ASSESSMENT OF ANTICANCER PROPERTIES OF ESSENTIAL OILS FROM AROMATIC PLANTS OF THE SAND DUNES OF PENICHE (PORTUGAL)

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escola superior de turismo
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“The journey of a thousand miles begins with a single step”

Lao Tzu

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Resumo

O cancro é um problema de saúde crescente no mundo e é a segunda causa de morte depois das doenças cardíacas. De acordo com a Agência Internacional de Investigação em Cancro (IARC) existem atualmente mais de 10 milhões de casos de cancro por ano no mundo.

Os produtos naturais oferecem oportunidades de inovação na descoberta de novos fármacos. Neste sentido, os compostos naturais isolados a partir de plantas medicinais, como potenciais fontes de novas drogas anticancerígenas, têm tido um interesse crescente.

Os Óleos Essenciais (OEs) são sintetizados pelas plantas e têm sido estudados pelas suas inúmeras atividades biológicas, incluindo anticancerígena, anti-inflamatória, antimicrobiana, antiviral, antioxidante e repelente de insetos.

Este estudo tem como objetivos determinar a eficácia de OEs de seis espécies de plantas das dunas de Peniche (Portugal), como potenciais agentes terapêuticos anticancerígenos em linhas celulares de cancro da mama (MCF7) e do colo-rectal (RKO), assim como perceber o mecanismo de ação destes OEs.

Neste estudo, partes aéreas de *Artemisia campestris* subsp. *maritima, Crithmum maritimum*, *Eryngium maritimum, Juniperus turbinata* subsp. *turbinata, Otanthus maritimus* e *Seseli tortuosum* foram colhidas na praia da Consolação, em Peniche (Portugal), e os seus OEs isolados através de hidrodestilação. A composição química dos OEs foi investigada por cromatografia gasosa (GC) e por cromatografia gasosa com espetrofotometria de massa (GC-MS) e os compostos maioritários foram descritos para cada óleo.

Para avaliar a atividade anticancerígena nas linhas celulares MCF7 e RKO, o método MTS (3- (4, 5-dimethyl- 2 -thiazolyl) - 2, 5-dyphenyl-2H-tetrazolium bromide) foi usado e a viabilidade celular avaliada, através de diluições sucessivas, a concentrações iniciais de 5 µL/mL e 1 µL/mL, com diluição de 1:2 e 1:10, respetivamente, comparando com o controlo (DMSO). De todos os OEs testados, a atividade anticancerígena foi descrita, em ambas as linhas celulares, como observado pela diminuição da viabilidade/proliferação celular – exceto o OE *Eryngium maritimum* a uma concentração inicial de 5 µL/mL.

Com o objetivo de avaliar o mecanismo biológico de ação dos OEs, foi realizado um *western blot* para marcadores relativos ao bloqueio do ciclo celular e apoptose (p53, p21 e caspase 3 clivada), para *Seseli tortuosum* e *Otanthus maritimus.* Foi observado um aumento do nível proteína p53 nas células tratadas com estes OEs, sugerindo a indução de stress celular nas células cancerígenas testadas. No entanto, não foi observada caspase 3 clivada, sugerindo que a apoptose não terá sido a causa para a diminuição da viabilidade/proliferação celular observada. Foi ainda observado o aumento da expressão da p21 com os OEs selecionados, sugerindo que o tratamento com OE está associado ao bloqueio do ciclo celular. Para validar estas observações, a análise realizada por FACS, depois do tratamento indica um possível bloqueio do ciclo celular na fase G_1.

Concluindo, a concentração inicial de 5 µL/mL revelou ser muito tóxica para as linhas celulares testadas. No entanto, a uma concentração final de 1 µL/mL foi demonstrada uma diminuição da viabilidade/proliferação celular para todos os OEs.

No estudo preliminar do mecanismo de ação dos OEs, foi demonstrado, face à presença da p21, que os óleos de *Seseli tortuosum* e *Otanthus maritimus* atuam bloqueando o ciclo celular. Para comprovar estes resultados, o FACS realizado (apenas no OE de *Seseli tortuosum*) revelou que este bloqueio pode ocorrer, pelo aumento da percentagem de células observadas, na fase G_1.

Estes resultados demonstram o interesse destes OEs de Peniche na procura de novos agentes quimo preventivos contra a progressão do cancro da mama e colo-rectal.

Palavras-passe: Plantas Aromáticas, Óleo Essencial, Propriedades Anticancerígenas.

Abstract

Cancer is a growing health concern around the world and is the second leading cause of death after heart disease. According to the International Agency for Research on Cancer (IARC), there are now more than 10 million cases of cancer per year worldwide.

Novel natural products offer opportunities for innovation in drug discovery. In this sense, natural compounds isolated from medicinal plants, as potential sources of novel anticancer drugs, have been of increasing interest.

Essential Oils (EOs) are synthesised by plants and have been studied regarding their numerous biological activities, including anticancer, anti-inflammatory, antimicrobial, antiviral, antioxidant and insect repellent.

This study aims to determine the efficacy of the EOs from six selected plant species from the dunes of Peniche (Portugal), as potential anticancer therapeutic agents in breast (MCF7) and colorectal (RKO) cancer cell lines, as also understand the mechanism of action of these EOs.

In this work, aerial parts of *Artemisia campestris* subsp. *maritima, Crithmum maritimum*, *Eryngium maritimum, Juniperus turbinata* subsp. *turbinata, Otanthus maritimus* and *Seseli tortuosum* were harvested in Consolação beach, in Peniche (Portugal), and the EOs isolated by hidrodistillation. The chemical composition of EOs was investigated by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) and major compounds were reported for each EO.

To evaluate anticancer activity in MCF7 and RKO cell lines, MTS (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dyphenyl-2H-tetrazolium bromide) assay was carried out and cell viability was evaluated, through successive dilutions, at starting concentrations 5 μL/mL and 1 μL/mL with a dilution 1:2 and 1:10, respectively, comparing to vehicle (DMSO). For all the EOs tested, anticancer activity was observed in both cell lines, as indicated by a decrease in cell viability/proliferation – except for *Eryngium maritimum* EO at starting concentration of 5 μL/mL.

With the aim to evaluate the mechanism of biological effect of the EOs, a Western blot was carried out for markers of cell cycle arrest and apoptosis (p53, p21 and cleaved caspase 3), for *Seseli tortuosum* and *Otanthus maritimus*. Were observed an increase of p53 protein levels in cells treated with these EOs, suggesting that cellular stress was

induced in the cancer cells tested. However, cleaved caspase 3 was not observed, suggesting that apoptosis was not the cause for the observed decrease in cellular viability/proliferation. It was also observed the increased of p21 expression with the selected EOs, suggesting that EO treatment is associated with cell cycle arrest. To validate these observations, FACS analysis performed, indicating a possible G_1 phase cell cycle arrest after treatment.

In conclusion, the starting concentration 5 μL/mL revealed to be very toxic to the cell lines tested. However, at a starting concentration 1 μL/mL showed a decrease in cellular viability/proliferation for every EOs.

In the preliminary study of the mechanism of action of EOs, was demonstrated, due to presence of p21, *Seseli tortuosum* and *Otanthus maritimus* EOs can act by arresting cell cycle. To comprove this results, the FACS performed (just for *Seseli tortuosum* EO) revealed this arrest may be occur, by the increase of percentage of cells observed, in G_1 phase.

Our results show the interest of these EOs from Peniche in search of new chemo preventive agents against breast and colorectal cancer progression.

Keywords: Aromatic Plants, Essential Oil, Anticancer Properties.

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List of Abbreviations

ADP - Adenosine diphosphate

APS – ammonium persulphate

ATM – Ataxia Telangiectasia mutated

ATR – ATM and rad3 related

BSA - Bovine Serum Albumin

Cdc25 – Cell division cycle 25

Cdk – Cyclin dependent kinase

Chk1 – checkpoint kinase 1

Chk2 – checkpoint kinase 2

DMAPP- dimethylallyldiphosphate

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DOX - doxorubicin

DPPH - 2,2-diphenyl-1-1-picrylhydrazyl

DXP - deoxyxylulose phosphate patways

ECL - Enhanced Chemiluminescence

EO – Essential Oil (s)

EtOH - Ethanol

FACS – Fluorescence-activated cell sorting

FBS – Foetal bovine serum

FDA - Food and Drug Administration

FITC - Fluorescein Isothiocyanate

G0- Gap phase 0 of cell cycle

G1 – Gap phase 1 of cell cycle

G2 – Gap phase 2 of cell cycle

HSV-1 - Herpes simplex virus type 1

HSV-2 - Herpes simplex virus type2

IC_{50} – Median inhibitory concentration

IPP - Isopentenyl diphosphate

M- Mitosis

M-Cdk – Mitosis/cyclin-dependent kinase complex

MCF7 AdrR - MCF7 multi-drug resistant

MCF7 WT – MCF7 Wild Type

Mdm2 - Mouse double minute 2 homolog

MDR - multi-drug resistant

MTS - 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

MVA - mevalonate

PAGE - Polyacrylamide Gel Electrophoresis

PARP - Poly(ADP-ribose)-polymerase

PI – Propidium Iodide

PBS – phosphate buffered saline

S – S phase of cell cycle

SDS – Sodium Dodecyl Sulphate

TBS-T - Tris-Buffered Saline Tween

TEMED – tetramethylethylenediamine

1. Introduction

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1.1 Essential Oils

1.1.1 Plant metabolic pathways

Plants possess metabolic pathways through which sugars, amino acids, fats, nucleotides are synthesised (Evans 2002). These pathways constitute the plants primary metabolism since the compounds produced have an essential role in the plant metabolism and are universally present in all plants — these molecules are designated as primary metabolites (Azcon-Bieto & Talon 1996).

Plants can also have other metabolic pathways that lead to the production of compounds characteristic of a taxonomic group (*i.e.* family, genus and species). These pathways constitute the secondary metabolism and their products are designated as secondary metabolites. The biosynthesis of these metabolites is restricted to specific stages of the development and specialized cells of the plant, and can be induced by the stress caused by nutrient deficiency or by the attack by organisms (Azcon-Bieto & Talon 1996).

Unlike primary metabolites, secondary metabolites do not have a direct role in the cells that produce them. Secondary metabolites are involved in functions such as providing characteristic odors, pungencies and colors; plant-plant relations and plant-animal interaction, providing culinary, medicinal or poisonous properties to plants. Although the functions of many secondary metabolites are already well known, for the greatest part of these the value to the plant is still unknown (Evans 2002).

Essential oils (EOs) are secondary metabolites and they are produced in a wide range of plant species, called aromatic plants.

1.1.2 Secretory structures in plants

EOs are found in only 10% of the plant kingdom and can be synthetized by all plant organs, including buds, flowers, leaves, stems, twigs, seeds, fruits, roots and wood or bark (Gershenzon 1994; Ahmadi *et al.* 2002; Bezić *et al.* 2009; Djilani & Dicko 2010). Different organs can share the same kind of secretory structures or possess different types (Fahn 1979). These structures can be classified into six groups: secretory cells, osmophores, secretory cavities, secretory ducts, glandular trichomes (figure 1.1.2.1) and epidermal cells (Svoboda & Svoboda 2000; Fahn 1979).

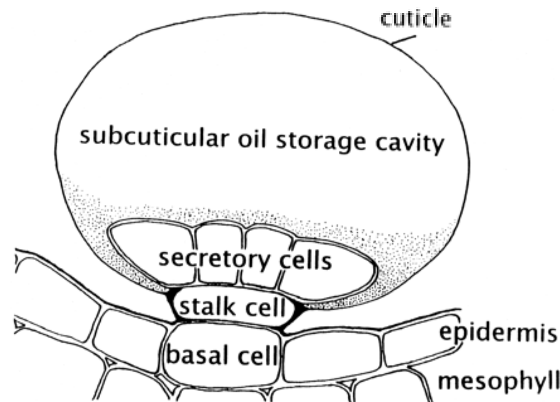

Figure 1.1.2.1 – Schematic diagram of a glandular trichome illustrating the placement of this epidermal structures and the relationship of the disc of secretory cells to the stalk, basal cells and to the subcuticular storage space (Turner *et al.* 2014).

Secretory cells are the simplest secretory structures comprising a single secretion-containing cell where it is only the actual content that distinguishes it from adjacent non-secretory cells (Svoboda & Svoboda 2000). This cell type is found in many different plant tissues, such as in the rhizome pith and cortex of ginger, and in the perisperm and embryo of nutmeg (Svoboda & Svoboda 2000).

Osmophores are areas of flower tissues with secretory cells differing structurally from adjacent cells (e.g. isodiametric in orchids) (Fahn 1979).

Secretory cavities are spherical structures that can be formed in two ways. In one mechanism, the parenchyma cells (thin-walled, relatively undifferentiated cells that may vary in structure and function) can separate one from another, leaving intercellular spaces called lumina or lacuna. The other, an actual cell can disintegrate and leave a cavity within the tissue (Fahn 1979; Svoboda & Svoboda 2000). These spaces are lined with secretory cells or an epithelium that produces the essential oils. In high oil-yielding plants, several layers of these secretory cells are formed. The cavities continually enlarge and some become filled with cells with thin, convoluted walls that also store the oil produced from within their plastids (a class of cytoplasmic organelles) (Fahn 1979; Azcon-Bieto & Talon 1996; Svoboda & Svoboda 2000).

Secretory ducts are elongated cavities. They can often branch to create a network extending from the roots through the stem to the leaves, flowers and fruits. They are

composed of an epithelium that surrounds a central cavity. Several predisposed cells within the parenchyma undergo asynchronous division and in doing so, expand the initial space in the middle, where the cells are adjacent, to form a cavity. Some of the cells forming the wall of the cavity will change into secretory epithelial cells. The oils are biosynthesized within their leucoplasts and move via the endoplasmic reticulum into the cavity. These cavities join up to form ducts (Fahn 1979; Svoboda & Svoboda 2000).

Glandular trichomes are modified epidermal hairs and can be found covering leaves, stems, and even flower parts (Svoboda & Svoboda 2000). The secretory cells are attached by a single stem or basal cell in the epidermis. The outer surface of the gland is heavily cutinised (Fahn 1979). A cuticle, in which no pores or perforations are present, usually completely covers the trichome (Svoboda & Svoboda 2000). The EOs accumulate in subcuticular spaces and are thought to diffuse outwards through the cuticle (Fahn 1979). The glandular cells differ from normal plant cells in that they have a very large nucleus and dense protoplasm that lacks a large central vacuole (Svoboda and Svoboda 2000). There are numerous plasmodesmata (cytoplasmic threads running through cell walls, connecting cytoplasm of adjacent cells) across the walls of the gland cells, especially between the stalk cell and the collecting cell (Fahn 1979). In the very young gland the intracellular organization is almost identical to that of the adjacent cells, but as the secretory cells develop, complex changes occur (Svoboda & Svoboda 2000). The membrane system progressively degenerates and in the fully developed glands only a thin granular cytoplasm (the living cell parts within the membrane, except the nucleus) remains (Fahn 1979; Svoboda & Svoboda 2000).

Secretory cells also occur in flower petals – for example in jasmine and rose. As in this cases glandular hairs and glandular trichomes are not present, the volatile oil diffuses through the **epidermal cells** and the cuticle to reach the atmosphere (Svoboda & Svoboda 2000).

1.1.3 Chemical composition of essential oils

Since ancient times, EOs have been recognised for their medicinal value and are considered very interesting and powerful natural plant products (Baris *et al.* 2006). EOs continue to be of paramount importance until the present day (Margaris *et al.* 1982; Tisserand 1997). Recording findings in Mesopotamia, China, India, Persia and ancient Egypt show their uses for many treatments in various forms (Guenther 1948; Wei &

Shibamoto 2010). For example, in ancient Egypt, the population extracted oils by infusion for medicinal purposes (Burt 2004; Peeyush *et al.* 2011).

EOs naturally occur in aromatic plants, which make these species very valuable. Among many other well-known families rich in species bearing EOs are Apiaceae, Asteraceae, Geraniaceae, Lamiaceae, Pinaceae, Solanaceae, Verbenaceae (Figueiredo *et al.* 1997; Ahmadi *et al.* 2002; Elias *et al.* 2003).

According to the International Standard Organization on Essential Oils, ISO 9235 (1997) of the ISO/TC and the Portuguese Standard, NP 90 (1987) of the IPQ-CT 5, the definition of EO is restricted to the products obtained exclusively by distillation of plant material, with or without water steam, or by mechanical processes applied to the pericarp of fruits of the genus *Citrus* L. EOs are volatile, natural, complex compounds characterised by a strong odour. They are hydrophobic, soluble in alcohol, DMSO and methanol, non-polar or weakly polar solvents, waxes and oils (Gupta *et al.* 2010). EOs are limpid and rarely coloured, most are liquid and of lower density than water (Ciccarelli *et al.* 2008; Martín *et al.* 2010). Due to their molecular structures (presence of olefenic double bonds and functional groups such as hydroxyl, aldehyde, and ester) EOs are readily oxidizable by light, heat and air (Gershenzon 1994; Liolios *et al.* 2010; Djilani & Dicko 2010).

Essential oils are very complex natural mixtures that can contain about 20-60 components at varied concentrations (Miguel 2010). They are composed by two or three major components at fairly high concentrations (20-70%), with other components present in trace amounts. Generally, these major components determine the biological properties of the EOs (Skaltsa *et al.* 2003; Sell 2006; Thomar 2011).

These oils may comprise volatile compounds that have carbon, hydrogen, and oxygen as their building blocks. The major volatile constituents are: hydrocarbons, alcohols, acids, aldehydes, cyclic aldehydes, ketones, lactones, phenols, phenolic ethers, oxides and esters (Bakkali *et al.* 2008). These are subdivided into two categories: of **terpenoid origin**, for compounds composed almost exclusively of terpenes (monoterpenes, sesquiterpenes and diterpenes), and **nonterpenoid origin**, for compounds containing phenylpropanoid and aliphatic skeletons. Some compounds may also contain nitrogen (antharanilates, pyrazines) or sulphur (thiols, sulphides) (Bakkali *et al.* 2008; Baser & Demirci 2007; Kalemba & Wajs 2011). All of them are hydrocarbons and their oxygenated derivatives (Proença da Cunha *et al.* 2003).

Terpenoids (figure 1.1.3.1) are the most structurally varied class of plant natural products (Bakkali *et al.* 2008). In nature, they play significant roles in plant-environment interactions, plant-plant communication and plant-animal interactions (Pichersky & Gershenzon 2002). They are commercially important due to their wide application in a vast number of industrial products, such as flavouring agents, pharmaceuticals, perfumes, insecticides and anti-microbial agents (Martin *et al.* 2003).

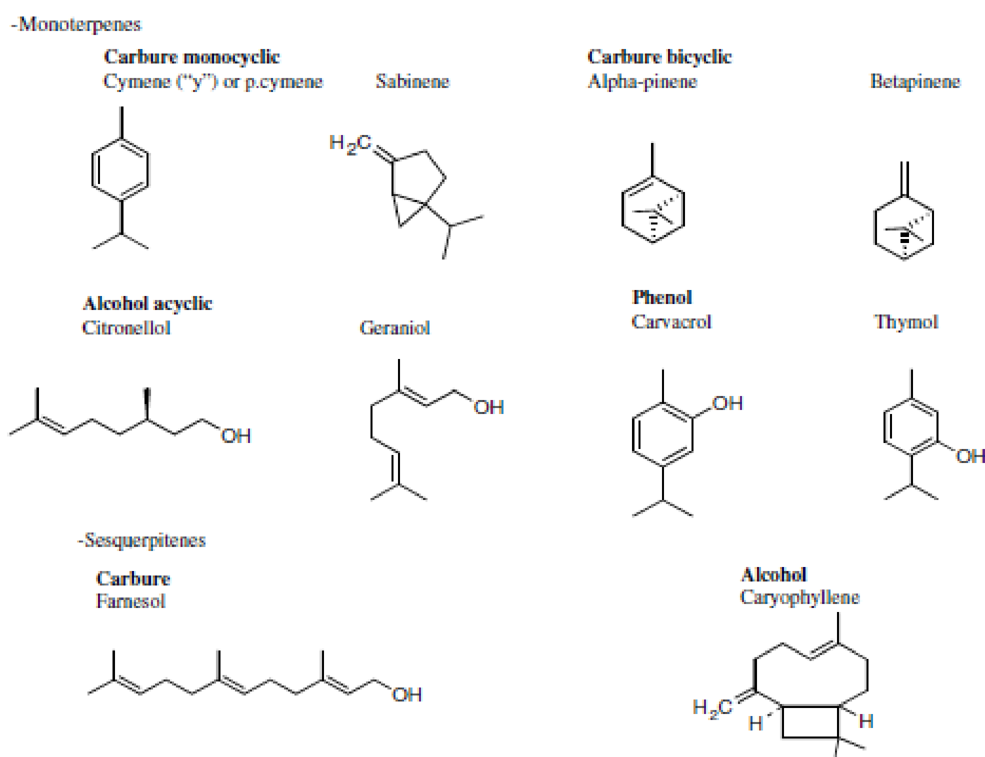

Figure 1.1.3.1 – Chemical structures of terpenoids (selected compounds of essential oils). Monoterpenes have a variety of structures, for example - Carbures, Alcohols, Aldeydes, Ketones, Esters and Ethers. The structures of the sesquiterpenes - Carbures, Alcohols, Ketones and Epoxides - are similar comparing to the monoterpenes (modified after Bakkali *et al.* 2008).

Terpenes result from the condensation of the pentacarbonate unit, 2-methilbutadiene or isoprene (Dubey *et al.* 2003). For this reason they are also called isoprenoides. These are classified according to the isoprene units (five carbons) in their structure: hemiterpenes C5 (1 isoprene unit), monoterpenes C10 (2 isoprene units), sesquiterpenes C15 (3 isoprene units), diterpenes C20 (4 isoprene units), sesterpenes C25 (5 isoprene units), triterpenes C30 (6 isoprene units), tetraterpenes C40 (8 isoprene units) and polyterpenes (C5) n (Dubey *et al.* 2003). Dimers and trimers of isoprene, monoterpenes with 10

carbons and sesquiterpenes with 15 carbons, respectively, are predominant in essential oils (Gershenzon & Kreis, 1999; Proença da Cunha *et al.* 2003).

In higher plants, terpenes are synthesised *via* two isopentenyl diphosphate (IPP) generating pathways. Isopentenyl diphosphate (IPP) and dimethylallyldiphosphate (DMAPP) serve as universal precursors for the biosynthesis of terpenes. They are biosynthesised through the *mevalonate* (MVA) and the *non-mevalonate* or *deoxyxylulose phosphate pathways* (DXP) (Croteau 2000; Sangwan *et al.* 2001; Dubey *et al.* 2003; Baser & Demirci 2007) (Figure 1.1.3.2).

