE + SB203580 vs. HSE).

The transmigration ability of RPMI 8226 cells was affected by ERK inhibitor and the passage through the membrane was reduced about 70% compared with untreated cells (Fig. 4B U0126 vs. CT). When RPMI 8226 were treated simultaneously with HSE and U0126 a further reduction in transmigration capacity (about 80%) was evident compared with RPMI 8226 treated only with HSE (Fig. 4B: HSE+U0126 vs. HSE). p38 instead was not involved in RPMI 8226 transmigration and only a 14% reduction was observed in SB203580 treated cells compared with untreated cells (Fig. 6B: SB203580 vs. CT). SB203580 and HSE combination did not affect RPMI 8226 transmigration ability compared with cells treated only with HSE (Fig 6B: HSE+SB203580 vs. HSE).

## Discussion

In this study, we aimed at evaluating the potential antitumoral effect of HS (HS), a flower mainly cultivated in

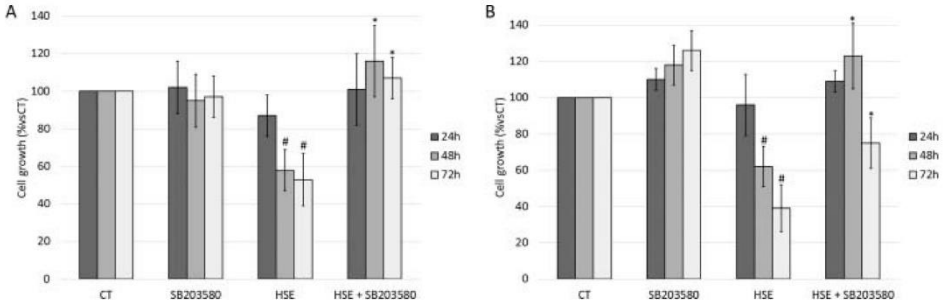


**Figure 8.** Involvement of p38 in cell viability. SCC-25 (A) and RPMI 8226 (B) cells were treated with HSE 5 mg/ml in the presence or absence of SB203580 1 $\mu$M. Cell viability was assessed by MTT assay after 24, 48, and 72 h. Data represent percentage of cell viability in comparison to untreated control cells (CT) that were arbitrarily set to 100% and are reported as mean ± SD of three independent experiments. # $P < 0.05$ vs. CT; * $P < 0.05$ vs. HSE.

Eastern Countries and used in traditional Chinese medicine thanks to its biological properties, i.e. anti-hypertensive, anti-inflammatory.

We have used two cancer cell lines that are completely different in terms of ontology and growth properties: the OSCC cell line SCC-25 and the MM cell line RPMI 8226. OSCC constitutes the large majority of the neoplasia arising in oral cavity and MM (MM) is the second most frequent hematological malignancy. Both tumors are highly metastatic (10,11). Despite recent improvements in OSCC and MM treatment (12,13), these tumors are a relevant health problem and development of novel therapeutic approaches is necessary.

In preliminary dose finding experiments we tested several HSE concentrations (0.5–1–3–5 mg/ml) on the basis of previous works where a concentration range of between 0.05 and 20 mg/ml HSE was used against different cancer cell lines (4,14,15).

Up to now in vivo animal and human studies have been performed with different HSE concentrations calculated as HSE mg/kg weigh of animal/day or in percentile or HSE mg/water or g/water (16,17), but no data exist about HSE pharmacokinetic. Only an article reports the pharmacokinetic parameters of several dietary anthocyanins following consumption of HS L. extract (18). Moreover, no information is available whether the concentrations of HSE used in our and other studies are biologically achievable. Lo et al. (19) affirm that in vitro HS anthocyanins 0.05–4 mg/ml should correspond to 0.2% of an animal standard diet, and to a dosage for human consumption of 1.5 g/day as diet supplement. These data suggest that the concentrations typically used in in vitro studies could be valid but other studies will be necessary to analyze this important topic.

In our hands, HSE proved to be effective in reducing viability of SSC-25 and RPMI 8226 cancer cells in a dose- and time-dependent manner, even if the RPMI 8226 myeloma cells were slightly more sensible.

Although up to our knowledge these are the first data available regarding HSE effect on oral carcinoma and myeloma multiple cell lines, the results obtained are in agreement with previous reported anti-proliferation effect of Hibiscus polyphenolic extract on gastric carcinoma cell line AGS and melanoma cells (4,20). A marked decrease of cell viability induced by one of the main HSE component, i.e. protocatechuic acid, was reported also by Tseng and colleagues (21) on HL-60 leukemia cells. In all of the above reported studies, the HSE effect was stronger than the one observed in our study, but this might depend on the different cell type used. Although the few data available in literature regarding the antitumoral effect of HSE on cancer cell lines have mainly pointed out a cytotoxic and pro-apoptotic effect, our results clearly demonstrated a non-cytotoxic, but rather cytostatic effect of HSE on both SCC-25 and RPMI 8226 cells. The disagreement of our data with other studies reporting a pro-apoptotic effect of HSE might be due not only to dissimilarities among the cell lines used, but also to the kind of HSE used. In fact, completely standardized methods to obtain HSE may turn out in a high variability in HSE component concentrations (22).

Furthermore, the HSE-induced cytostatic effect appears to be reversible, as HSE wash out resulted in an almost complete recovery of cell viability. Although reversibility might be considered a drawback for HSE potential clinical use, several antitumoral compounds have a reversible effect (23–25).