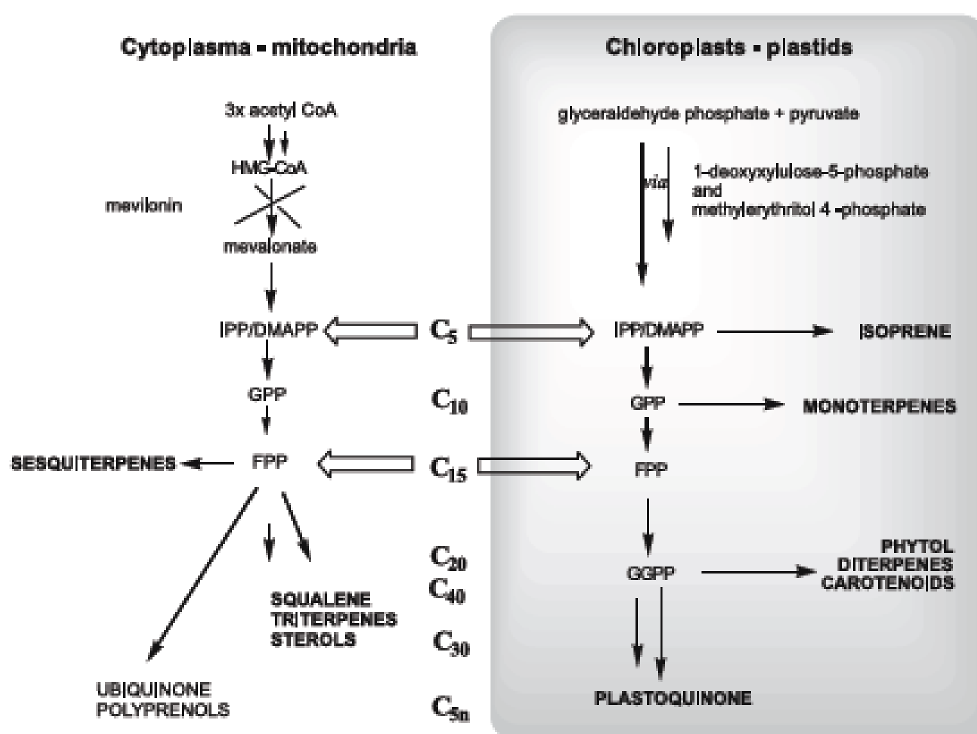

Figure 1.1.3.2 – The two different terpenoid classes in the mitochondria and plastids , respectively (from Baser & Demirci 2007).

Nonterpenoids or aromatic compounds (Figure 1.1.3.3) are derived from phenylpropane and occur less frequently than the terpenes. The biosynthetic pathways concerning terpenes and phenylpropanic derivatives generally are separated in plants but may coexist in some, with one major pathway taking over. Nonterpenoid compounds comprise: Aldeyde; alcohol, phenols, methoxy derivatives and methylene dioxy compounds (Bick & Lange 2003; Baser & Demirci 2007; Bakkali *et al.* 2008)

Nitrogenous or sulphured components such as glucosinolates or isothiocyanate derivatives (garlic and mustard oils) are also characteristic as secondary metabolites of diverse plants or of torrefied, grilled or roasted products (Bick & Lange 2003; Baser & Demirci 2007).

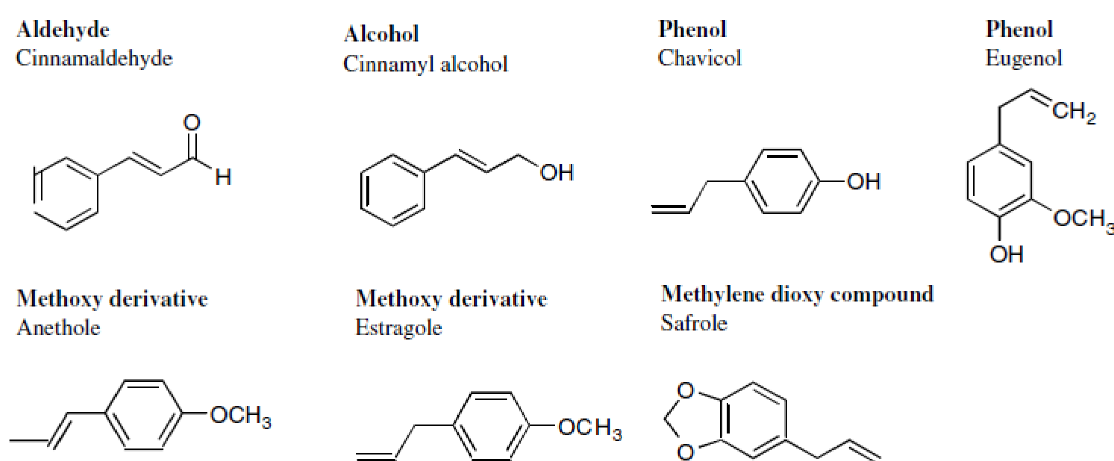

Figure 1.1.3.3 – Chemical structures of non-terpenoids compounds (selected compounds of essential oils) (modified after Bakkali *et al.* 2008).

1.1.4 Factors influencing the production and quality of essential oils

The fragrance and chemical composition of essential oils may vary according to the geo-climatic location and growing conditions (soil type, climate, altitude and amount of water available), season (before and after flowering), and time of day of harvesting (Djilani & Dicko 2010). Another important factor is the genetic composition of the plant. All these factors (genetic and epigenetic) influence the biochemical synthesis of EOs in a given plant (Bakkali *et al.* 2008; Adorjan & Buchbauer 2010). In this way, specimens from same species of plant can produce a similar essential oil but with different chemical composition, resulting in different therapeutic activities (Djilani & Dicko 2010).

1.1.5 Extraction techniques

The two main industrial processes used to obtain EOs are distillation and expression. However, there are more methodologies to obtain other aromatic extracts from aromatic plants, such as extraction by solvents, and adsorption techniques (Lawrence 1995; Başer & Demirci 2007; Figueiredo *et al.* 2007; Proença da Cunha *et al.* 2003).

The main method, at a laboratory scale that is used to obtain EOs from plant material is the distillation with water (hydrodistillation), using a Clevenger apparatus (figure 1.5.1.1). However, if not enough plant material is available to obtain the EOs by hydrodistillation, solvent extraction or solid phase micro-extraction can be used as alternative extraction processes to obtain a small amount of the volatile compounds and determine the chemical composition.

1.1.5.1 Hydrodistillation

The principle of this process is to boil a suspension of an aromatic plant material in water so that its vapour can be condensed. The oil, which is immiscible with the water, is then separated. In this extraction process, the plant material is always in direct contact with water. This technique has the advantage of only dragging volatile substances, and is simple and cheap (Lawrence 1995).

The apparatus (figure 1.1.5.1.1) described in the Portuguese Pharmacopoeia (2005), for dosage of essential oils in aromatic plants comprises the following parts: (1) a suitable round-bottomed flask with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end, where the plants and the water boil; (2) a condenser assembly that closely fits the flask; (3) a suitable heating device, allowing a fine control; and (4) a vertical support with a horizontal ring covered with insulating material (Farmacopeia Portuguesa 2005).

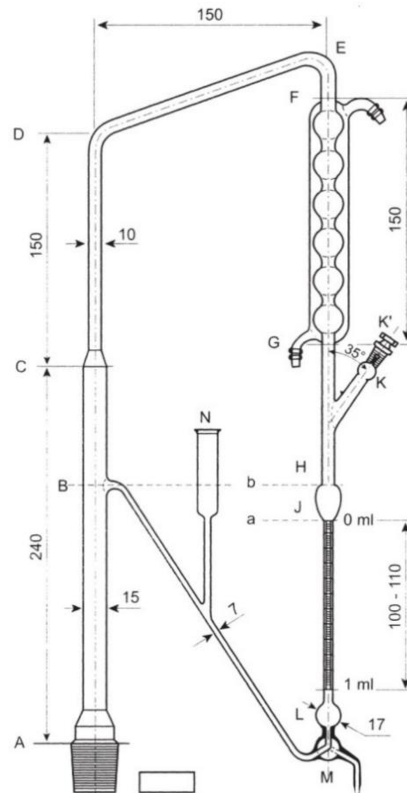

Figure 1.1.5.1.1 – Clevenger apparatus. Dimensions are indicated in millimetres (Farmacopeia Portuguesa 2005).

1.2 Biological Properties of Essential Oils

Approximately 3000 EOs have been described known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Djilani & Dicko 2010). For example, d-limonene, geranyl acetate or d-carvone are employed in perfumes, creams, soaps, as flavor additives for food, as fragrances for household cleaning products and as industrial solvents (Hajhashemi *et al.* 2003; Perry *et al.* 2003). However, the information available on the estimation of precisely number EOs is scarce (Silva *et al.* 2003; Djilani & Dicko 2010).

Essential oils are used in massage vegetable oil mixtures or added to baths but most frequently in aromatherapy (Perry *et al.* 2003). Importantly, EOs are also extremely useful as therapeutic agents for several pathologies. The use of EOs in medical applications has become very popular, both for the complex oil mixes, as well as the individual constituents as single compounds (Hajhashemi *et al.* 2003; Perry *et al.* 2003; Djilani & Dicko 2010).

Essential oils have been largely employed for their properties observed in nature, i.e. for their anti-nociceptive effect, insect repellent activity, antiviral activity, as well as other proprieties, such as antioxidant activity and anticancer activity.

1.2.1 Antinociceptive Effect

A nociceptor is a sensory receptor that responds to potentially damaging stimuli by sending nerve signals to the spinal cord and brain (Hucho & Levine 2007; Maham *et al.* 2013). The antinociceptive effect is a reduction in pain sensitivity made within neurons when endorphin or a similar opium-containing substance combines with a receptor (Maham *et al.* 2013).

Sousa and collaborators analysed the antinociceptive and anti-inflammatory effects of the EO extracted from *Eremanthus erythropappus* (DC.) McLeish (Asteraceae) leaves. In *in vivo* studies using mice, treatment with the *Eremanthus* EO led to a significant increase of the reaction time (reduction in pain sensitivity) after 30, 60 and 90 minutes of treatment, at doses of 200 mg/kg and 400 mg/kg (Sousa *et al.* 2008).

In 2008, the antinociceptive activity of the volatile oils of *Hyptis pectinatal* L. Pois (Lamiae) genotypes were analysed by Arrigoni-Blank and co-workers (Arrigoni-Blank *et al.* 2008) The use of *H. pectinata* is very common in Brazilian folk medicine for the treatment of inflammations, bacterial infections and pain. Six genotypes of volatile oil were investigated. The main compounds of all genotypes are sesquiterpenes. In the models used, all genotypes showed antinociceptive effect, for both central and peripheral nervous system (Arrigoni-Blank *et al.* 2008).

Takaki and colleagues investigated the anti-inflammatory and anti-nociceptive effects of *Rosmarinus officinalis* L. (Lamiaceae) EO in experiment in animal models (Takaki *et al.* 2008). In this study, it was shown that Eos extracted from *Rosmarinus officinalis*, at a dose of 500 mg/kg, led to a significant reduction of the volume of pleural exudate and slightly decreased the number of cells that had migrated in treated animals, when compared with control animals (not treated) (Takaki *et al.* 2008).

1.2.2 Insect Repellent Activity

Currently, the use of synthetic chemicals to control insects and arthropods raises several concerns related to the environment and human health. Therefore, there is a growing demand for alternative repellents or natural products. These products should possess good efficacy and be environmentally friendly (Nerio *et al.* 2010). Essential oils from several species have been extensively tested to assess their repellent and even insecticidal properties as a valuable natural source (Nerio *et al.* 2010).

In 2007, Rajkumar and colleagues investigated the repellent effect of selected EO against the malaria fever mosquito *Anopheles stephensi* in mosquito cages (Rajkumar *et al.* 2007). The five EOs tested were from *Centella asiatica*; *Ipomoea cairica*; *Momordica charantia; Psidium guajava* and *Tridax procumbens*. The oils were tested at three concentrations: 2, 4 and 6% (v/v). A dose-dependent effect was noticed and the highest concentration (6%) led to the highest repellent effect (Rajkumar *et al.* 2007).

In a study looking at the repellent effects of catmint (*Nepeta cataria* L., Lamiaceae) oil formulations against black flies (*Simulium decorum* Walker) and mosquitoes (primarily *Aedes intrudens* Dyar) in Maine and Florida showed that tested all formulations led to protection against mosquitoes for more than 4 hours (Rajkumar *et al.* 2007).

1.2.3 Antiviral Activity

A virus is a small infectious particle (20-300 nm) that is able to infect cells of another living organism, in which it can replicate itself (Saddi *et al.* 2007). Viruses cannot reproduce on their own: a virus is composed of genes and a protein coat, and some have an envelope of fat that surrounds them. Viruses can lead to infections, which provoke an immune response that usually eliminates the infecting virus (Saddi *et al.* 2007; Loizzo *et al.* 2008).

In 2007, Saddi and colleagues investigated the activities of the EOs from *Artemisia arborescens L.* (Asteraceae) against herpes simplex virus 1 and 2 (HSV-1 and HSV-2), since new prophylactic and therapeutic tools are needed. Using MTT assay, the result of this study showed that the IC_{50} values were 2.4 and 4.1 μg/mL for HSV-1 and HSV-2, respectively. Furthermore, the study showed that the antiviral activity of the EOs was principally due to direct virucidal effects, but also led to inhibition of the cell-to-cell virus diffusion of both HSV-1 and HSV-2 (Saddi *et al.* 2007).

An *in vitro* evaluation of the biological activity against HSV-1 was carried out with *Cedrus libani* A. Rich EO and ethanol extracts of cones, by Loizzo and collaborators (Loizzo *et al.* 2008). In this study, it was shown that ethanol extracts and EO possess anti-viral activity with IC_{50} values of 0.50 mg/mL and 0.44 mg/mL, respectively.

1.2.4 Antioxidant Activity

Antioxidant compounds in food play an important role as a health-protecting factor (Saeed *et al.* 2012). Antioxidants reduce the risk for chronic diseases including cancer and heart diseases (Camire *et al.* 2005; Saeed *et al.* 2012).

Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables (Saeed *et al.* 2012). Plant sources of food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce disease risk (Camire *et al.* 2005; Saeed *et al.* 2012). Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Some compounds, such as gallates, have strong antioxidant activity, while others, such as the monophenols are weak antioxidants (Camire *et al.* 2005; Saeed *et al.* 2012).

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative diseases (Saeed *et al.* 2012). Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxides, hydroperoxides or lipid peroxyl, and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Saeed *et al.* 2012).

In 2007, Sharififar and colleagues investigated the antioxidant and free radical scavenging activities of the EOs from flowers of *Otostegia persica* Boiss (Lamiaceae) (Sharififar *et al.* 2007). This study showed that this EO possesses high antioxidant and radical scavenging activity in both DPPH (2,2-diphenyl-1-1-picrylhydrazyl) free radical scavenging test and ammonium thiocyanate tests. In the DPPH free-radical scavenging protocol, the EO showed antioxidant activity with an IC_{50} value of 19.8 µg/mL. In the second protocol, the EO exhibited an inhibition rate of oxidation of linoleic acid of 93.5% (percentage of total inhibition of oxidation) (Sharififar *et al.* 2007)

Chaieb and colleagues investigated the antioxidant properties of the EO in clove (*Eugenia caryophyllata* (L. Myrtaceae) (Chaieb *et al.* 2007). The antioxidant activity was evaluated by the DPPH free-radical scavenging test and the IC_{50} value was 0.2 µg/mL, revealing that the oil has a very strong radical scavenging activity (Chaieb *et al.* 2007).

1.2.5 Anticancer Activity

Despite recent advances in treatment modalities, cancer remains a major source of morbidity and mortality throughout the world (Edwards *et al.* 2005; Hesketh 2013). In the United States, cancer is the leading cause of death for individuals less than 85 years of age (Jemal *et al.* 2006). Moreover, the incidence of many cancers, including cancers of the skin, prostate, breast, and kidney, continues to increase (Edwards *et al.* 2005). A recent study, led by Cancer Research UK (CRUK), indicated that one in two individuals born after 1960 will be diagnosed with cancer at some point in their lifetime (Ahmad *et al.* 2015)

Cancer is, in fact, a general term that refers to over 100 distinct diseases affecting many different tissues and cell types. However, all forms of cancer are characterised by abnormal cell growth resulting from a relatively small number of inherited or environmentally-induced genetic mutations (Hesketh 2013).

In their seminal review, Hanahan and Weinberg have argued that in order for a cell to become cancerous, it must acquire six unique traits as a result of altered cell physiology (Hanahan & Weinberg 2011). These defining traits of cancer cells are: (1) the ability to generate their own growth signals or respond to weak growth signals that are ignored by healthy cells; (2) insensitivity to anti-proliferative signals; (3) resistance to cell death by apoptosis; (4) the capacity for limitless replication; (5) the ability to stimulate new blood vessel development in order to allow for tumour growth; and (6) the capacity to invade tissues, at first locally, and later to spread or metastasize throughout the body (Hanahan & Weinberg 2011).. Although localized cancers can often be successfully treated by surgery and/or radiation therapy, chemotherapy remains the usual treatment of choice for advanced or metastatic disease (Espinosa *et al.* 2003). However, the use of conventional chemotherapeutic agents that typically target rapidly dividing cancer cells is often associated with deleterious side effects caused by inadvertent drug-induced damage to healthy cells and tissues (Cassidy *et al.* 2002).

Moreover, cancer cells that are quiescent or slowly proliferating are refractory to the cytotoxic effect of chemotherapeutic drugs that act at the level of DNA synthesis (Naumov *et al.* 2003). Cancer cells also frequently become resistant to chemotherapy as a consequence of cellular changes that include increased expression of drug-detoxifying enzymes and drug transporters, altered interactions between the drug and its target, altered DNA damage capacity, and defects in the cellular machinery that mediate apoptosis (Gatti & Zunino 2005).

Therefore, the development of a new class of anticancer drugs that lack the toxicity of conventional chemotherapeutic agents and are unaffected by common mechanisms of chemoresistance would be a major advance in cancer treatment (Gatti & Zunino 2005).

Some data strongly support the view that EOs have potential therapeutic applications in the prevention of cancer, but EOs remain mainly poorly researched.

Ravizza and colleagues investigated the anticancer properties of linalool, a plant-derivated monoterpene alcohol that is found in the essential oils from many aromatic plants (Ravizza *et al.* 2008). The cancer modules for this study were two human breast adenocarcinoma cell lines, MCF7 WT (wild type) and multi-drug resistant MCF7 AdrR (selected as being resistant to treatment with the chemotherapy agent Adriamycin, also known as doxorubicin). Linalool was used either as single agent or in combination with doxorubicin. Linalool treatment alone only mildly inhibited cell proliferation. However, at sub-toxic concentrations when in combination with doxorubicin, it led to increased doxorubicin-induced cytotoxicity and pro-apoptotic effects in both cell lines. It was therefore concluded that combination with linalool could lead to an improved therapeutic index of anthracyclines (such as doxorubicin) in the treatment of breast cancer, especially in multi-drug resistant tumours (Ravizza *et al.* 2008).

Another study analysed the induction of mitochondria-associated (or intrinsic) apoptosis by EO extracted from *Tanacetum gracile* Hook., (Asteraceae), an alpine aromatic herb that contains about 40 components (Verma *et al.* 2008). This EO led to an inhibition the proliferation of the HL-60 leukaemia cell line, with an IC_{50} value of 27 μg/mL, associated with induction of apoptosis. Furthermore, the study showed that this effect was mediated by the mitochondria, since it led to a decrease of mitochondrial membrane potential, release of cytochrome C, activation of caspases-9 and -3, and an increase poly(ADP-ribose)-polymerase (PARP) cleavage.

In another report, Medina-Holguín and colleagues reported on the chemo-typical variation of EOs in medicinal plant *Anemopsis californica* (Nutt.) Hook., Saururaceae (Medina-Holguín *et al.* 2009). The authors extracted EOs from roots/rhizomes of the *Anemopsis californica* (ACEO) plant by steam distillation. It was then analysed for its effect on AN3CA (uterine) and HeLa (cervical) human cancer cell lines. The study showed an antiproliferative activity of the EO against both cell lines *in vitro*, with IC_{50} values of 0.056% and 0.052% (v/v) for AN3CA and HeLa cells, respectively. The three main compounds, thymol, piperitone and methyleugenol, were tested independently for growth inhibition against the AN3CA and HeLa cells and also inhibited cell growth. The IC_{50} values for these three compounds against each cell line was determined and compared with the concentration of these compounds in the root oil of *A. californica* (Medina-Holguín *et al.* 2009). The inhibition may be the result of a synergistic relationship between the combined abundant compounds piperitone and methyleugenol, or also with a minor component in the oil. In conclusion, the study showed the specific bioactivity against uterine and cervical cancer cell lines of steam-distilled oil of *Anemopsis californica* root tissue, thus supporting the traditional and cultural use of ACEO to treat uterine cancer.

Finally, another study analysed the antibacterial, antifungal, and anticancer activities of volatile oils and extracts from stems, leaves and flowers of *Eucalyptus siderxylon* A. Cunn and *Eucalyptus torquata* grown in Egypt (Ashour 2008). To analyse the anticancer activity, the sulforhodamine B (SRB) assay was used, an evaluation of cell density based on the measurement of cellular protein content (Ashour 2008). The *in vitro* cytotoxic activities of the essential oils and extracts were evaluated using the hepatocellular carcinoma cell line HEPG2 and the breast adenocarcinoma cell line MCF7. The results indicated that the EOs extracted from *E. torquata* leaves and stems and of *E. sideroxylon* leaves exert cytotoxic effect activity against MCF7 cells, but no effect on HEPG2 cells (Ashour 2008).

1.3 Key concepts in cancer biology

An abnormal cell that grows (increases in mass) and proliferates (divides) out of control will give rise to a tumour, or *neoplasm* (Knowles & Selby 2005). As long as the neoplastic cells have not yet become invasive, however, the tumour is said to be benign. For most types of such neoplasms, removing or destroying the mass locally usually achieves a complete cure. A tumour is considered a true cancer if it is malignant; that is, when its cells have acquired the ability to invade the surrounding tissue, and invasiveness is an

essential characteristic of cancer cells. It allows them to loose, intravase (or enter) into blood or lymphatic vessels, and form secondary tumours called metastases at distant sites in the body (Figure 1.3.1) (Knowles & Selby 2005). Key to early tumourgenesis is a deregulation of cell cycle progression and defective responses to DNA damage (Bartkova *et al*. 2005; Gorgoulis *et al.* 2005).

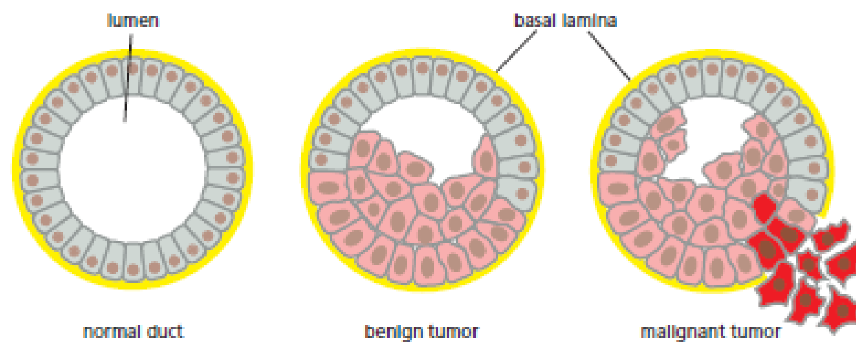

Figure 1.3.1 - Benign versus malignant tumours. A benign glandular tumour (pink cells; an adenoma) remains inside the basal lamina (yellow) that marks the boundary of the normal structure (a duct, in this example). In contrast, a malignant glandular tumour (red cells; an adenocarcinoma) can develop from a benign tumour cell, and it destroys the integrity of the tissue, as shown (from Alberts *et al*. 2008).

1.3.1 Cell cycle regulation

The normal cell-cycle checkpoints governs cell-cycle progression at three major regulatory transitions (Karp 2002; Knowles & Selby 2005). The first is Start (or the restriction point) in late G1, where the cell commits to cell-cycle entry and chromosome duplication (Knowles & Selby 2005). The second is the G2/M transition, where the control system triggers the early mitotic events that lead to chromosome alignment on the mitotic spindle in metaphase. The third is the metaphase-to-anaphase transition, where the control system stimulates sister-chromatid separation, leading to the completion of mitosis and cytokinesis (Knowles & Selby 2005). The control system blocks progression through each of these transitions if it detects problems inside or outside the cell. If the control system senses problems in the completion of DNA replication, for example, it will hold the cell at the G2/M transition until those problems are solved (Karp 2002; Knowles & Selby 2005). Similarly, if extracellular conditions are not appropriate for cell proliferation, the control system blocks progression through Start, thereby preventing cell division until conditions become favourable (Karp 2002; Knowles & Selby 2005).

Central components of the cell-cycle control system are members of a family of protein kinases known as cyclin-dependent kinases (CDKs) (Karp 2002). The expression and activity of these kinases rise and fall as the cell progresses through the cycle, leading to cyclical changes in the phosphorylation of intracellular proteins that initiate or regulate the major events of the cell cycle (Karp 2002). An increase in CDK activity at the G2/M transition, for example, increases the phosphorylation of proteins that control chromosome condensation, nuclear-envelope breakdown, spindle assembly, and other events that occur in early mitosis (Karp 2002).

Cyclical changes in CDK activity are controlled by a complex array of enzymes and other proteins (Knowles & Selby 2005). The most important of these CDK regulators are proteins known as cyclins. Cdks, as their name implies, are dependent on cyclins for their activity: unless they are bound tightly to a cyclin, they have no protein kinase activity. Cyclins were originally named because they undergo a cycle of synthesis and degradation in each cell cycle (Knowles & Selby 2005; Karp 2002). Cyclical changes in cyclin protein levels result in the cyclic assembly and activation of cyclin–CDK complexes at specific stages of the cell cycle (Karp 2002).

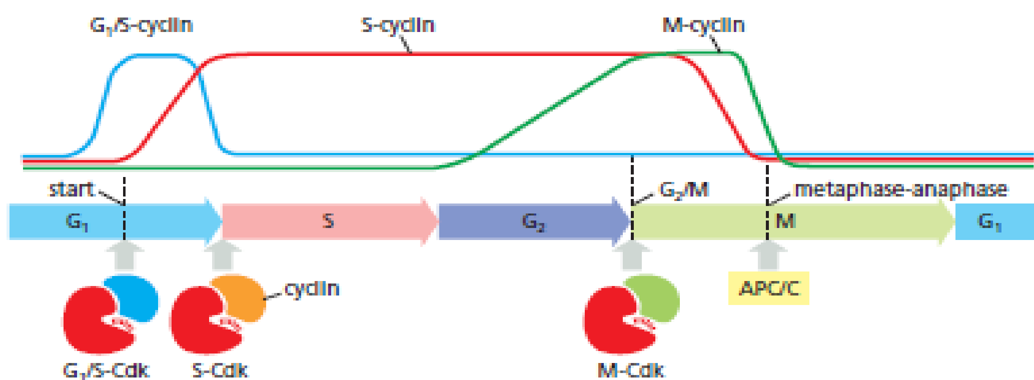

Figure 1.3.1.1 - Cyclin–CDK complexes of the cell-cycle control system. The concentrations of the three major cyclin types oscillate during the cell cycle, while the concentrations of CDKs (not shown) do not change and exceed cyclin amounts. In late G1, rising G1/S-cyclin levels lead to the formation of G1/S-Cdk complexes that trigger progression through the Start transition. S-Cdk complexes form at the start of S phase and trigger DNA replication, as well as some early mitotic events. M-Cdk complexes form during G2 but are held in an inactive state; they are activated at the end of G2 and trigger entry into mitosis at the G2/M transition. A separate regulatory protein complex, the APC/C, initiates the metaphase- to- anaphase transition (from Alberts *et al*. 2008).

There are four classes of cyclins, each defined by the stage of the cell cycle at which they bind CDKs and function. All eukaryotic cells require three of these classes (Karp 2002) (Figure 1.3.1.1):

1. G_1/S-cyclins activate Cdks in late G_1 and thereby help trigger progression through Start, resulting in a commitment to cell-cycle entry. Their levels fall in S phase.

2. S-cyclins bind Cdks soon after progression through Start and help stimulate chromosome duplication. S-cyclin levels remain elevated until mitosis, and these cyclins also contribute to the control of some early mitotic events.

3. M-cyclins activate Cdks that stimulate entry into mitosis at the G_2/M transition.M-cyclin levels fall in mid-mitosis.

4. The G_1-cyclins helps govern the activities of the G_1/S-cyclins, which control progression through Start in late G1.

1.3.2 The DNA damage response

Progression through the cell cycle, and thus the rate of cell proliferation, is controlled not only by extracellular mitogens but also by other extracellular and intracellular signals (Morgan 2007). A key factor for induction of cell cycle arrest is the presence of DNA damage, which can occur as a result of spontaneous chemical reactions in DNA, errors in DNA replication, or exposure to radiation or exogenous chemicals (Morgan 2007). It is essential that damaged DNA and chromosomal damage are repaired before attempting to duplicate or segregate them (Morgan 2007; Bertoli *et al.* 2013). The cell-cycle control system can readily detect DNA damage and arrest the cycle at three DNA damage checkpoints transitions: at the transition of G_1 and S (G_1/S), which prevents entry into the cell cycle and into S phase; during S-phase, when DNA replication stalling can be detected (intra-S); and at the G_2/M transition (G_2/M), which prevents entry into mitosis (Morgan 2007; Bertoli et al. 2013).

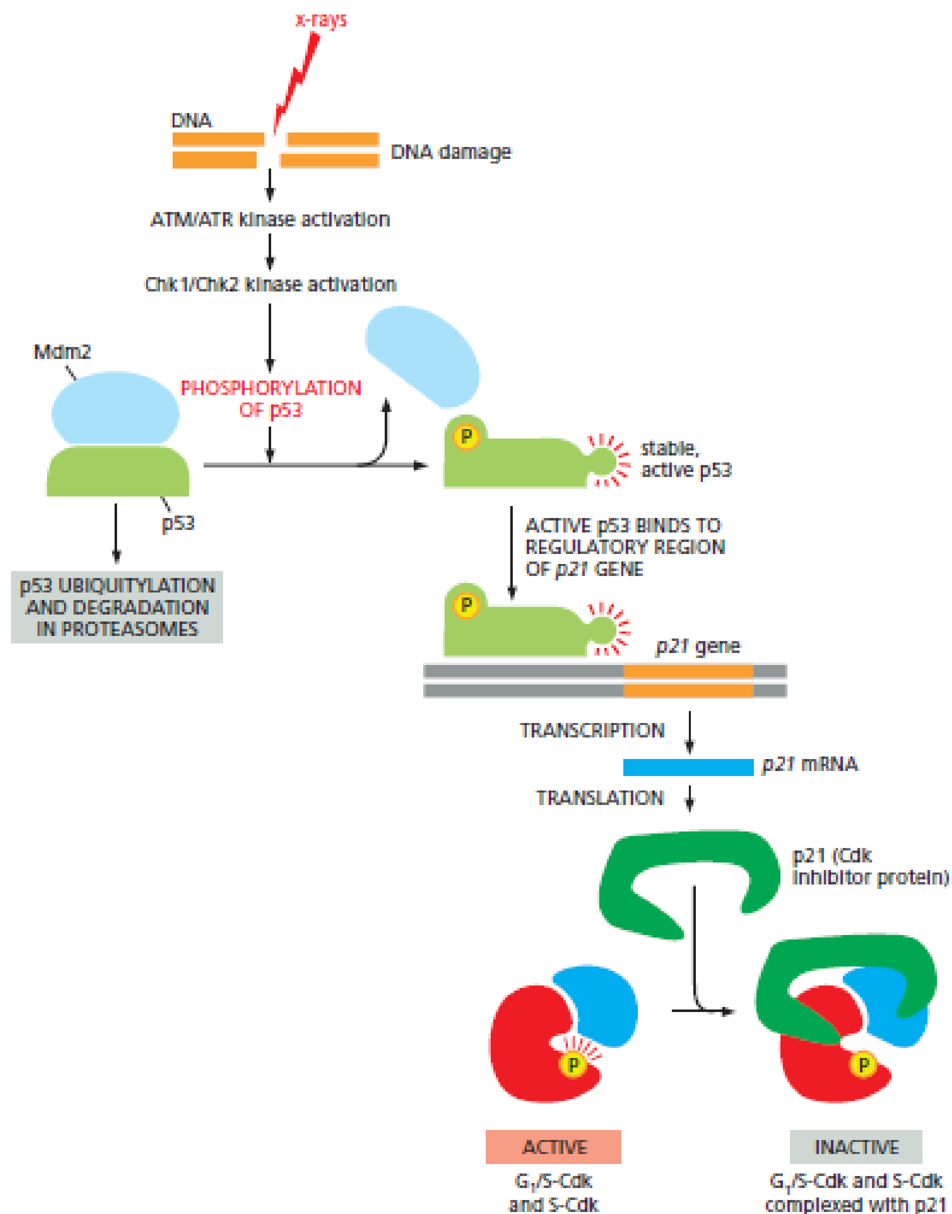

Figure 1.3.2.1 - How DNA damage arrests the cell cycle in G_1 phase. When DNA is damaged, various protein kinases are recruited to the site of damage and initiate a signalling pathway that causes cell-cycle arrest. The first kinase at the damage site is either ATM or ATR, depending on the type of damage. Additional protein kinases, called Chk1 and Chk2, are then recruited and activated, resulting in the phosphorylation of the transcription regulatory protein p53. Mdm2 normally binds to p53 and promotes its ubiquitylation and destruction in proteasomes. Phosphorylation of p53 blocks its binding to Mdm2; as a result, p53 accumulates to high levels and stimulates transcription of numerous genes, including the gene that encodes the CKI protein p21. The p21 binds and inactivates G1/S-Cdk and S-Cdk complexes, arresting the cell in G1. In some cases, DNA damage also induces either the phosphorylation of Mdm2 or a decrease in Mdm2 production, which causes a further increase in p53 (not shown) (from Alberts *et al.* 2008).

DNA damage is detected by a series of protein complexes, which are recruited to the site of damage and activate the apical protein kinases ATM (Ataxia Telangiectasia mutated) and ATR (ATM and rad3 related). The DNA damage response (DDR) signalling continues via the ATM/ATR-mediated phosphorylation of various target proteins, including two other protein kinases, Chk1 (checkpoint kinase 1) and Chk2 (checkpoint kinase 2) (Karp 2002; Knowles & Selby 2005). These kinases phosphorylate further target proteins leading to various response pathways, including cell-cycle arrest, apoptosis and DNA repair. A key DDR target in both cell cycle arrest regulation and apoptosis is the transcription factor p53, which stimulates transcription of the gene encoding p21, a CKI (cyclin-dependent kinase inhibitor) protein. p21 binds to G1/S phase-CDK/Cyclin and S phase-CDK/cyclin complexes and inhibits their activity, thereby inhibiting the entry into the next stage of the cell cycle (figure 1.3.2.1) (Knowles & Selby 2005).

DNA damage activates p53 by an indirect mechanism. In undamaged cells, p53 concentration is kept at very low level via proteasomal degradation mediated by the E3 ligase MDM2 (Mouse double minute 2 homolog) (Knowles & Selby 2005). Phosphorylation of p53 by ATM, ATR, Chk1 and Chk2 after DNA damage reduces its affinity to MDM2, leading to a decrease in p53 degradation and a marked increase in p53 concentration in the cell, also know as p53 stabilisation. In addition, the decreased binding to MDM2 enhances the ability of p53 to stimulate gene transcription (Knowles & Selby 2005).

The protein kinases Chk1 and Chk2 also block cell-cycle progression by phosphorylating members of the CDC25 (cell division cycle 25) family of protein phosphatases, thereby inhibiting their function. These phosphatases are particularly important in the activation of mitotic CDKs at the beginning of mitosis. Chk1 and Chk2 phosphorylate CDC25 at inhibitory sites that are distinct from the phosphorylation sites that stimulate CDC25 activity. The inhibition of CDC25 activity by DNA damage helps block entry into mitosis (Knowles & Selby 2005).

1.3.3 Study of cell cycle progression

One simple method to determine where in the cell cycle a specific cell is, is it to observe living cells with a microscope (Knowles & Selby 2005). A glance at a population of mammalian cells proliferating in culture reveals that a fraction of the cells have rounded up and are in mitosis. Others can be observed in the process of cytokinesis (Knowles &

Selby 2005). Is possible to gain additional information about the position on the cell cycle by staining the cells with DNA-binding fluorescent dyes (which reveal the condensation of chromosomes in mitosis) or with antibodies that recognize specific cell components such as the microtubules (revealing the mitotic spindle), or other mitotic markers. Cells in S phase can be identified in the microscope by labelling with molecules that are incorporated into newly synthesized DNA, such as the thymidine halogenated analogue BrDU (BromoDeoxyUridine) and can be detected by immunofluorescence (Knowles & Selby 2005).

Another widely used methodology to assess the stage that a cell has reached in the cell cycle is by measuring the DNA content, which doubles during S phase (Knowles & Selby 2005). This approach is greatly facilitated by the use of fluorescent DNA-binding dyes and a flow cytometer, which allows the rapid and automatic analysis of large numbers of cells (figure 1.3.3.1). Using flow cytometry allows to determine the lengths of G_1, S and G_2+M phases, by measuring the DNA content in asynchronous cell populations (Knowles & Selby 2005).

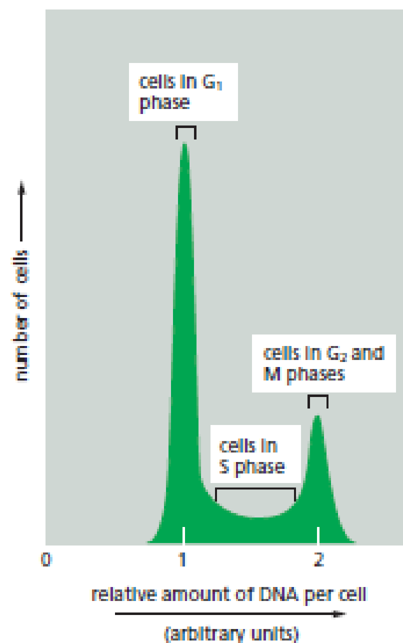

Figure 1.3.3.1 - Analysis of DNA content with a flow cytometer. This graph shows typical results obtained for a proliferating cell population when the DNA content of its individual cells is determined in a flow cytometer. A flow cytometer, also called a fluorescence-activated cell sorter, or FACS, can also be used to sort cells according to their fluorescence. The cells analysed here were stained with a dye that becomes fluorescent when it binds to DNA, so that the amount of fluorescence is directly proportional to the amount of DNA in each cell. The cells fall into three categories: those that have an unreplicated complement of DNA and are therefore in G1, those that have a fully replicated complement of DNA (twice the G1 DNA content) and are in G2 or M phase, and those that have an intermediate amount of DNA and are in S phase. The distribution of cells indicates that there are greater numbers of cells in G1 than in G2 + M phase, showing that G1 is longer than G2 + M in this population (from Alberts *et al.* 2008).

1.4 Dune Plant Species selected in this study

Vegetation patterns in coastal sand dunes hold particular interest for ecologists, in part because of their clear interaction with the dune geomorphology (Cowles 1911). Coastal sand dunes are subjected to severe environmental stresses and disturbance, caused by salinity, drought, nutrient limitation, substrate instability, sand burial, wind abrasion, erosion of the coastline and storms (Gornish & Miller 2010).

Sand-dune species, due to their understudied and unexplored stress adaptation mechanisms and strategies present an opportunity for the discovery of new bioactive molecules (Murray *et al.* 2013). However, although work in this field has expanded in the last few years, the role of EOs as potential anti-cancer agents is still not fully explored (Gautam *et al.* 2014; Bhalla *et al.* 2013).

The aromatic plants present in the sand dunes of Peniche, Portugal chosen for this research are described below:

1.4.1 *Crithmum maritimum* L.

This species, commonly known as sea fennel or rock samphire, is a perennial member of the Apiaceae family (Meot-Duros *et al.* 2010). This halophytic plant grows in sand hills and is often found on rocky cliffs (figure 1.4.1.1). It has been attributed many interests in traditional medicine, including diuretic, antiscorbutic, digestive and purgative properties, and can be consumed as a condiment (Atia *et al.* 2006).

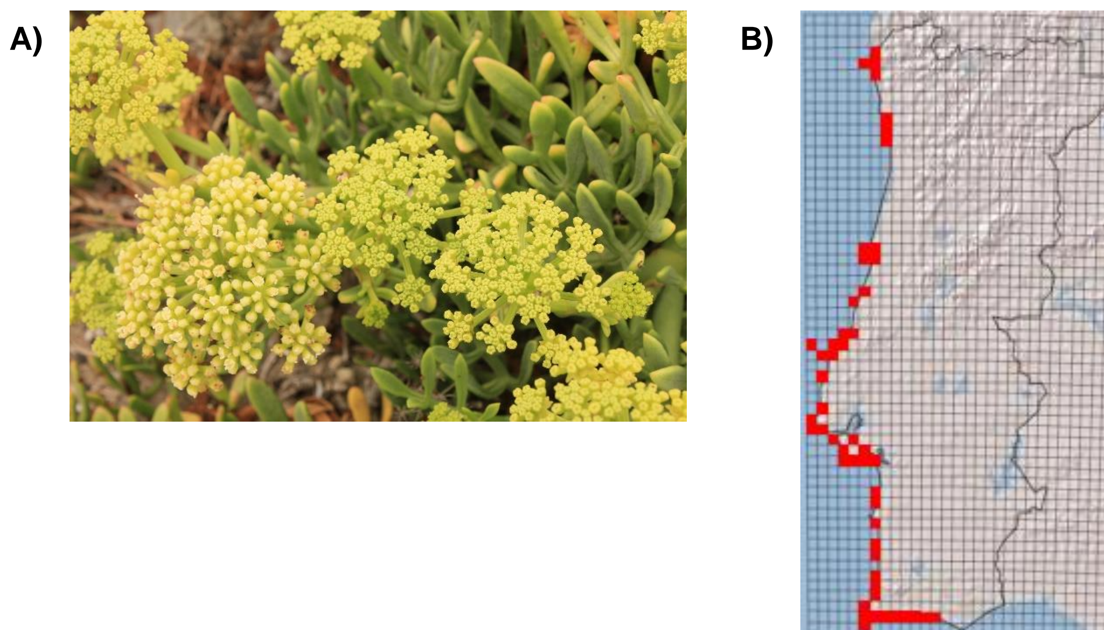

Figure 1.4.1.1 – *Crithmum maritimum.* A) Photography of *Crithmum maritimum* in Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http://www.flora-on.pt/#/1crithmum+maritimum).

1.4.2 *Seseli tortuosum* L.

This species belongs to the family Apiaceae, which is composed of aromatic herbs and economically important species that are used as foods, spices, condiments and ornamentals (Lawrence 1995; Crowden *et al.* 1969; Pimenov & Leonov 1993). It is possible to find this species in psammophilous bushes in the secondary dune, less frequent in maritime rocks and slopes of saline (Pimenov & Leonov 1993). Several *Seseli* species are reported in ancient literature for various healing effects, namely herbal remedy for human inflammation, swelling, rheumatism, pain and common cold (Hu *et al.* 1990). In Turkish folk medicine, the fruit of *Seseli tortuosum* (figure 1.4.2.1) is used as emmenagogue and antiflatulent (Baytop 1999). This EO have been investigated for its various biological properties including anti-inflammatory, antinociceptive (Küpeli *et al.* 2006; Tosun *et al.* 2006) and antifungal activities (Gonçalves *et al.* 2012).

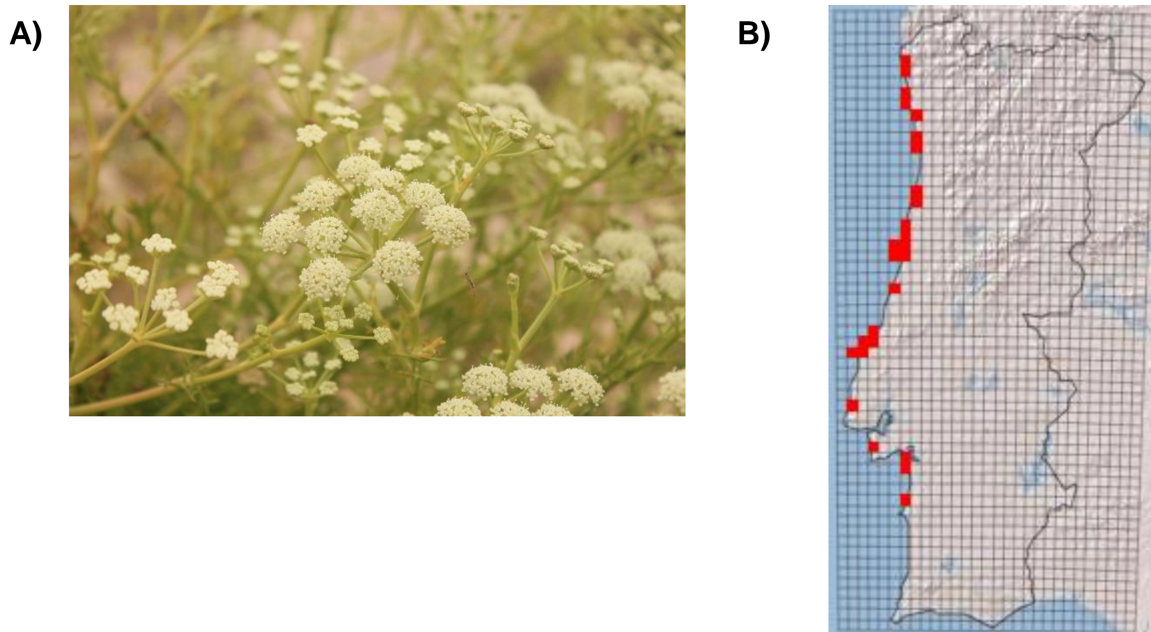

Figure 1.4.2.1 – *Seseli tortuosum*. A) Photography of *Seseli tortuosum* in Consolação beach, Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http:// www.flora-on.pt/#wSeseli+tortuosum).