One of the pivotal events in cancer development is the cancer cell's ability to move away from the primary tissue of origin, leading to cancer spreading and metastasization. HSE inhibited cell motility and migration/invasion of SSC-25 and RPMI 8226 cells. Up to now, data regarding HSE effect on cancer cell migration have been reported only for human prostate cancer cells (26). Moreover, some of HSE components have proved to inhibit cancer cell migration such as delphinidin, quercetin, and epigallocatechin-3-gallate (EGCC), respectively, on breast cancer and oral squamous carcinoma cells (27–30).

Although HSE has been attracting research attention for many years now, few data are available regarding the molecular mechanisms underlying its antitumoral activity. Several studies have clearly demonstrated the involvement of MAPKs in multiple processes regulating to cancer cell growth (31). Therefore, we evaluated the potential involvement of MAPKs in HSE effect. In particular, our efforts focused on MAPKs ERK1/2 and p38, which play a central role in several cellular processes such as cell survival, proliferation, and motility (7,32–37).

HSE treatment significantly affected ERK1/2 and p38 phosphorylation both in SCC-25 and RPMI 8226 cells. In SCC-25 cells the decreased ERK1 phosphorylation seems to be responsible for the reduced cell proliferation and motility observed upon HSE treatment. These findings are supported by the observation that when the cells (not stimulated with HSE) are treated with U0126, an inhibitor of ERK phosphorylation, reduction of proliferation and motility was evident. Furthermore, an additive effect occurred when cells were treated with HSE and ERK inhibitor simultaneously. Our results are in agreement with Kim et al. (38) who reported an inhibitory action of HSE on ERK1/2 in 3T3-L1 preadipocytes. Furthermore, there is very little information on the contribution of individual form of ERK, and an important but unresolved question is whether ERK1 and ERK2 have a unique physiological function or are they used

redundantly to reach a threshold of global ERK activity. Our results suggest a role of ERK1 and not ERK2 in HSE effect on SCC-25 cells and this finding could represent an example of specific ERK isoform involvement in cellular processes. Further studies are necessary to elucidate this point.

The basal activation of ERK pathway in RPMI 8226 cells seems crucial for both cell proliferation and migration, as in the presence of U0126 a reduction of both processes was observed. In presence of HSE an increase of ERK1/2 phosphorylation was evident after 24–48 h of treatment. On the contrary the effects of HSE on RPMI 8226 proliferation and migration occur at early time. Furthermore, U0126 inhibition of ERK did not prevent the HSE effects. These results suggest that ERK1/2 pathway is not involved in HSE effects on RPMI 8226 cells.

Both SCC-25 and RPMI 8226 cells had a basal p38 phosphorylation that was not involved in cell proliferation due to presence of a specific p38 inhibitor. In contrast, cell migration was altered in presence of SB203580 in SCC-25 but not in RPMI 8226. In SCC-25 treated with HSE an increase of p38 phosphorylation (compared to control cells) occurred at early time (at 30 min) lasting 6 h. p38 phosphorylation plays a role in inhibition of cell proliferation that occurred in presence of HSE since chemical inhibition of p38 resulted in the complete prevention of HSE effect. Also in RPMI 8226 cells HSE treatment induced a marked increase in p38 phosphorylation (compared to control cells) evident at 30 min after HSE exposure and lasting 4 h. As SCC-25 cells, in RPMI 8226 cells p38 was strongly associated to HSE-induced cell proliferation inhibition, as HSE effect was completely abrogated by chemical inhibition of p38.

Lin et al. (4) have also reported an increase in p38 phosphorylation in AGS gastric carcinoma cells treated with HSE beginning 6 h and lasting 24 h; the authors suggest that p38 phosphorylation could be associated to apoptosis induction. In accordance with Lin et al. (4) we demonstrated an involvement of p38 in HSE effect but we observed a cytostatic and not cytotoxic effect of HSE. The different time courses of p38 phosphorylation and the different cell lines used could be an explanation of this discrepancy; moreover, a non-classical pathway associated to p38 activation, which may be due to involvement in cell cycle blockage without cell death (39,40), could support our hypothesis.

In conclusion, our results demonstrated the antitumoral properties of HS in vitro, as it proved to prevent both cell proliferation and invasiveness of OSCC and MM cells through MAPKs modulation. It is interesting to highlight that HSE was effective against two very different tumor cell lines. In fact, RPMI 8226 cells are of hematopoietic origin and grow in suspension, whereas SCC-25 cells derive from epithelium and are characterized by adherent cell growth. Furthermore, the cytostatic effect observed and the non-toxic profile of HS might be of interest for its use as chemopreventive agent to prevent tumor reoccurrence alone or in a combination therapy.

It is important to keep in mind that our study is a pure in vitro experiment, and this could be a limitation (41). However, undoubtedly our results will provide key information to improve the knowledge of the anticancer properties of HS extract and to perform future in vivo studies in appropriate animal models of MM and oral carcinoma during human clinical trials.

## Acknowledgments

We are grateful to Dr. Anna Rognoni for her language assistance. We would like to thank Prof. Marco Parenti for kindly providing us the Boyden chamber and Dr. F. Guzzi for assistance in Boyden Chamber assay. This work was funded by School of Medicine and Surgery at the University of Milan-Bicocca, Monza, Italy.

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