1.4.3 *Artemisia campestris* subsp. *maritima* (DC.) Arcang.

The genus *Artemisia* L. is widespread throughout the world, growing wild over the Northern Hemisphere and belongs to the Asteraceae family. *Artemisia campestris* subsp. *maritima* (Figure 1.7.3.1) grows in Coastal sands, usually in primary dunes (figure 1.4.3.1) (Djeridane *et al.* 2007). This species has been used as febrifuge, vermifuge, against digestive troubles, gastric ulcer, menstrual pain (Dob *et al.* 2005; Djeridane *et al.* 2007) and for medicinal uses, such as antispasmodic and antihelmintic (Naili *et al.* 2010).

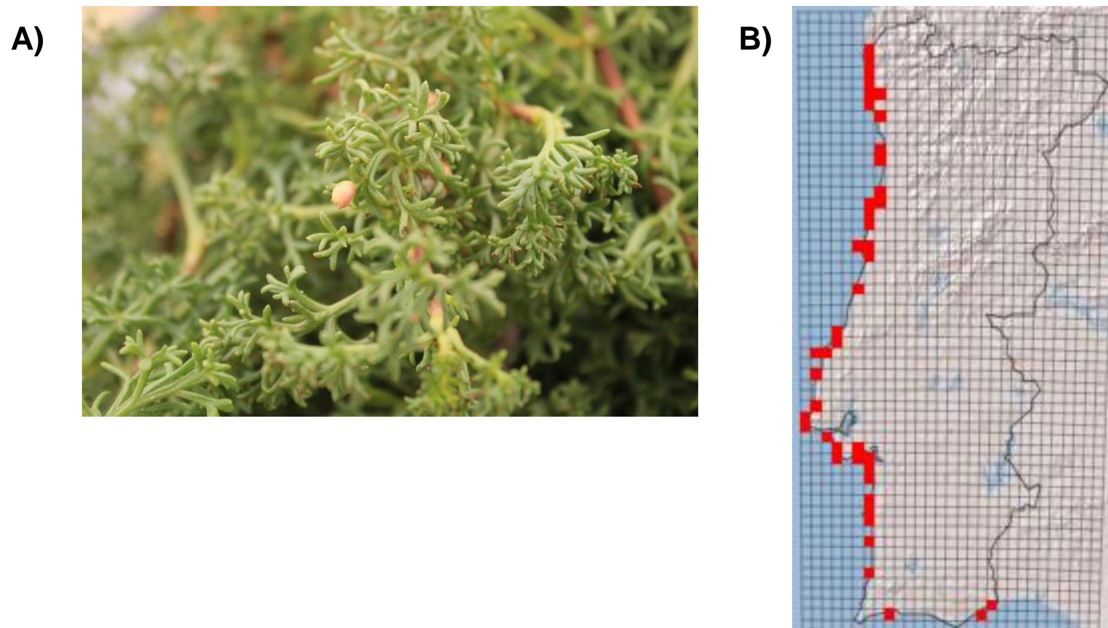

Figure 1.4.3.1 - *Artemisia campestris* subsp. *maritima*. A) Photography of *Artemisia campestris* subsp. *maritima* in Consolação beach, Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http:// www.flora-on.pt/#wArtemisia+campestris).

1.4.4 *Juniperus turbinata* Guss. subsp. *turbinata*

The genus *Juniperus* L. (Cupressaceae) is represented approximately by 70 species in the Northern Hemisphere (Nakanishi *et al.* 2004; Seca & Silva 2005). *Juniperus turbinata* subsp. *turbinata* (figure 1.4.4.1) is a bush growing in relatively dry conditions in stabilized dunes and coastal cliffs, but also on rocky slopes thermophilic, limestone outcrops and embedded river valleys (figure 1.4.4.1) (Seca & Silva 2005). Studies regarding the proprieties of *Juniperus* species occurring in Portugal have been even more limited and the focus again was on its EOs (Cavaleiro *et al.* 2001; Cavaleiro *et al.* 2002; Cavaleiro *et al.* 2003). Plants of the genus *Juniperus* are used in different European cuisines as spice and flavouring alcoholic drinks, as well as in cosmetics (Loizzo *et al.* 2008). These plants have an extensively history of use in global folk medicine for various disorders, such as common colds, urinary and kidney infections and dermatological disorders (Allen & Hatfield, 2004). Many biological activities have been reported for *Juniperus* sp. These include anti-inflammatory (Akkol *et al.* 2009; Lesjak *et al.* 2011), diuretic, antiseptic (bacterial and fungal) (Cavaleiro *et al.* 2006; Ennajar *et al.* 2009), anthelmintic (Kozan *et al.* 2006), hypoglycaemic (Ju *et al.* 2008), hypotensive, abortifacient, antinociceptive

(Akkol *et al.* 2009), antiviral (Sassi *et al.* 2008), anticancer (Kusari *et al.* 2011), anti-oxidant (Lesjak *et al.* 2011) and analgesic properties (Lesjak *et al.* 2011).

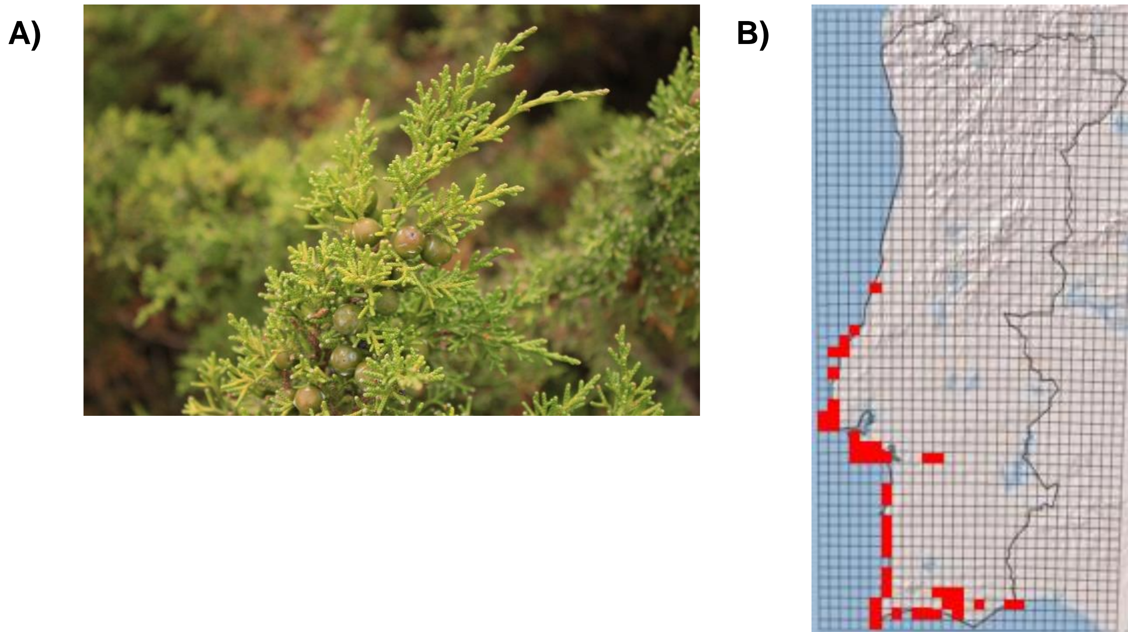

Figure 1.4.4.1 – *Juniperus turbinata* subsp. *turbinata*. A) Photography of *Juniperus turbinata* in Consolação beach, Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http://www.flora-on.pt/#wJuniperus+turbinata).

1.4.5 *Otanthus maritimus* (L.) Hoffmans. & Link

This species belongs to the family Asteraceae and it is a strongly aromatic perennial herb, 15–30 cm tall, growing on maritime sands along the coasts of South and West Europe, northwards to South-East Ireland (figure 1.4.5.1). The plant has been employed in folk medicine in decoctions as tonics, dyspeptics and for the treatment of toothache, asthmatic bronchitis, dysentery and inflammation of the urinary bladder. Dry specimens have been traditionally used as decoration and as insect repellent (Reutter 1923; Tsoukatou *et al.* 2000).

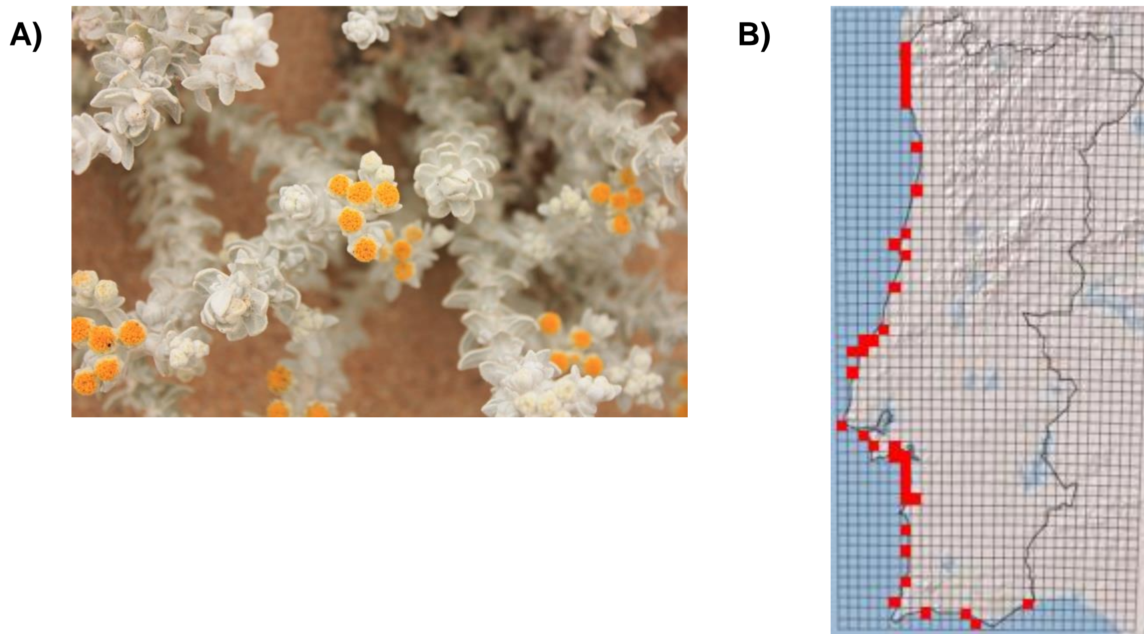

Figure 1.4.5.1 – *Otanthus maritimus*. A) Photography of *Otanthus maritimus* in Consolação beach, Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http:// www.flora-on.pt/#wOtanthus+maritimus).

1.4.6 *Eryngium maritimum* L.

The genus *Eryngium* L. belongs to the family Apiaceae and includes around 250 species that are widespread throughout the world (Darriet *et al.* 2014). Among them, several *Eryngium* species have been used as ornamental plants, condiments or in traditional medicine (Küpeli *et al.* 2006; Darriet *et al.* 2014). *Eryngium maritimum*, usually named 'sea holly' in England or 'Panicaut desmers' in France, is a perennial plant (30–60 cm high) with mauve flowers (blossoming time, June–September), growing wild on the sandy beaches of western Europe, the Mediterranean basin and the Black Sea (figure 1.4.6.1) (Küpeli *et al.* 2006; Darriet *et al.* 2014). The plant is one of the typical dune species implicated in the plant network that contributes to sand dune edification and restoration (Darriet *et al.* 2014). *Eryngium maritimum* has also been reported to exhibit different therapeutic uses in folk medicine (Küpeli *et al.* 2006).

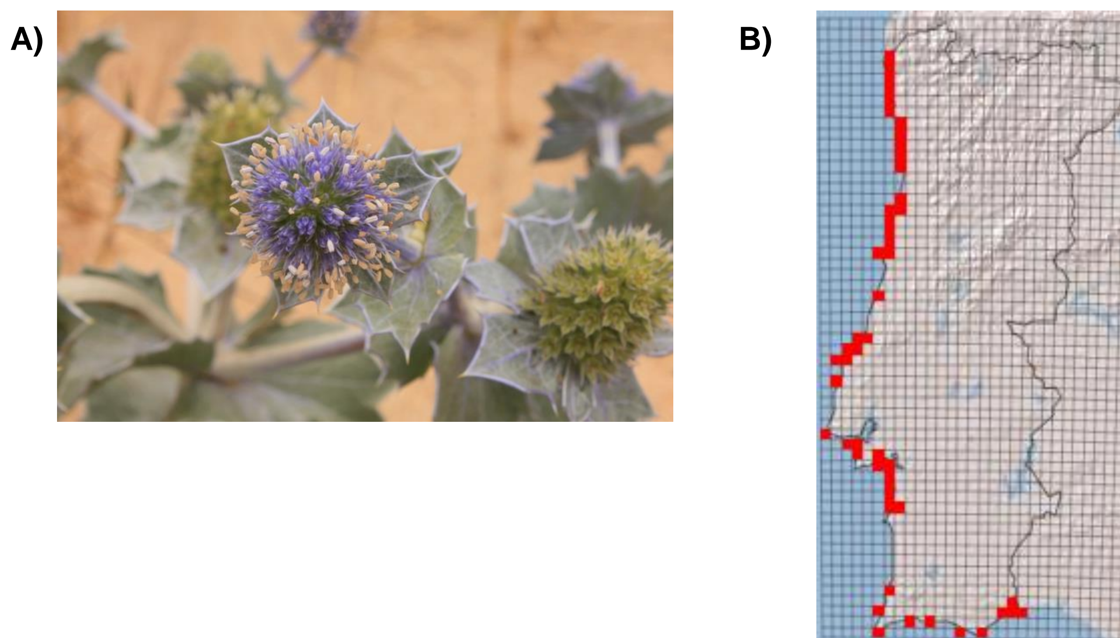

Figure 1.4.6.1 – *Eryngium maritimum*. A) Photography of *Eryngium maritimum* in Consolação beach, Peniche and B) geographic distribution map of this species in Portugal (extracted from Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica, http:// www.flora-on.pt/#wEryngium+maritimum).

1.5 Aim of this study

Essential oils have been widely used for medicinal purposes, due to their variety of biological properties such as antibacterial, antiviral, antioxidant, antiparasitical and anti-inflammatory properties. Their potential anticancer properties have not yet been properly clarified.

This study has the objective to determine the efficacy of the essential oils from six selected taxa from the sand dunes of Peniche (Portugal): *Artemisia campestris* subsp. *maritima*, *Crithmum maritimum, Eryngium maritimum*, *Juniperus turbinata* subsp. *turbinata, Otanthus maritimus* and *Seseli tortuosum* as potential cytotoxic/cytostatic therapeutic agents in breast (MCF7) and colorectal (RKO) cancer cell lines.

In a first stage, for every EOs enumerated, the cellular viability will be evaluated, in both cell lines, by MTS assay using serial dilutions with starting concentrations of 5 µL/mL and 1 µL/mL. From these results, the concentration of the EOs required for 50% inhibition *in vitro* (IC_{50}) will be calculated for each species, in both cancer cell lines.

The two EOs that present the lowest IC_{50}, representing the EOs that could have a decrease in cell viability more pronounced, will be selected and will be part of a preliminary study of mode of action the EOs. Western blot will be carried out in order to understand the effects of the EOs on cell death. According to the results obtained, we will proceed to the next stage, in order to study in more detail the mechanism of action of EOs in cancer cells. For this, the cell cycle progression will be studied, in order to understand the effect of the EOs in cell cycle arrest, using fluorescence-activated cell sorting (FACS) technique.

2. Materials and Methods

2.1 General materials and reagents

All general reagents were purchased from Fisher Scientific (UK), Sigma-Aldrich (UK) or VWR (UK), unless stated. All tissue culture reagents were purchased from PAA (UK), Gibco (UK) or Lonza (US).

2.2 Plant Material

Aerial parts of *Artemisia campestris* subsp. *maritima, Crithmum maritimum*, *Eryngium maritimum, Juniperus turbinata* subsp. *turbinata, Otanthus maritimus* and *Seseli tortuosum* were collected during the flowering stage in July 2013, in the sand dunes of Consolação beach, Peniche (Portugal).

Voucher specimens were deposited in the Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra, under the numbers: J. Poças 02013 (*Artemisia campestris* subsp. *maritima*), J. Poças 06013 (*Crithmum maritimum)*, J. Poças 02013 (*Eryngium maritimum),* J. Poças 05013 (*Juniperus turbinata* subsp. *turbinata),* J. Poças 01013 (*Otanthus maritimus)* and J. Poças 04013 (*Seseli tortuosum).*

2.3 Essential oils isolation

Essential oils were isolated by hydrodistillation for 3h using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe 1997) and stored in appropriate vials at 4ºC.

2.4 Chemical characterization of essential oils

2.4.1 Gas chromatography (GC)

Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm, film thickness 0.20 μm),

and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm, film thickness 0.20 μm). Oven temperature program: 70–220ºC (3ºC/min), 220 ºC (15 min); injector temperature: 250ºC; carrier gas: helium, adjusted to a linear velocity of 30 cms^{-1}; splitting ratio 1:40; detectors temperature: 250ºC.

2.4.2 Gas chromatography-mass spectrometry (GC-MS)

GC–MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm, film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described above; interface temperature: 250ºC; MS source temperature: 230ºC; MS quadrupole temperature: 150 ◦C; ionization energy: 70 eV; ionization current: 60μA; scan range: 35–350 units; scans per second: 4.51.

2.4.3 Qualitative and quantitative analyses

Components of the volatile oil were identified by their retention indices on both SPB-1 and SupelcoWax-10 columns, calculated by linear interpolation relative to retention times of C_8–C_{24} of *n*-alkanes and compared with those of reference compounds included in CEF laboratory database or literature data (Adams 2004), and by their mass spectra by matching with reference spectra from the CEF laboratory own spectral database, Wiley/NIST database or literature data (Joulain & Konig, 1998; Adams, 2004; Wiley Registry, 2006). Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

2.5 Cell Culture

Human cancer cell lines RKO (colorectal) and MCF7 (breast) (ATCC -US- or ECCAC -UK-), were maintained in DMEM (Dulbecco's Modified Eagle's) medium supplemented with 10% foetal bovine serum (FBS) and 1% sodium pyruvate. Cells were grown in a 37˚C incubator, at a 5% CO_2 humidified atmosphere (90% humidity). The subculture was made three times a week in T75 flasks. Initially, the medium was removed from the flask and

cells were washed with 1x Phosphate Buffered Saline (PBS) without calcium or magnesium. The PBS was then removed and Trypsin was added for promoting cell detachment. After incubation for 2-5 mins at 37˚C, cells were resuspended in fresh media and subcultured at a ratio of 1:6 to 1:12. Cells were regularly tested for Mycoplasma infection absence.

2.6 Treatment of cancer cells with EOs

The serial dilution of the EO was prepared in DMSO. For stock solution were used a proportion 1:4 of EO per DMSO. The starting concentrations were prepared at 5 µL/mL (figure 2.6.1) and 1 µL/mL (figure 2.6.2), using dilutions ranges of 1:2 and 1:10, respectively.

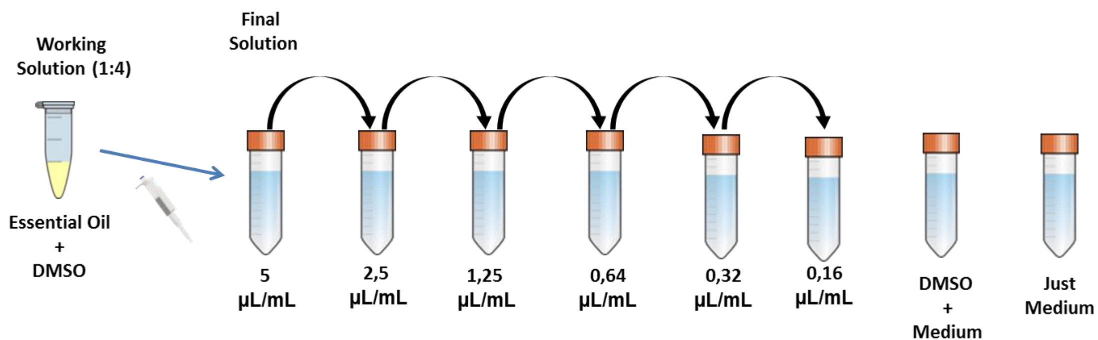

Figure 2.6.1 – Diagram relative to the preparation of dilutions of EOs. Initially, the working solution was prepared at a ratio 1:4 (EO per DMSO). 80 µL were pipetted for 4 mL of medium, thus making the final concentration 5 µL/mL. The serial dilutions (until 0, 16 µL/mL) was made in dilution 1:2. The last samples were prepared by 80 µL DMSO added with 4 mL of medium and just medium, respectively. Previously, RKO and MCF7 cell lines were seeded into 96 well microtiter plates, at a density of $1x10^4$ and $5x10^3$ cells per well, respectively. For each EO and cancer cell line was made two intra replicas.

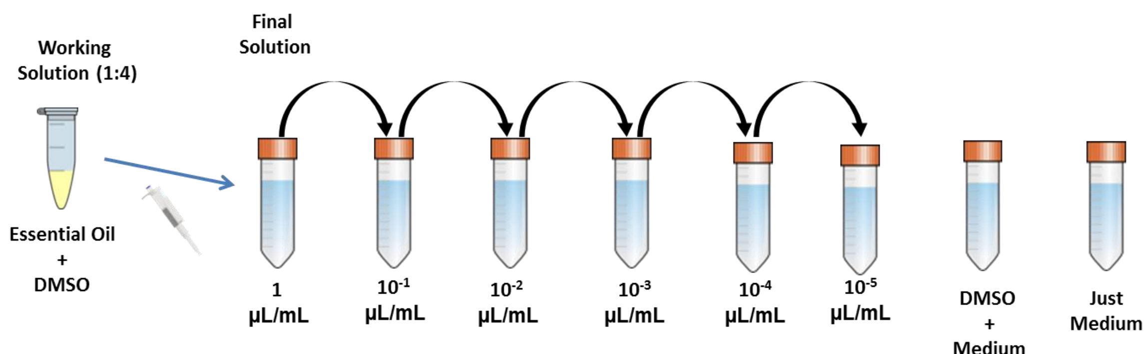

Figure 2.6.2 – Diagram relative to the preparation of dilutions of EOs. Initially, the working solution was prepared at a ratio 1:4 (EO per DMSO). 16 µL were pipetted for 4 mL of medium, thus making the final concentration 1 µL/mL. The serial dilutions (until 10^{-5} µL/mL) was made in dilution 1:10. The last samples were prepared by 16 µL DMSO added with 4 mL of medium and just medium, respectively. Previously, RKO and MCF7 cell lines were seeded into 96 well microtiter plates, at a density of $1x10^4$ and $5x10^3$ cells per well, respectively. For each EO and cancer cell line was made two intra replicas.

2.7 MTS (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) Assay for cellular viability

The assay is dependent on the reduction of the tetrazolium salt MTS (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product (Figure 2.6.1). This conversion is presumably accomplished by NADPH or NADH produced by these dehydrogenase enzymes (Berridge & Tan 1993). The assay measures cell respiration and the amount of formazan produced is proportional to the number of living cells present in culture (Mosmann 1983; Gerlier & Thomasset 1986). It is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells (Vega-Avila & Pugsley 2011). Due to toxicity associated with exposure to MTS assay, is impossible to conduct follow-up cell culture assessments.

MTS assay (CellTiter 96® AQueous (Promega)) was performed according to manufacturer's instructions. In brief, the spent medium of each well was removed and fresh medium was added as well as MTS reagent solution. After 4 hours incubation, the absorbance was determined using a microplate reader (Bioteck ELx800, UK) at a test wavelength of 490 nm.

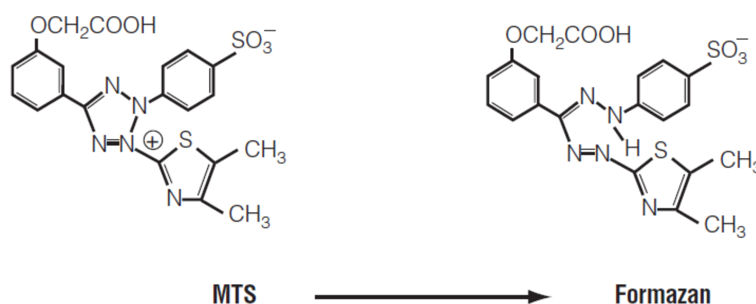

Figure 2.7.1 – Structures of MTS tetrazolium and its formazan product (CellTiter 96® AQueous One Solution Cell Proliferation Assay).

2.8 Half maximal inhibitory concentration (IC_{50}) calculations

A dose-response curve describes the relationship between response to drug treatment and drug dose or concentration. The IC_{50} measures how effective a drug is. It indicates how much of a particular drug treatment is needed to inhibit a given biological process (i.e. an enzyme, cell or microorganism, etc.) by half (50 %).

According to the Food and Drug Administration (FDA), IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro. To calculate the IC_{50} values, Graph Pad Prism Software (GraphPad Software, Inc.) was used. The tools used in this software were "Dose- Response Inhibition" and "Log (Inhibition) vs response (three responses)". These tools were selected because it was necessary to create a curve that better describes the concentration tested versus inhibition of cellular viability by MTS assay, and also to normalize the concentrations in Log_{10}, in order to calculate the correct IC_{50} values.

2.9 Protein Lysis and Protein Concentration Determination

After incubation, the medium on treated or untreated cell cultures was removed, and cells were washed in 1x PBS. Cells were harvested into PBS using a cell scrapper. The cells were collected in microcentrifuge tubes and pelleted by centrifugation at high speed for 10 seconds and the supernatant was removed. The pellet was resuspended with UTB lysis buffer (9M Urea, 75mM Tris-HCl pH 7.5 and 0.15M β-mercaptoethanol). Lysates were then sonicated during 5 minutes. After, samples were centrifuged at 500g during 15 min (at 4°C) and the resulting supernatant was transferred to a falcon tube. Protein concentration was determined using on NanoDrop spectrophotometer software (ND-1000 version 3.5.2).

2.10 SDS-PAGE and Western Blot

Samples were prepared for loading into an SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) gel at a final protein amount of 50 µg.

Due to the molecular weight of the proteins used subsequently, 10% SDS-PAGE gels were prepared. For this, the running gel was prepared with 1.5 M Tris pH 8.8, Acrylamide, 10% SDS, 10% APS and TEMED (Tetramethylethylenediamine), and stacking gel with 0.5 M Tris pH 6.8, Acrylamide, 10% SDS, 10% APS and TEMED. For the running buffer 10x was needed Tris base, glycine and 10% SDS, for a final volume 1 L of water. SDS-PAGE gels were run in 1x running buffer at 120 volts over 90 minutes.

The Blotting buffer 10x was prepared with Tris base and glycine, to a final volume of 1 L of water. The nitrocellulose membranes were cut and placed in 1x blotting buffer to equilibrate.

After electrophoresis, membranes were place, correctly, between 2 sponges and 2 set of pieces and blotting paper onto sponge. The gels were placed onto blotting paper (all bubbles removed) and membrane onto gel. After the membrane was covered with blotting paper, the set of pieces were closed with sponge between membrane and blotting paper, and placed in blotting tank half filled with blotting buffer. This way, blotting was prepared to run at 100 volts, during 90 minutes. After, the membranes were washed in Tris-Buffered Saline - 0.1% Tween (TBS-T) and blocked with 5% milk in TBS-T during 1 hour.

The membranes were incubated with primary antibodies (prepared in 1% dried milk powder in TBS-T), overnight at 4°C, and after that washed again with TBS-T. Membranes were then incubated in secondary antibody solution (prepared in 1% milk in TBS-T) for 1 hour at room temperature. After further washes with TBS-T, membranes were incubated before using Enhanced Chemiluminescence (ECL) reagent (Biorad) and developed using the Fluorescent Imager system (Biorad) using the Imager Lab software (Image Lab software, version 5.1). The antibodies used for Western Blot analysis are described in Table I.

Table I – List of antibodies used for western blot technique.

Antibody	Branch/ catalogue number	Dilution	Animal Origin
p53	Santa Cruz Biotechnology / sc-126	1:1000	Mouse
p21	EMD Millipore / 05655	1:1000	Mouse
Cleaved Caspase 3	Cell Signalling Technology / #9661	1:1000	Rabbit
β-Actin	Santa Cruz Biotechnology / sc-130656	1:10000	Mouse
HRP-tagged Anti-mouse secondary antibody	Dako / P 0260	1:2000	Rabbit
HRP-tagged Anti-rabbit secondary antibody	Dako / P 0448	1:2000	Goat

2.11 Determination of DNA content by Fluorescence-activated cell sorting (FACS)

The nuclear DNA content of a cell can be quantitatively measured at high speed by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilised single cells or nuclei (Ormerod 1992). The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA (Ormerod 1992). The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered a measurement of the cellular DNA content (Ormerod 1992).

To measure the DNA content, cells need to be stained with a fluorescent dye that binds to DNA in a manner that reflects the amount of DNA present. The most widely used dye is a propidium iodide (PI), which has red fluorescence and can be excited at 488 nm.

Cells were trypsinised and resuspended in order to obtain a single cell suspension (collected cold media from each dish to avoid the clumping of cells) and were centrifuged (at 500g, during 5 minutes). The cells were resuspended in ice cold PBS and centrifuged again. The pellet was resuspended in a small volume of ice cold PBS and ice cold 70% ethanol (EtOH) was added dropwise while vortexing. Fixed cells were stored at -20°C.

For staining, samples centrifuged at 500g, during 5 minutes. After, they were resuspended for blocking in 2% BSA (Bovine Serum Albumin) (Cell Signalling Technology) in 0.1% tween/PBS and, and centrifuged. The cell pellet was resuspended in 10 µg/mL PI (Fisher Scientific) and 10 µg/mL RNAse A (Fisher Scientific) in PBS and were incubated on ice up to an hour and transferred to FACS tube. Samples were then analysed using a BD Cell Quest™ Pro FACS instrument.

3. Results

3.1 Essential oils composition

Through hidrodistillation technique, every EOs tested, were extracted. To maintain their properties, they were saved in appropriate vials and were protected by light and air.

EOs chemical composition was studied though gas chromatography (GC) and gas chromatography-mass spectrophotometry (GC-MS) to understand which major compounds exist in each EO tested.

For this, a small volume sample (1 µL) of each EO was injected into the GC/ GC-MS and analyzed individually.

The yield and main compounds of the six species studied are listed in the table I.

Table II - Species with respective yield and main compounds of the essential oils.

Species	Yield (%)	Main compounds (above 10%, except *Eryngium maritimum*)
Artemisia campestris subsp. *maritima*	0.47	β-pinene, sesquiterpenes
Crithmum maritimum	0.36	γ-terpinene, thymol methyl ether, sabinene
Eryngium maritimum	0.08	germacrene D and other sesquiterpene hidrocarbons (all below 10%)
Juniperus turbinata subsp. *turbinata*	0.29	α-pinene, β-phellandrene, α-terpinyl acetate
Otanthus maritimus	0.12	filifolone, chrysanthenone, cis-chrysantenyl acetate
Seseli tortuosum	0.66	α-pinene, β-pinene, (Z)- β –ocimene

3.2 Antitumor Activity

To determine the antitumor activity of the EOs extracted from these species, MCF7 (breast) and RKO (colorectal) cancer cells were treated with the various extracted EOs for 3 days at two serial dilution ranges: 1:2 (starting at 5 µL/mL) and 1:10 (starting at 1 µL/mL). After this, an MTS assay was carried out, and cell viability was calculated

comparing to the vehicle (DMSO), corresponding to 100% cellular viability. Each EO was initially diluted in DMSO (vehicle) and further diluted in growing medium.

Cells were treated with serial dilutions of 1:2 starting at a concentration of 5 µL/mL (figure 2.6.1).

The results of the MTS assay for all EOs on the RKO cell line treated at a starting concentration of 5 µL/mL are shown in Figure 3.2.1.

For MCF7 cell line, the results of MTS assay in the serial dilutions at a starting concentration of 5 µL/mL are shown in figure 3.2.2.

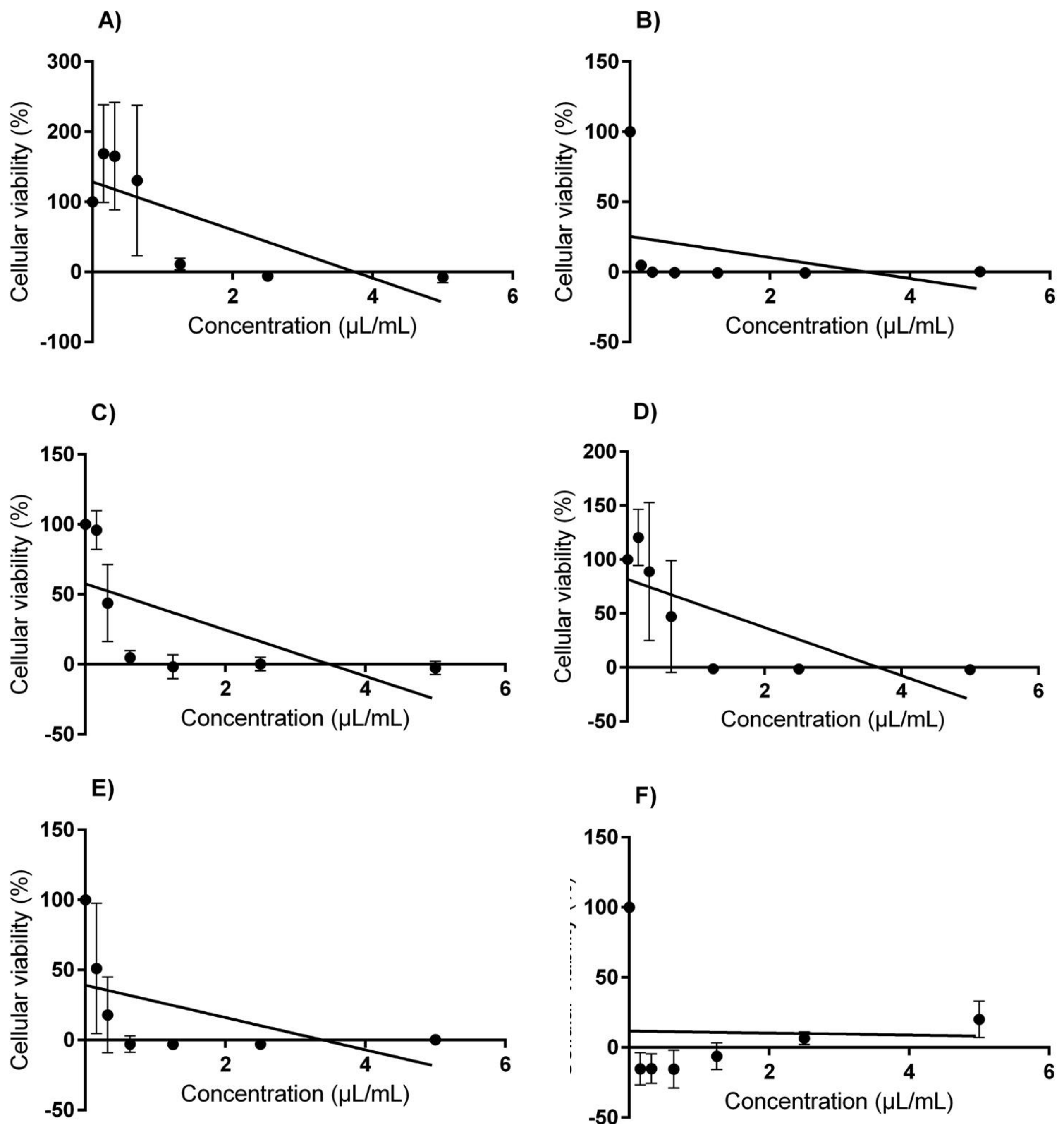

Figure 3.2.1 - Effect of EO treatment on RKO cells on cell viability (serial dilutions 1:2 starting at 5 µL/mL concentration). Oils used in this study were extracted from the Peniche dune plants A) *Crithmum maritimum* (n=3), B) *Seseli tortuosum* (n=3), C) *Artemisia campestris* (n=2), D) *Juniperus turbinata* (n=4), E) *Otanthus maritimus* (n=4) and F) *Eryngium maritimum* (n=3). The starting concentration of each EO was 5 µL/mL, further serially diluted 1:2 up to 0.16 µL/ml. RKO cells were seeded in a 96 well plate at a density $1x10^4$ cells per well and treated with the diluted oil solutions. After 72 hours of incubation, an MTS assay was performed for each plate, according to manufacturers instructions. Values represent the % of cellular viability as assessed by the MTS assay.

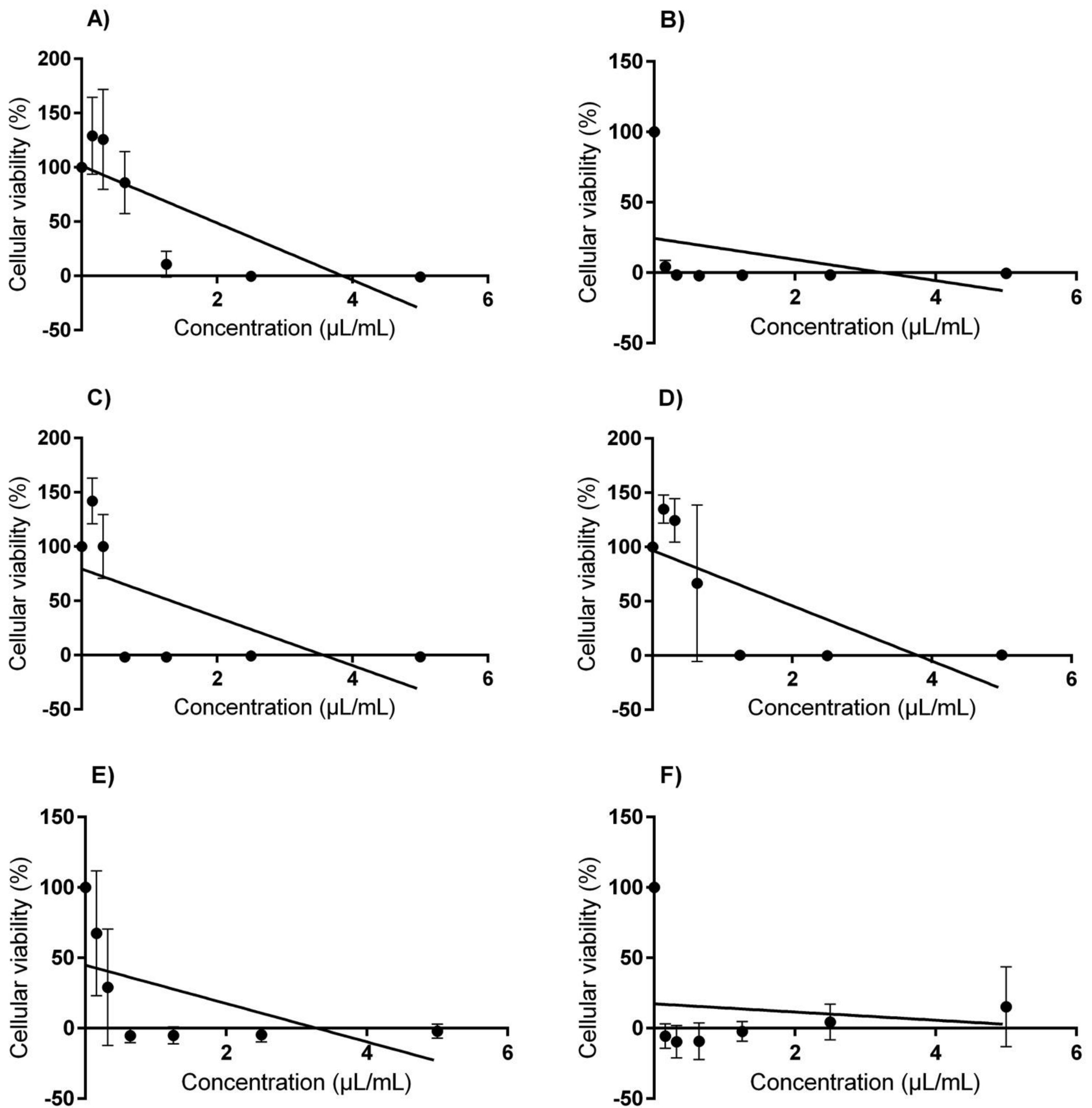

Figure 3.2.2 - Effect of EO treatment on MCF7 cells on cell viability (serial dilutions 1:2 starting at 5 µL/mL concentration). Oils used in this study were extracted from the Peniche dune plants A) *Crithmum maritimum* (n=3), B) *Seseli tortuosum* (n=3), C) *Artemisia campestris* (n=2), D) *Juniperus turbinata* (n=4), E) *Otanthus maritimus* (n=4) and F) *Eryngium maritimum* (n=3). The starting concentration of each EO was 5 µL/mL, further serially diluted 1:2 up to 0.16 µl/ml. RKO cells were seeded in a 96 well plate at a density 5x10^3 cells per well and treated with the diluted oil solutions. After 72 hours of incubation, an MTS assay was performed for each plate, according to manufacturers instructions. Values represent the % of cellular viability as assessed by the MTS assay.

All the EOs exhibited anticancer activity against RKO cells, as shown by a clear decreased in cell viability as measured by the MTS assay. *Artemisia campestris*, *Otanthus maritimus*, *Seseli tortuosum* and *Eryngium maritimum* EOs, treatment with our lowest dose for this dilution range (0.16 µL/mL, the lowest concentration tested) led to a clear decrease in cellular viability. However, *Seseli tortuosum* and *Eryngium maritimum* EOs treatment had a less toxic effect on the cells as the other species of EOs, as can be observed in the graphs.

Against MCF7 cell line, every EOs exhibited a decrease in cellular viability as measured by the MTS assay. The effect of *Seseli tortuosum* EO on cellular viability was the less toxic of all the species, with other EOs inducing a clear decrease in cellular viability at the lowest dose used. Interestingly, *Eryngium maritimum* EO promoted an increase in cellular viability or proliferation at the same dose range.

As the dilution range starting at 5 µL/mL concentration were shown to be very toxic to the cells for the majority of EOs (figures 3.2.1 and 3.2.2), it was decided to test a lower concentration range of EO dilutions, with a wider dilution ratio. The starting concentration chosen was 1 µL/mL with a dilution 1:10 series.

MCF7 and RKO cells were treated with the various EOs already described for three days, after which MTS assay was carried out, and cell viability was calculated comparing to the vehicle (DMSO), corresponding to 100% cellular viability, as described before, except the staring concentration was 1 µL/mL, with a dilution ratio of 1:10 (figure 2.6.2).

The results of the MTS assay for all EOs on the RKO cell line treated at a starting concentration of 1 µL/mL are shown in figure 3.2.3.

For MCF7 cell line, the results of MTS assay in the serial dilutions at a starting concentration of 1 µL/mL are shown in figure 3.2.4.

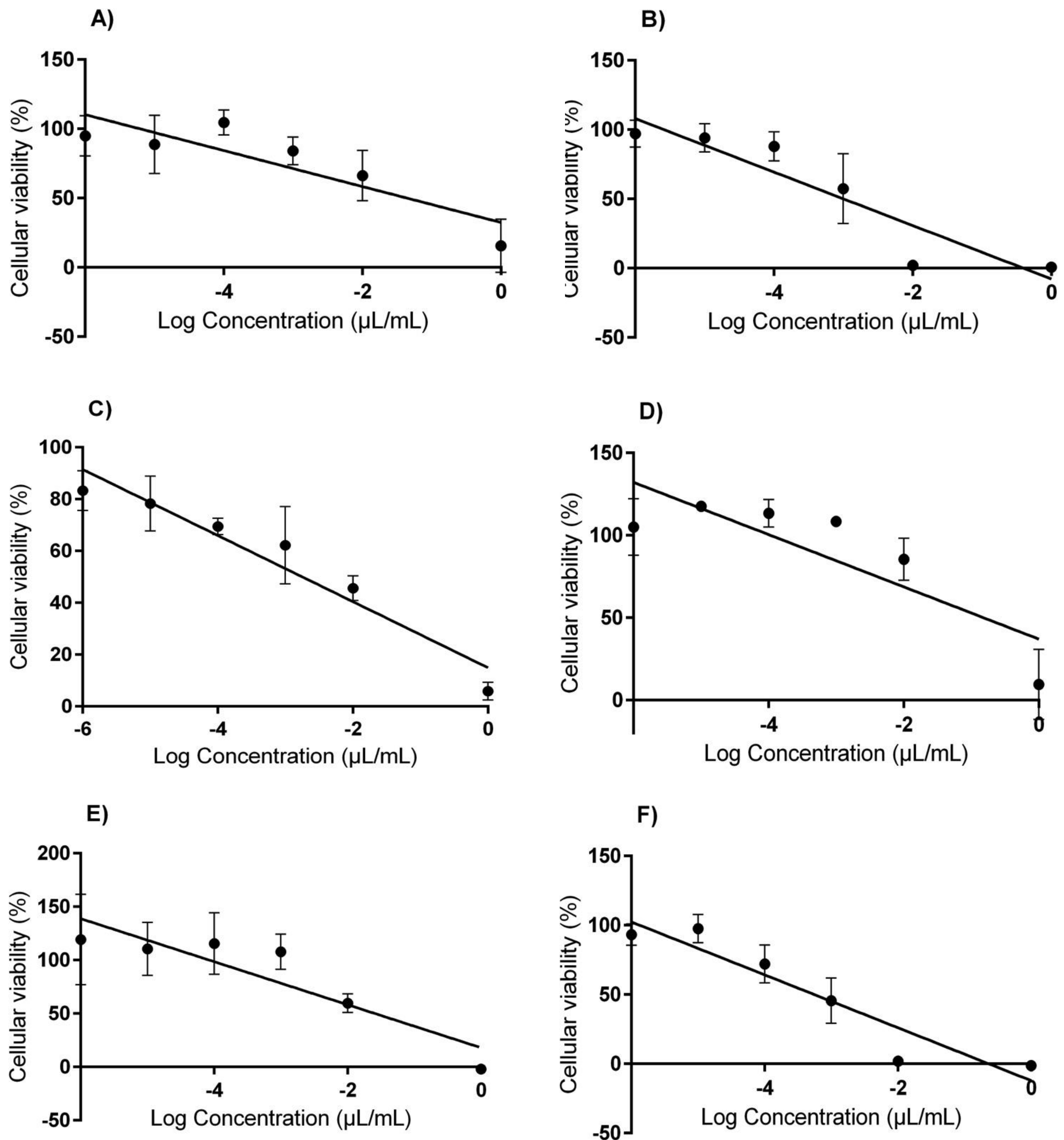

Figure 3.2.3 - Effect of EO treatment on RKO cells on cell viability (serial dilutions 1:10 starting at 1 µL/mL concentration). Oils used in this study were extracted from the Peniche dune plants A) *Crithmum maritimum* (n=3), B) *Seseli tortuosum* (n=3), C) *Artemisia campestris* (n=2), D) *Juniperus turbinata* (n=4), E) *Otanthus maritimus* (n=4) and F) *Eryngium maritimum* (n=2). The starting concentration of each EO was 1 µL/mL, further serially diluted 1:10 up to 10^{-6} µl/ml. RKO cells were seeded in a 96 well plate at a density $1x10^{4}$ cells per well and treated with the diluted oil solutions. After 72 hours of incubation, an MTS assay was performed for each plate, according to manufacturers instructions. Values represent the % of cellular viability as assessed by the MTS assay.

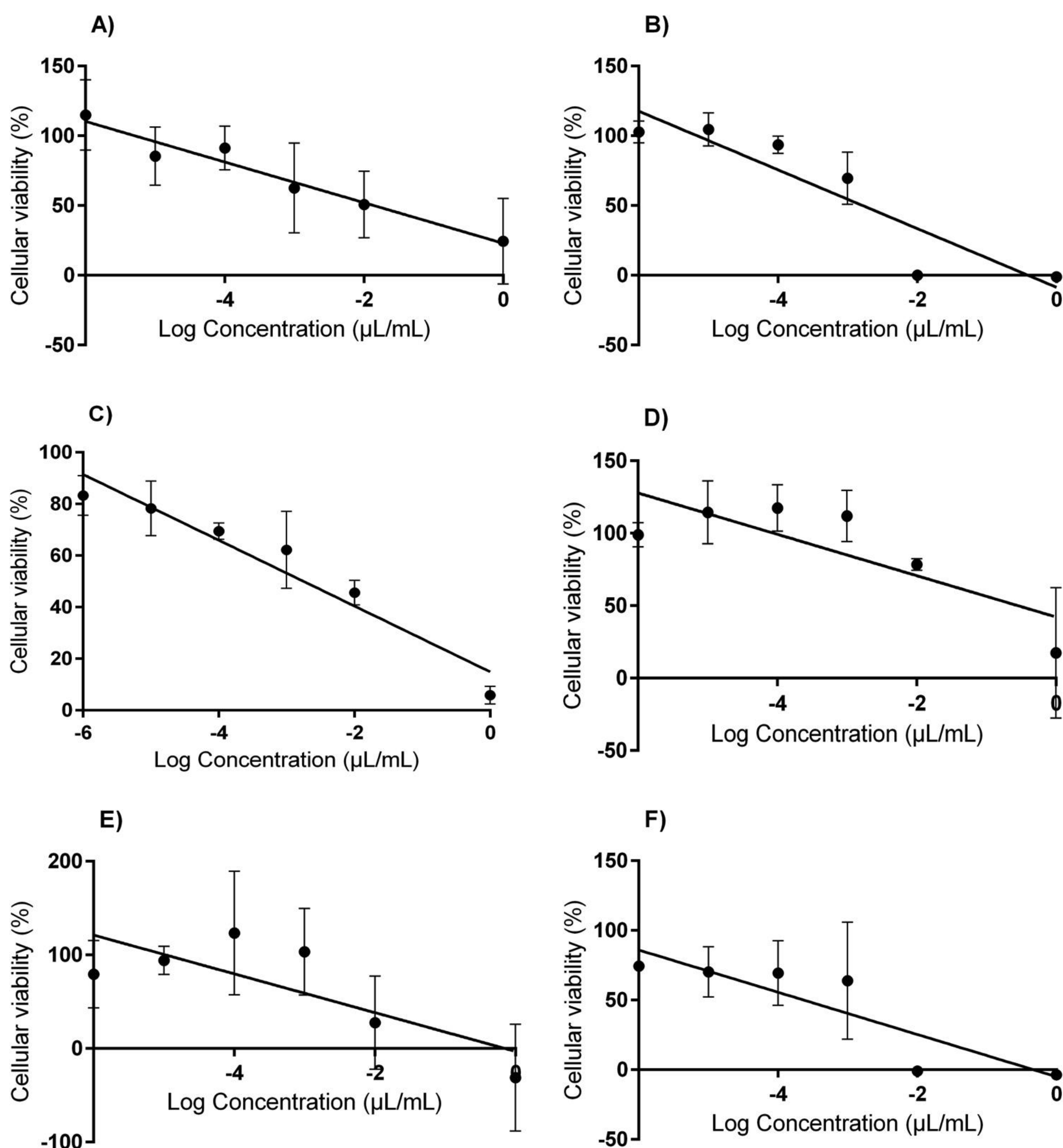

Figure 3.2.4 - Effect of EO treatment on MCF7 cells on cell viability (serial dilutions 1:10 starting at 1 µL/mL concentration). Oils used in this study were extracted from the Peniche dune plants A) *Crithmum maritimum* (n=3), B) *Seseli tortuosum* (n=3), C) *Artemisia campestris* (n=2), D) *Juniperus turbinata* (n=4), E) *Otanthus maritimus* (n=4) and F) *Eryngium maritimum* (n=2). The starting concentration of each EO was 1 µL/mL, further serially diluted 1:10 up to 10^{-6} µl/ml. RKO cells were seeded in a 96 well plate at a density $5x10^3$ cells per well and treated with the diluted oil solutions. After 72 hours of incubation, an MTS assay was performed for each plate, according to manufacturers instructions. Values represent the % of cellular viability as assessed by the MTS assay.

In serial dilution with a final concentration 1 μL/mL, all the EOs exhibited anti-proliferative activity against RKO cells, as shown by a clear decreased in cell viability. *Seseli tortuosum* and *Eryngium maritimum* EOs induced the highest decrease of cellular viability.

For MCF7 cell line, the results of MTS assay in the serial dilutions at a starting concentration of 1 μL/mL are shown in figure 3.2.4. All EOs exhibited a decrease in cellular viability against MCF7 cells. *Seseli tortuosum*, *Otanthus maritimus* and *Eryngium maritimum* EOs led to the strongest decrease in cellular viability, as is show in figure 3.2.4.

After the determination of the potential antitumor activity of each EO, by MTS assay, it was necessary to calculate the half maximal inhibitory concentration (IC_{50}) values, in order to compare more directly the effect of the different EOs, for each cell line. It is important to note that the lowest the IC_{50}, the strongest the effect of the drug/EO/compound, and low IC_{50}s are desirable due to reduced likeliness of toxicity to non-cancerous tissue (Ganot *et al.* 2013).

Dose-response curve fitting for all EOs for both cell lines, at a starting concentration of 5 μL/mL using a dilution 1:2 are displayed in figure 3.2.5. The respective IC_{50} values are shown in table III.

Table III - IC_{50} mean values (± SD) for *Crithmum maritimum*, *Seseli tortuosum*, *Artemisia campestris*, *Juniperus turbinata*, *Otanthus maritimus* and *Eryngium maritimum* EOs from Peniche (Portugal), at a final concentration 5 μL/mL with dilution 1:2. To calculate the IC_{50} values, MTS assay was carried out and was performed using the Graph Pad Prism software.

Final concentration 5 μL/mL			
Essential Oil	**Average IC_{50} (μL/mL) ± SD**		**Number of inter-experimental repeats**
	RKO	**MCF7**	
Crithmum maritimum	8,35E-01 ± 5,31E-01	6,04E-01 ± 3,25E-01	n = 3
Seseli tortuosum	2,00E-05 ± 1,68E-05	9,69E-06 ± 1,62E-05	n = 3
Artemisia campestris	4,88E-02 ± 6,90E-02	3,74E-02 ±3,96E-02	n = 2
Juniperus turbinata	2,65E-01 ± 3,06E-01	4,47E-01 ± 3,89E-01	n = 4
Otanthus maritimus	1,74E-05 ± 1,19E-05	4,48E-03 ± 6,70E-03	n = 4
Eryngium maritimum	1,13E+04 ± 1,96E+04	2,35E+04 ± 4,03E+04	n = 3

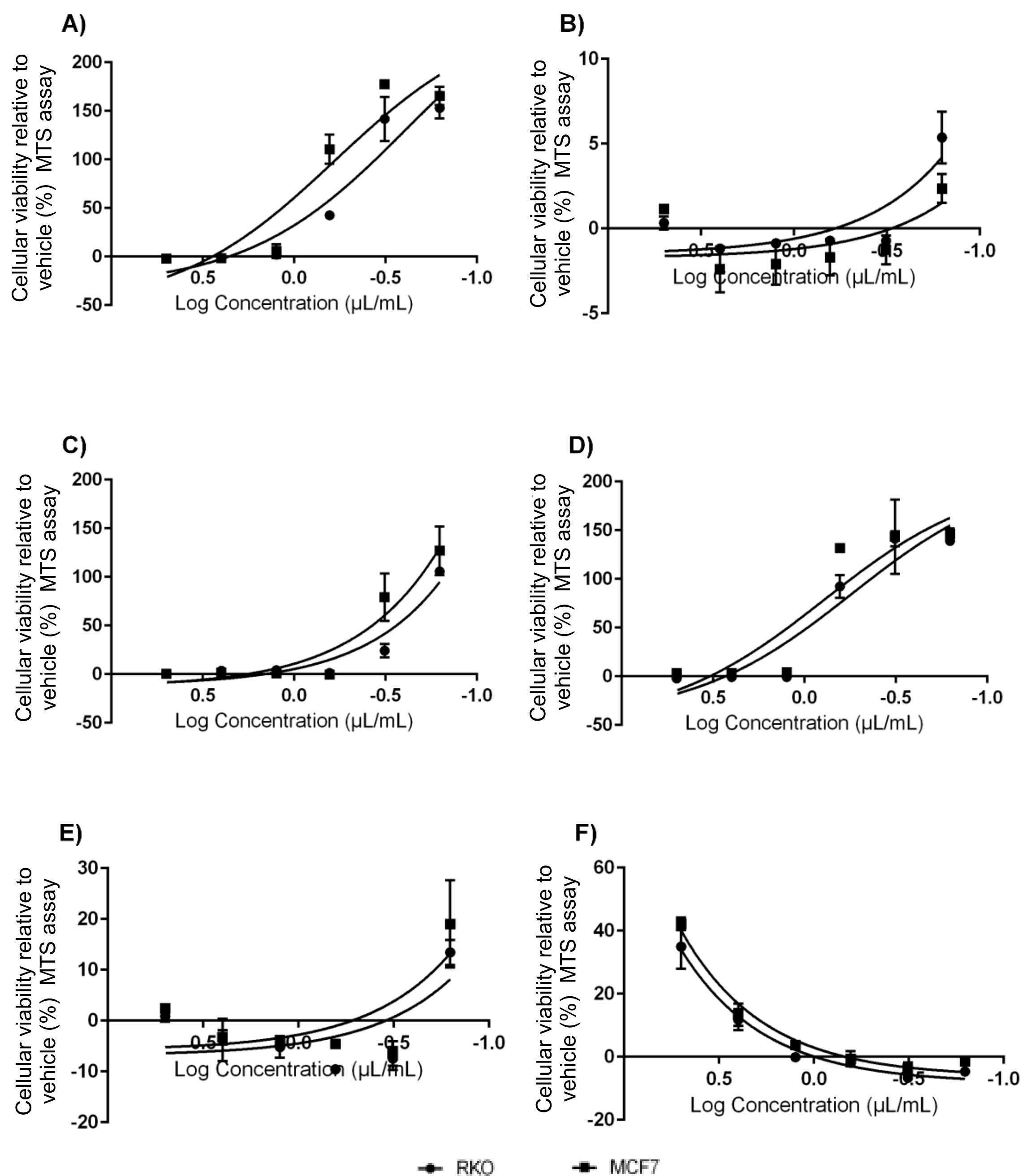

Figure 3.2.5 – Estimated dose-response parameter of each EO tested against RKO and MCF7 cancer cell lines. The species used were A) *Crithmum maritimum*, B) *Seseli tortuosum*, C) *Artemisia campestris*, D) *Juniperus turbinata*, E) *Otanthus maritimus* and F) *Eryngium maritimum* Essential Oils, against RKO (●) and MCF7 (■) cell lines viability detected by MTS Assay, at a concentration 5 µL/mL and dilution 1:2. MTS assay was carried out as described before (Figures 3.2.1 and 3.2.2) and Graph pad prism software was used to plot the curve fitting for each oil.

When comparing the IC_{50} values obtained for all EOs tested for the RKO cell line data set, *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* EOs treatment showed the highest decrease of cellular viability (as indicated by the lowest IC_{50}), followed by *Juniperus turbinata* (leaves) and *Crithmum maritimum* EOs (figure 3.2.5 and table III).

The EOs that shown the highest activity against MCF7 cells (again, as indicated by the lowest IC_{50}) were *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris*, followed by *Juniperus turbinata* and *Crithmum maritimum* EOs. *Eryngium maritimum* EO had the lower activity against MCF7 and RKO cell lines.

Dose-response curves fitting for all EOs for both cell lines, at a starting concentration of 1 µL/mL using a dilution 1:10 are displayed in figure 3.2.6. The respective IC_{50} values are shown in table IV.

Table IV - IC_{50} mean values (± SD) for *Crithmum maritimum*, *Seseli tortuosum*, *Artemisia campestris*, *Juniperus turbinata* (leaves), *Otanthus maritimus* and *Eryngium maritimum* EOs from Peniche (Portugal), at a final concentration 5 µL/mL with dilution 1:2. To calculate the IC_{50} values, MTS assay was carried out and was performed using the Graph Pad Prism software.

Final concentration 1 µL/mL			
Essential Oil	**Average IC_{50} (µL/mL) ± SD**		**Number of inter-experimental repeats**
	RKO	**MCF7**	
Crithmum maritimum	8,07E-02 ± 1,26E-01	5,36E-03 ± 7,06E-03	n = 3
Seseli tortuosum	3,29E-03 ± 2,58E-03	1,85E-03 ±8,38E-04	n = 3
Artemisia campestris	1,14E-02 ±2,85E-03	9,81E-02 ± 1,28E-01	n = 2
Juniperus turbinata	5,78E-02 ± 4,35E-02	1,13E-01 ±1,88E-01	n = 4
Otanthus maritimus	1,52E-02 ± 8,77E-03	1,23E-02 ±4,02E-03	n = 4
Eryngium maritimum	8,59E-04 ±5,58E-04	2,72E-03 ±2,15E-03	n = 2

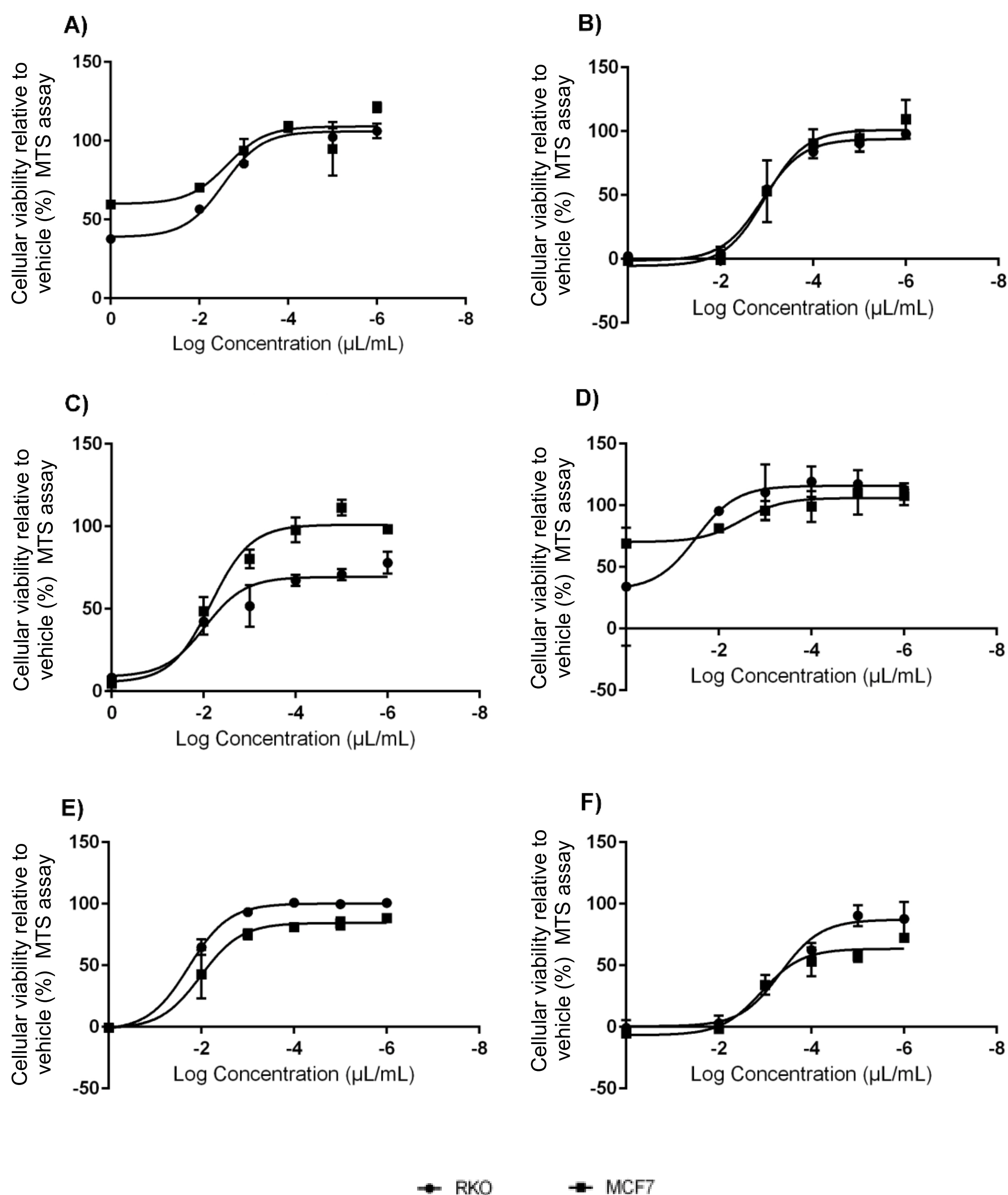

Figure 3.2.6 – Estimated dose-response parameter of each EO tested against RKO and MCF7 cancer cell lines. The species used were A) *Crithmum maritimum*, B) *Seseli tortuosum*, C) *Artemisia campestris*, D) *Juniperus turbinata*, E) *Otanthus maritimus* and F) *Eryngium maritimum* Essential Oils, against RKO (●) and MCF7 (■) cell lines viability detected by MTS Assay, at a concentration 1 µL/mL and dilution 1:10. MTS assay was carried out as described before (Figures 3.2.3 and 3.2.4) and Graph pad prism software was used to plot the curve fitting for each oil.

When comparing the IC_{50} values obtained for all EOs tested for RKO cell line data set, *Seseli tortuosum*, *Eryngium maritimum* and *Crithmum maritimum* EOs treatment showed the highest decrease of cellular viability (as indicated by the lowest IC50), followed by *Juniperus turbinata* (leaves), *Otanthus maritimus* and *Artemisia campestris* EOs (figure 3.2.6 and table IV).

The EOs that showed the highest activity against MCF7 cells (again, as indicated by the lowest IC50) were *Crithmum maritimum, Eryngium maritimum* and *Seseli tortuosum* EOs, followed by *Artemisia campestris*, *Otanthus maritimus* and *Juniperus turbinata* (leaves) EOs.

3.3 Evaluation of mechanism of biological effects of EOs

After the evaluation of the efficacy of the EOs extracted in decreasing cancer cell viability the EOs from the species that showed the highest potential as anticancer agents (*Seseli tortuosum* and *Otanthus maritimus*) were selected for a study concerning the mode of action.

Seseli tortuosum EO was diluted to the IC_{50} concentrations of 6 μL/mL (for RKO cells) and 8.5 μL/mL (for MCF7), and *Otanthus maritimus* EO was diluted to the IC_{50} concentrations of 4 μL/mL and 7.5 μL/mL (for RKO and MCF7 cell lines, respectively). DMSO was used as a vehicle control.

MCF7 and RKO cells were incubated for 8 h, 24 h, 48 h and 72 h with the EO solutions in media or vehicle control diluted in media, after which cells were harvested and protein expression analysed by Western blotting. The proteins selected for analysis are associated biological processes relevant to decreased viability/proliferation in cells, namely programmed cell death/apoptosis by looking for cleaved caspase 3, and cell cycle arrest and DNA damage response by looking at p21 and p53 expression. β-Actin was used as a loading control.

The results of Western blot technique, for *Seseli tortuosum* treatment are shown in figure 3.3.1 and for *Otanthus maritimus* treatment are shown in figure 3.3.2.

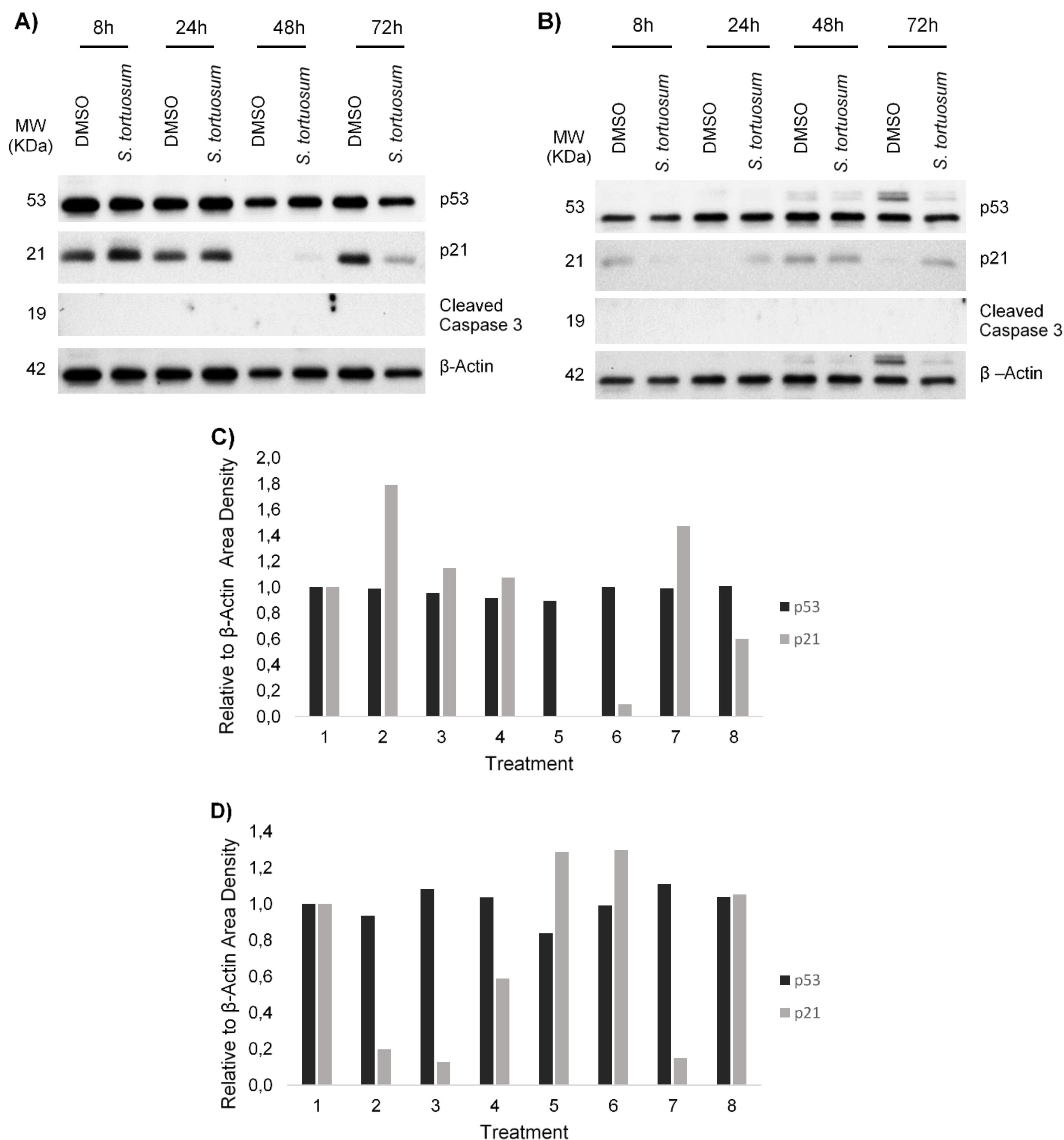

Figure 3.3.1 – Western blot analysis for *Seseli tortuosum* EO treatment in MCF7 (A) and RKO (B). MCF7 and RKO cells were treated with 8.5 μL/mL and 6 μL/mL (IC_{50} values represented in table III), respectively. Cells were incubated for 8, 24, 48 and 72 hours before harvesting. Western blotting was carried out for p53, p21, cleaved Caspase-3 and β-Actin (loading control). Bar charts represent densitometry analysis of the protein bands (y axis) was performed using ImageJ software (calculated relative to β-Actin band intensity) for MCF7 (C) and RKO (D) cell lines, of the proteins bands in p53 and p21 in treatment (x axis) 1) DMSO at 8 hours (Control); 2) *Seseli tortuosum* EO at 8 hours; 3) DMSO at 24 hours; 4) *Seseli tortuosum* EO at 24 hours; 5) DMSO at 48 hours; 6) *Seseli tortuosum* at 48 hours; 7) DMSO at 72 hours and 8) *Seseli tortuosum* at 72 hours treatments.

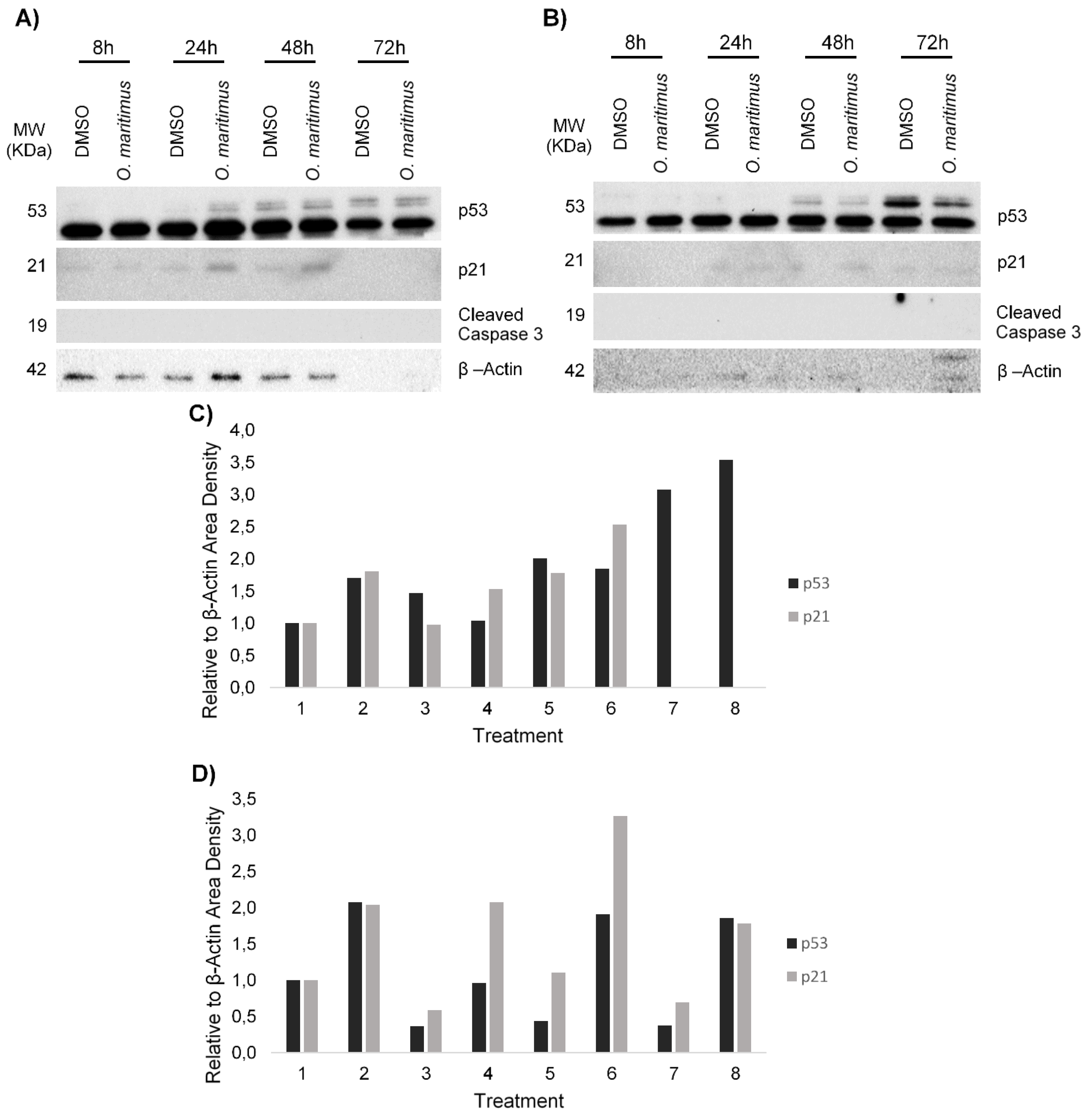

Figure 3.3.2 – Western blot analysis for *Otanthus maritimus* EO treatment in MCF7 (A) and RKO (B). MCF7 and RKO cells were treated with 7.5 µL/mL and 4 µL/mL (IC_{50} values represented in table IV), respectively. Cells were incubated for 8, 24, 48 and 72 hours before harvesting. Western blotting was carried out for p53, p21, cleaved Caspase-3 and β-Actin (loading control). Bar charts represent densitometry analysis of the protein bands (y axis) was performed using ImageJ software (calculated relative to β-Actin band intensity) for MCF7 (C) and RKO (D) cell lines, of the proteins bands in p53 and p21 in treatment (x axis) 1) DMSO at 8 hours (Control); 2) *Otanthus maritimus* EO at 8 hours; 3) DMSO at 24 hours; 4) *Otanthus maritimus* EO at 24 hours; 5) DMSO at 48 hours; 6) *Otanthus maritimus* at 48 hours; 7) DMSO at 72 hours and 8) *Otanthus maritimus* at 72 hours treatments.

Western blot analysis showed that treatment with the *Seseli tortuosum* EO, in MCF7 cells affected the expression of p53, showing a small decrease until treatment 5 (DMSO at 48h) and after a small increase. In RKO cell line, the expression of p53 showed a stabilization in their amount, except in treatment 5 (DMSO at 48h).

Treatment with *Seseli tortuosum* EO affected the expression of p21, leading to an increase of p21 amount in MCF7 cells, except in treatment 5 (DMSO at 48 hours). In RKO cells the expression of p21 showed a variation in their amount. At 8 hours and 48 hours of treatments p21 showed a stabilization in their levels, but at 24 hours and 72 hours, the amount of p21 had a small amount followed by an increased amount (DMSO and EO treatments, respectively.

Western blot analysis showed that treatment of MCF7 cells with *Otanthus maritimus* EO affected the expression of p53, leading to an increased in its amount until 72 hours of treatment. In RKO cell line, the expression of p53 showed an increase in amount in EO treatments than DMSO (vehicle).

In treatment with the *Otanthus maritimus* EO, western blot showed that EO affected the expression of p21, showing an increase of their amount in EO treatments than DMSO (vehicle), by the times tested in MCF7 cells, except at 72 hours of treatment (p21 was not observed). In RKO cells the expression of p21 showed an increase of their amount in EO treatments than DMSO (vehicle), by the times tested, and in treatment 6 (EO treatment at 48 h) the amount showed be very high.

For *Seseli tortuosum* and *Otanthus maritimus* EO treatment of both cancer cell lines tested cleaved caspase 3 was not observed.

In the previous result dataset, it was observed that treatment with *Seseli tortuosum* EO led to an increase in p21 protein level in the first two days of treatment (and to a certain degree, this was also observed for *Otanthus maritimus* EO treatment). Since it is known that p21 accumulation could be an indicative of cell cycle arrest (as mentioned in the Introduction, section 1.3.2), it was decided to analyse cell cycle progression of both MCF7 and RKO cells after treatment with *Seseli tortuosum* EO using Fluorescence-activated cell sorting (FACS) to detect changes in DNA content.

The results regarding the evaluation of the effect of *Seseli tortuosum* EO treatment in cell cycle progression are presented in figure 3.3.3 and table V, for RKO cells, and in figure 3.3.4 and table VI, for MCF7 cells.

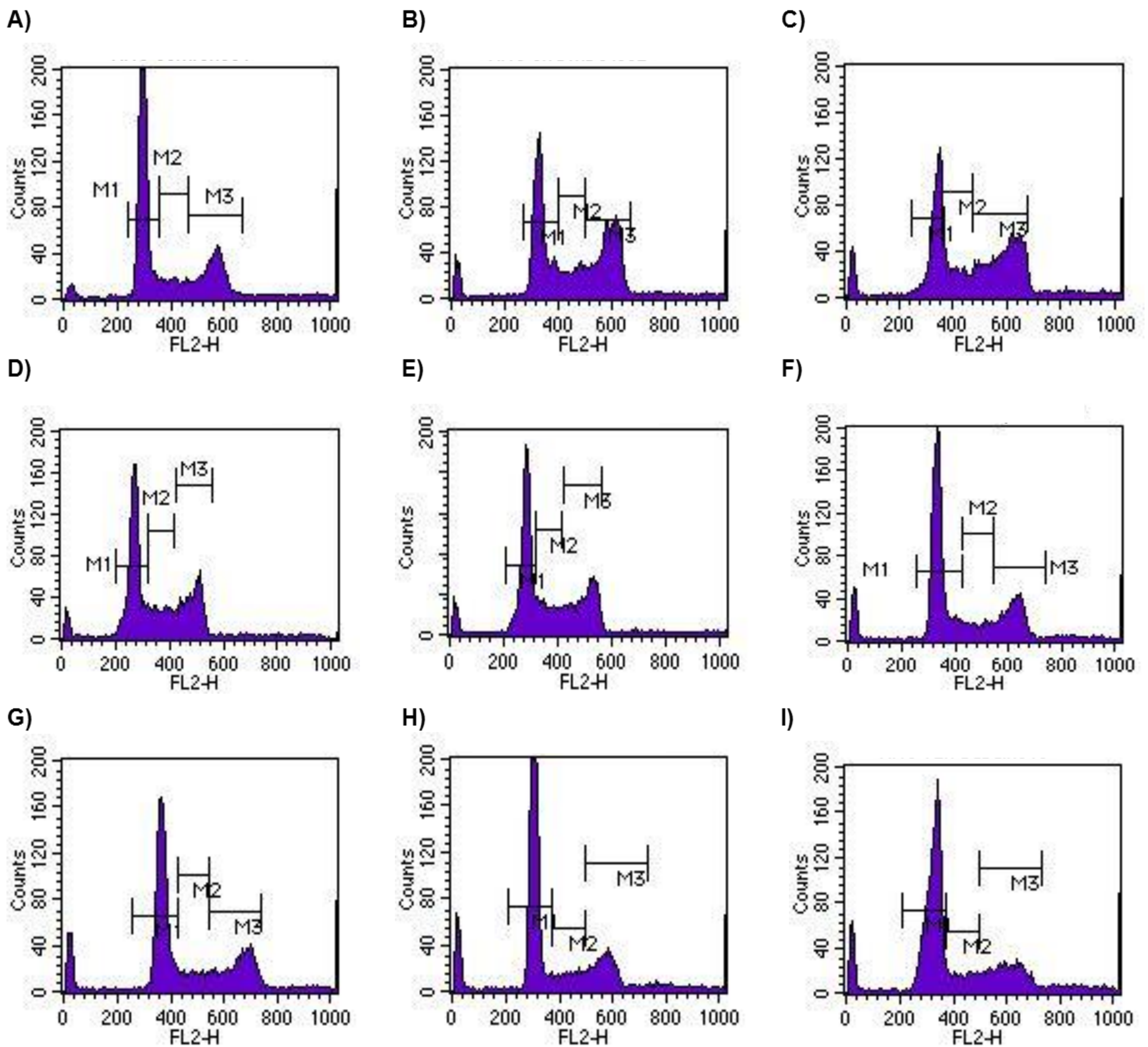

Figure 3.3.3 – DNA content histograms from RKO cell line after treatment with *Seseli tortuosum*. RKO cells was treated with 6 µL/mL, respectively, of *Seseli tortuosum* EO and DMSO (vehicle). The cells were incubated for 8, 24, 48 and 72 hours and fixed in 70% ethanol. After PI staining, FACS analysis (section 2.11) was done for all samples. Sample key: A) Control; B) DMSO – 8 hours; C) *Seseli tortuosum* – 8 hours; D) DMSO for 24h; E) *Seseli tortuosum* – 24 hours; F) DMSO – 48 hours; G) *Seseli tortuosum* – 48 hours; H) DMSO for 72h; I) *Seseli tortuosum* – 72 hours. The different phases of the cell cycle were represented by M1: G1 Phase; M2- S Phase and M3 – G2 and M Phase. In the FL2-H (x axis) the intensity of emitted fluorescent from PI (DNA dye) is represented, as a surrogate of DNA content.

Table V – Flow cytometry analysis of % of cells per stage of the cell cycle in RKO cells treated with *Seseli tortuosum* EO. The different phases of the cell cycle were represented by M1: G1 Phase; M2- S Phase and M3 – G2 and M Phase and were obtained from the histograms shown in figure 3.3.3)

RKO

		8 hours		**24 hours**		**48 hours**		**72 hours**	
Phase	Control	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*
M1	60.29	41.23	32.36	50.16	46.84	57.76	53.30	59.88	57.92
M2	9.02	13.26	14.43	17.12	17.31	10.61	10.44	9.97	9.65
M3	25.26	38.17	45.00	26.55	29.37	22.81	26.80	26.55	22.45

In the RKO cell line the percentage of cells in each cell cycle stage, as quantified by FACS analysis, is represented in table V.

The first phase (corresponding to G_1 phase) had higher percentage of cells in all treatments. By the times tested, it was possible to observe that in G_1 phase the percentage of cells had increased. The M2 phase, corresponding to S phase, the percentage of cells were lower comparing to G_1 phase, and by the times points tested, the percentage of number of cells decreased, except at 24 hours of treatments (DMSO and EO). The percentage of cells in phase G_2 is lower than G_1 phase, except at 8 hours with EO treatment and by the time points tested is possible observe a decrease of the percentage of cells, except for DMSO treatment at 72 hours.

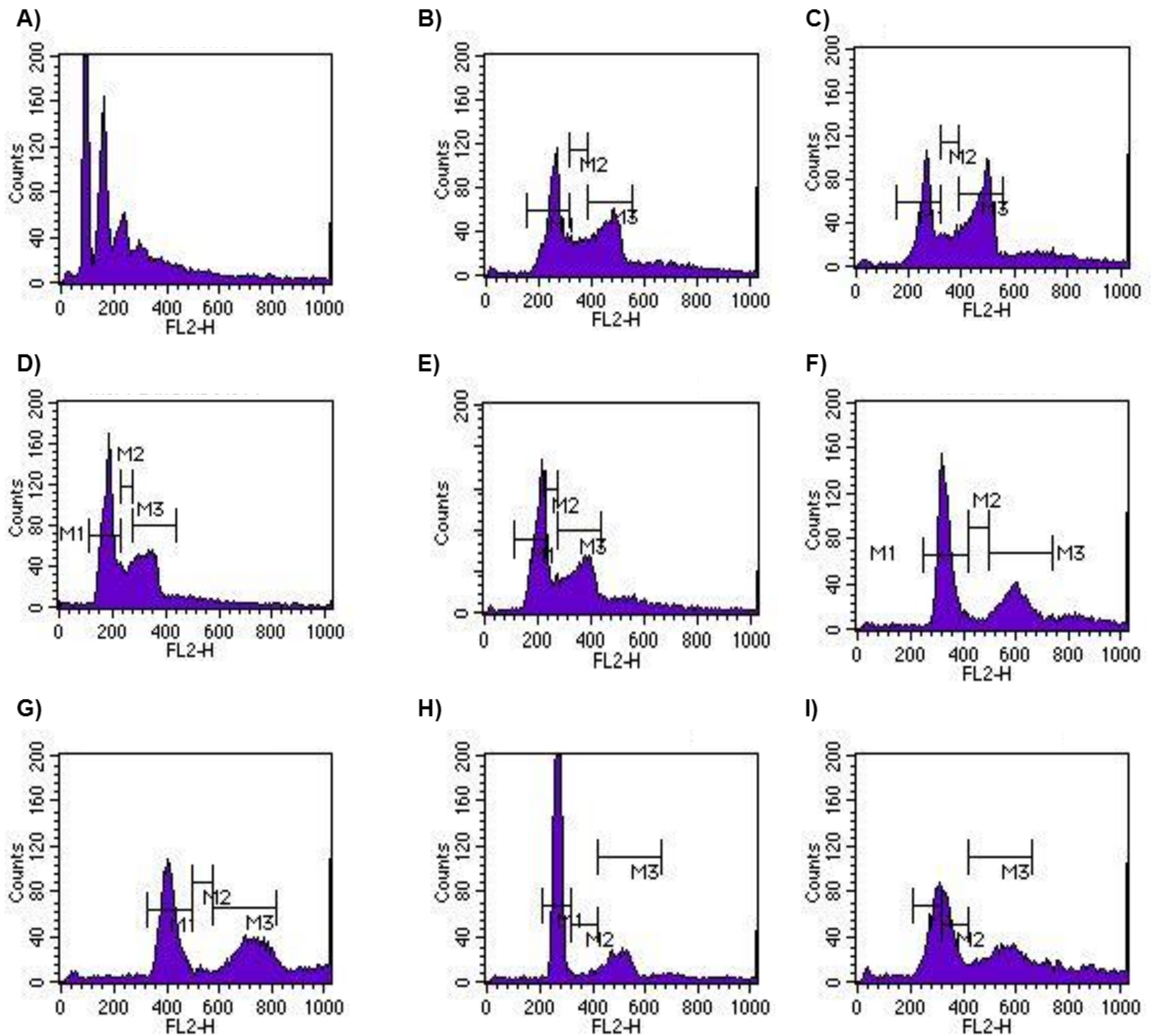

Figure 3.3.4 - DNA content histograms from MCF7 cell line after treatment with *Seseli tortuosum*. RKO cells was treated with 8.5 µL/mL, respectively, of *Seseli tortuosum* EO and DMSO (vehicle). The cells were incubated for 8, 24, 48 and 72 hours and fixed in 70% ethanol. After PI staining, FACS analysis (section 2.11) was done for all samples. Sample key: A) Control; B) DMSO – 8 hours; C) *Seseli tortuosum* – 8 hours; D) DMSO for 24h; E) *Seseli tortuosum* – 24 hours; F) DMSO – 48 hours; G) *Seseli tortuosum* – 48 hours; H) DMSO for 72h; I) *Seseli tortuosum* – 72 hours. The different phases of the cell cycle were represented by M1: G1 Phase; M2- S Phase and M3 – G2 and M Phase. In the FL2-H (x axis) the intensity of emitted fluorescent from PI (DNA dye) is represented, as a surrogate of DNA content.

Table VI – Flow cytometry analysis of % of cells per stage of the cell cycle in RKO cells treated with *Seseli tortuosum* EO. The different phases of the cell cycle were represented by M1: G1 Phase; M2- S Phase and M3 – G2 and M Phase and were obtained from the histograms shown in figure 3.3.4)

MCF7

		8 hours		**24 hours**		**48 hours**		**72 hours**	
Phase	Control	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*	DMSO	*Seseli tortuosum*
M1		38.51	28.96	48.92	39.78	50.85	37.25	71.64	22.98
M2		13.06	10.03	10.94	8.95	2.86	2.54	3.26	19.78
M3		39.49	45.20	33.51	35.85	27.30	27.33	19.47	24.80

In MCF7 cell line the percentage of cells in each cell cycle stage, as quantified by FACS analysis, is represented in table VI.

The first phase (corresponding to G_1 phase) had higher percentage of cells in all treatments, except at 8 hours in both treatments (DMSO and EO) and at 72 hours in EO treatment. Is possible observe in G_1 phase the percentage of cells increased in 8 hours and 24 hours, but in 48 hours and 72 hours, this value decreased. The M2 phase, corresponding to S phase, the percentage of cells was lower comparing to G_1 phase, and by the time points tested, the percentage of cells had a decrease, except at 72 hours of EO treatment. Relatively to the percentage of cells in phase G_2, this value is lower than G_1 phase, except at 8 hours with both treatments and 72 hours in EO treatment. By the time points tested is possible to observe a decrease of the percentage of cells.

4. Discussion

4.1 Overview of this work

In recent years, the use of aromatic and medicinal plants has increased (Burt 2004; Kordali *et al.* 2005; Gonçalves *et al.* 2010; Miguel 2010; Chen *et al.* 2011; Koba *et al.* 2011;). Some EOs have also been used in food preservation, aromatherapy and fragrance industries and other oils have been used in cancer treatment (Sharma *et al.* 2009; Sylvestre *et al.* 2006; Kapoor *et al.* 2008; Cooke & Ernst 2000; Carnesecchi *et al.* 2001).

In this study, EOs of the six selected species – *Artemisia campestris* subsp. *maritima* *Crithmum maritimum*; *Eryngium maritimum*; *Juniperus turbinata* subsp. *turbinata*; *Otanthus maritimus* and *Seseli tortuosum* - were investigated in order to determine their efficacy as potential cytotoxic/cytostatic agents in human breast adenocarcinoma (MCF7) and colorectal carcinoma (RKO) cell line models.

As shown in section 3.2, treatment with all EOs tested led to a decrease in cellular viability for both cell lines tested (as measured by MTS assay). The only exception was for the effect of treatment with *Eryngium maritimum* EO (1:2 dilution starting at a concentration of 5 µL/mL).

In order to realise if the reduction in cell viability is concentration-dependent, and in particular for achieving compare the potency of the effects demonstrated by the various EOs studied, it was decided to carry out a test concentration dependence for each EO, showing a reduction in capacity less than 50% in different tested concentrations of 5 µL/mL (figure 3.2.5) and 1 µL/mL (figure 3.2.6). The lowest IC_{50} values represent the EOs that need a lower concentration to administer and obtain a stronger effect in cells tested. Instead, the highest IC_{50} values obtained represent a required highest concentration of the EO to obtain a stronger effect in MCF7 and RKO cells.

As showned in table III, for RKO cell line, *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* EOs presented the lowest IC_{50} values. *Juniperus turbinata* subsp. *turbinata* (leaves) and *Crithmum maritimum* EOs presented the highest IC_{50} values, for RKO cell line (table III). *Eryngium maritimum* EO obtained an IC_{50} value too high. For MCF7 cell line, represented in table III, *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* EOs had the lowest IC_{50} values. Namely, Juniperus *turbinata* subsp. *turbinata* and *Crithmum maritimum* EOs had the highest IC_{50} values. *Eryngium maritimum* EO had an IC_{50} value very high.

As reported in table IV, relative to RKO cell line, *Seseli tortuosum*, *Eryngium maritimum* and *Crithmum maritimum* EOs had the lowest IC_{50} values. Conversely, *Juniperus turbinata* subsp. *turbinata* (leaves), *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* EOs obtained the highest IC_{50} values. In MCF7 cell line, *Crithmum maritimum, Eryngium maritimum* and *Seseli tortuosum* EOs showed the lowest values and *Artemisia campestris* subsp. *maritima*, *Otanthus maritimus* and *Juniperus turbinata* subsp. *turbinata* (leaves) EOs obtained the highest IC_{50} values.

In section 3.3 the mechanism of action of selected EOs (*Seseli tortuosum* and *Otanthus maritimus*) was explored by western blot, using the determined IC_{50} values, in order to understand which mechanisms that EOs follow to inhibit cellular viability/proliferation. This study revealed that p53 was activated by cellular stress and not by programmed cell death, as cleaved caspase 3 was not observed. p21 was observed in present study, and to comprove the results, FACS was performed concluding that EOs tested possibly had a potential to induce cell cycle arrest, namely in G_1 phase.

4.2 Chemical composition of EOs tested

In the present study, EO extraction led to yields ranging from 0.08% to 0.66%, depending on the species. A wide range of compounds were identified above 10% concentration (compounds with a reasonable concentration), except for *Eryngium maritimum* EO. Main constituents were summarised in table I.

Different major compounds in each species studied are represented in table VII. This table allowed a comparison between different studies and major compounds in present study.

Table VII – Major constituent(s) for each specie studied with respective author and year of relevant studies.

Species	Major constituent(s) in present study	Major constituent(s)	Plant part(s) used / Extraction technique	Relevante Reference
Artemisia campestris	β-pinene and Sesquiterpenes	β-pinene, Limonene and Germacrene D	Aerial parts/ Hydrodistillation	Akrout *et al.* 2011
Crithmum maritimum	γ-terpinene, Thymol methyl ether and Sabinene	p-cymene, β-phellandrene, γ-terpinene, Thymol methyl ether and dillapiole	Aerial parts/ Supercritical CO_2 Extraction	Marongiu *et al.* 2007
Eryngium maritimum	Germacrene D and other Sesquiterpene hidrocarbons	Germacrene D, 4bH- cadin-9-en-15-al, 4bH-cadin-9-en-15-ol and 4bH-muurol-9-en-15-al	Aerial parts/ Hydrodistillation	Darriet *et al.* 2014
Juniperus turbinata	α-pinene, β-phellandrene and α-terpinyl acetate	α-pinene, β-phellandrene and α-terpinyl acetate	Leaves/ Hydro-distillation	Cavaleiro *et al.* 2006
Otanthus maritimus	Filifolone, Chrysanthenone and cis-chrysantenyl acetate	Chrysanthenone, Filifolone and cis-chrysantenyl acetate	Aerial parts/ Hydrodistillation	Cabral *et al.* 2013
Seseli tortuosum	α-pinene, β-pinene and (Z)-β -ocimene	α–pinene, β-pinene and (Z)- β-ocimene	Ripe Umbles with mature seeds/ Hydro-distillation	Gonçalves *et al.* 2012

In the present study, *Artemisia campestris* subsp. *maritima* EO was identified to be predominantly composed of β-pinene (monoterpene) and sesquiterpenes. In another study based in Tunisia (table VII), *Artemisia campestris* subsp. *maritima* was characterized by Akrout and collaborators as having as main compounds β-pinene (monoterpene) (34.2%), limonene (monoterpene) (8.2%) and germacrene D (sesquiterpene) (7.3%). The authors reported in this EO monoterpene hydrocarbons as the more representative group (72.2%) followed by sesquiterpene hydrocarbons (15%) (Akrout *et al.* 2011). Comparing to *Artemisia campestris* subsp. *maritima* study from Portugal, β-pinene is the major compound, and the sesquiterpenes were present in both species in highest concentrations.

The EO extracted from *Crithmum maritimum* was shown to be composed primarily by γ-terpinene, thymol methyl ether and sabinene. Marongiu and colleagues investigated the chemical composition of this species harvested in Buggerru (Sardinia Island, Italy), Figueira da Foz (Portugal), and S. Pedro de Moel (Portugal). The authors reported the major compounds to be p-cymene, β-phellandrene, γ-terpinene, thymol methyl ether and dillapi-

ole (Marongiu *et al.* 2007). This is in accordance to the composition of *Crithmum maritimum* EO extracted from Peniche in the present study, where γ-terpinene and thymol methyl ether were present in high concentration.

The EO extracted from *Eryngium maritimum* was identified in the present study as having lower yield, with several compounds below 10% concentration. The main compounds observed were germacrene D (sesquiterpene), as well as other sesquiterpene hidrocarbons. In another study, EO extracted from *Eryngium maritimum* from Corsica had as main components germacrene D (33.9%), 4bH- cadin-9-en-15-al (26.1%), 4bH-cadin-9-en-15-ol (9.5%) and 4bH-muurol-9-en-15-al (5.2%) (Darriet *et al.* 2014). Comparing to *Eryngium maritimum* EO from Peniche, gremacrene D is the only compound found in both species.

In the present study, the EO mixture extracted from *Juniperus turbinata* subsp. *turbinata* contained α-pinene, β-phellandrene and α-terpinyl acetate as main compounds, all present in concentration over 10%. When compared to the composition described by Cavaleiro *et al.* (2006), of *Juniperus turbinata* subsp. *turbinata* (also collected in Portugal), the sample collected for this study has similar compounds, namely, α-pinene (48.2%), β-phellandrene (23.1%) and α-terpinyl acetate (5.6%) (Cavaleiro *et al.* 2006).

The extracted EO mixture composition for *Otanthus maritimus* from Quiaios beach, Figueira da Foz, Comporta beach and Baleal beach (Portugal) were previously investigated in the study by Cabral *et al.* in 2013. There, it was reported that there was no significant difference in chemical composition of EOs extracted from specimens harvested in the different locations, and the main compounds were the same for all EO mixtures, namely chrysanthenone (40.4–57.2%), filifolone (12.2–15.5%) and cis-chrysantenyl acetate (10.1 – 11.2%) (Cabral *et al.* 2013). Importantly, the EOs extracted from the specimens collected for the present study (Consolação beach, Peniche, Portugal) had a similar chemical composition.

The chemical composition of *Seseli tortuosum* EOs was previously studied by Gonçalves and collaborators, in 2012 (Gonçalves *et al.* 2012). The study reported that *Seseli tortuosum* EOs from two different locations, Vieira de Leiria (Portugal) and Baleal (Portugal), had as major constituents α–pinene (24.9% and 24.8%, respectively), β-pinene (23.9% and 23.5%, respectively) and (Z)-β-ocimene (13.3% and 16.0%, respectively). *Seseli tortuosum* EO extracted from specimens harvested at Consolação beach (Peniche, Portugal) for the present study revealed a similar composition as reported by Gonçalves *et al.* (2012).

Biological activity of EOs may be due to one of the compounds presents in their chemical composition or due to the entire mixture. Effects of different classes of compounds present in essential oils together with their major properties and their potential therapeutic activities are described:

α–pinene, β-pinene, γ-terpinene, sabinene, germacrene D, p-cymene, β-phellandrene, (Z)-β-ocimene and limonene are example compounds of hydrocarbons category. This category is characterized to contain molecules of hydrogen and carbon only, and are classified into terpenes (mono-, sesqui-, and diterpenes) (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). The hydrocarbons may be acyclic, alicyclic (mono-, bi- and tricyclic) or aromatic. These compounds have been associated with various therapeutic activities as antiviral, antitumor, antibacterial and hepatoprotective (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010).

Phenols are aromatic components. These compounds are among the most reactive, potentially toxic and irritant, especially for the skin and the mucous membranes. They possess antimicrobial, rubefacient properties, stimulate the immune and nervous systems and may reduce cholesterol (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). Phenols are often found as crystals at room temperature, and the most common ones are thymol, eugenol, carvacrol and chavicol (Djilani & Dicko 2010).

Esters are sweet smelling and give a pleasant smell to the oils and are very commonly found in a large number of EOs. They include for example, α-terpinyl acetate, cis-chrysantenyl acetate, linalyl acetate, geraniol acetate, eugenol acetate and bornyl acetate. Esters are anti-inflammatory, spasmolytic, sedative, and antifungal (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010).

Oxides or cyclic ethers are the strongest odorants, and by far the most known oxide is 1,8- cineole, as it is the most omnipresent one in essential oils. Other examples of oxides are bisabolone oxide, linalool oxide, sclareol oxide and ascaridole. Their therapeutic benefits are expectorant and stimulant of nervous system (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). In present study any of this compounds category was described in any specie.

Lactones are of relatively high molecular weight and are usually found in pressed oils. Some examples of lactones are nepetalactone, bergaptene, costuslactone, dihydronepetalactone, alantrolactone, epinepetalactone, aesculatine, citroptene, and psoralen (Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). They may be used for antipyretic, sedative

and hypotensive purposes, but their contraindication is allergy, especially such involving the skin (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). In this study was not reported any of this compounds of this category.

In addition to their pleasant fragrance, **alcohols** are the most therapeutically beneficial of essential oil components with no reported contraindications. They are antimicrobial, antiseptic, tonifying, balancing and spasmolytic (Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). Examples of EO alcohols are linalol, menthol, borneol, santalol, nerol, citronellol and geraniol (Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). In present study, any of this compounds was reported.

Aldehydes are common EO components that are unstable and oxidize easily. Many aldehydes are mucous membrane irritants and are skin sensitizers (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). They have characteristically sweet, pleasant fruity odours and are found in some culinary herbs such as cumin and cinnamon. Therapeutically, certain aldehydes have been described as: antiviral, antimicrobial, tonic, vasodilators, hypotensive, calming, antipyretic and spasmolytic (Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). Common examples of aldehydes in EOs include citral (geranial and neral), myrtenal, cuminaldehyde, citronellal, cinnamaldehyde, benzaldehyde (Djilani & Dicko 2010) and any of this compounds were reported, in present study.

Ketones are not very common in the majority of EOs; they are relatively stable molecules and are not particularly important as fragrances or flavor substances (Djilani & Dicko 2010). They may be mucolytic, cell regenerating; sedative, antiviral, analgesic and digestive. Due to their stability, ketones are not easily metabolized by the liver (Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). Common examples of ketones found in EOs include carvone, menthone, pulegone, fenchone, camphor, thujone and verbenone (Bakkali *et al.* 2008; Adorjan & Buchbauer, 2010; Djilani & Dicko 2010). Any of this EOs compounds were described in this study.

4.3 Cell viability assessment by MTS Assay

Evaluation of the anticancer activity of exposure to EOs can be achieved by the MTS assay. The MTS assay is a colorimetric viability assay based on enzymatic reduction of the MTS molecule to formazan when it is exposed to viable cells. The outcome of the reduction is a colour change of the MTS molecule. Absorbance measurements relative to a con-

trol determine the percentage of remaining viable cancer cells following their treatment with varying concentrations of a tested EO, which is translated to the EO anticancer activity and its IC_{50} values.

All the EOs exhibited anticancer activity against RKO and MCF7 cells, as shown by a clear decrease in cell viability, as measured by the MTS assay and by IC_{50} values calculated.

4.3.1 *Crithmum maritimum* L.

Treatment with *Crithmum maritimum* EO at a concentration 5 µL/mL (figure 3.2.1 A and 3.2.2 A), led to a decrease in cellular viability, however at this concentration, EOs treatments were highly toxic for both cell lines, and were not suitable for accurate determination of the IC_{50}s. The maximum concentration tested *in vitro* should be until 100% of the cells in tests lose their completely cellular activity (not more than that). This way, the dose-response of the EO/compound/drug, and sub consequently IC_{50} values, calculated through by MTS assay (as in present study) will be more efficiently and correctly. As is possible to observe, at 5 µL/mL concentration, in MTS assay results and IC50 values, the cellular viability was below of 0% cellular viability. Therefore, it was decided to repeat the experiments using serial dilutions 1:10 at a starting concentration 1 µL/mL.

At a final concentration 1 µL/mL with a dilution 1:10, *Crithmum maritimum* EOs treatment, again led to a decrease in cellular viability (figure 3.2.3 A and 3.2.4 A). That suggests the EOs from this species have potential anticancer properties.

Comparing to a final concentration 5 µL/mL with a dilution 1:2, *Crithmum maritimum* EO treatment in the serial dilutions, at a final concentration 1 µL/mL had lower IC_{50} values. The IC_{50} values in this concentration were 8.07E-02 µL/mL and 5.36E-03 µL/mL for RKO and MCF7 cell lines, respectively (figure 3.2.6 A and table III). These values allow us to compare how the cell viability is affected by dilution of the concentration of essential oils. Is possible to conclude that a lower maximal concentration is necessary to decrease viability in 50% of cell culture, *in vitro*.

Various authors have studied the effects of *C. maritimum* EO in several aspects of cell biology, uncovering antioxidant and antibiotic activities (Jallali *et al.* 2014; Meot-Duros *et al.* 2008; Meot-Duros & Magné 2009). However, to our knowledge, studies are scarce regarding any potential anticancer properties. One study, by Meot-Duros and collabora-

tors reported that falcarindiol showed cytotoxicity against IEC-6 (normal rat small intestine) cells with an IC_{50} value of 20 μM after 48 h of exposition (Meot-Duros *et al.* 2010). Falcarindiol is a compound reported in *Crithmum maritimum* that exhibits various biological properties, such as antibacterial and anti-mutagenic properties (Meot-Duros *et al.* 2010; Jin *et al.* 2012; Jallali *et al.* 2014). However, Meot-Duros isolated this compound by solvent extraction. By hydrodistillation, the isolation of this compound is difficult to perform. This compound was not found in the chemical composition in *Crithmum maritimum* EO from Peniche.

4.3.2 *Seseli tortuosum* L.

At a final concentration 5 μL/mL, *Seseli tortuosum* EO treatment had a significant decrease in cellular viability, in RKO (figure 3.2.1 B) and MCF7 (figure 3.2.2 B) cell lines. The cellular viability decrease proved to be very pronounced. Because of this situation serial dilutions were performed at starting concentration 1 μL/mL and dilution 1:10.

In concentration 1 μL/mL (figure 3.2.3 B and 3.2.4 B), the IC_{50} values were higher than the concentration 5 μL/mL, namely, the IC_{50} values for RKO and MCF7 cells are 3.29E-03 μL/mL and 1.85E-03 μL/mL, respectively (figure 3.2.6 B and table III). It is possible to conclude that a lower maximal concentration was necessary to decrease viability in 50% of cell culture, *in vitro*, when tested at starting final concentration 5 μL/mL.

Gonçalves and colleagues investigated the cytotoxic properties in human keratinocytes (HaCaT) cell line with *Seseli tortuosum* EO, at different concentrations – 0.32 μL/mL; 0.64 μL/mL and 1.25 μL/mL (Gonçalves *et al.* 2012). It was showed in this study that *Seseli tortuosum* EOs had a cytotoxic effect on human keratinocytes, especially when used in concentrations above 0.64 μL/mL. The authors suggested the attention for the application of the oil for health promoter activities, specifically in the selection of concentrations with fungicidal activity and without cytotoxicity (Gonçalves *et al.* 2012). The results in the present study are in agreement with results shown by Gonçalves and collaborators, as in both studies at 0.64 μL/mL the cellular viability had a clear decrease. However, a decrease in cellular viability was observed at lower concentrations than the ones used in Gonçalves *et al.* 2012, indicating that *Seseli tortuosum* EOs contain compounds that can decrease viability in cancer cell lines and that these are more sensitive to the EOs than primary cells (such as the keratinocytes).

4.3.3. *Artemisia campestris* subsp. *maritima* (DC.) Arcang.

Treatment with *Artemisia campestris* subsp. *maritima* EO treatment led to a decrease in cellular viability in both RKO and MCF7 cell lines, for final concentration range tested, at 5 µL/mL (figures 3.2.1 C and 3.2.2 C, respectively). However, at this range this EO treatment showed to be toxic to cancer cell lines tested. For this reason, the same treatment was performed with different starting final concentration 1 µL/mL and dilution 1:10.

The cellular viability are reported in figures 3.2.3 C and 3.2.4 C for RKO and MCF7, respectively. The IC_{50} values are 1.14E-02 µL/mL and 9.81E-02 µL/mL in RKO and MCF7 cell lines, respectively, at a final concentration 1 µL/mL in a dilution 1:10 (figure 3.2.6 C and table III). Comparing both concentrations tested, RKO cell line had a decrease in cellular viability as expected (comparison between 5 µL/mL and 1 µL/mL). However, MCF7 cell line had a small increase in cellular viability (as proved by IC_{50} values*)*, which was not expected. Thus indicates, that for RKO cell line a lower maximal concentration was necessary to decrease viability in 50% of cell culture, *in vitro*. But, for MCF7 cell line, the maximal concentration to inhibit viability to half needs to be higher. Comparing to other EOs treatments tested, *Artemisia campestris* subsp. *maritima* had the lower number of inter-replicas (n=2). To confirm this results, more inter-experimental replicas should be done.

Whilst there are many studies demonstrating antioxidant properties for *A. campestris* extracts (Aniya *et al.* 2000; Djeridane *et al.* 2006), the literature regarding possible anti-tumour action is scarce (Kaneshiro *et al.* 2005). EOs extracted from *Artemisia campestris* L. from southern Tunisia using ethanol-water (EtOH), hexane extract and infusion extract approaches were investigated by Akrout and colleagues (Akrout *et al.* 2011) The authors investigated the antitumor activity of EOs from this species against human colon cancer (HT-29) cell line using MTT assay to determine net growth inhibition. Akrout and collaborators showed the most significant growth inhibitory action was performed by *Artemisia campestris* L. EO (comparing to EtOH and hexane extracts) with a GI_{50} (estimation of growth inhibition of 50%) value 46.82 µg/mL, while EtOH presented a GI_{50} value higher than 100 µg/mL and the hexane extracts presented a GI_{50} value higher that 1920 µg/mL (Akrout *et al.* 2011). Akrout *et al.* described that *Artemisia campestris* EOs contained high concentration of β-pinene, α-pinene and germacrene D, myrcene and limonene – all terpenes, which have been described by their antitumor activity against several types of human cancers, such as breast cancer, liver cancer and melanoma (Li *et al.* 2009; Darmanin

et al. 2009; Crowell 1999). A hypothesis proposed by Akrout and his collaborators is that terpenes might be responsible for all or part of the antitumor activity (Akrout *et al.* 2011). Importantly, these compounds were present in *Artemisia campestris* subsp. *maritima* EO harvested from Peniche and used in the present study.

4.3.4 *Juniperus turbinata* Guss. subsp. *turbinata*

For both final concentrations used - 5 µL/mL (figure 3.2.1 D and 3.2.2 D) and 1 µL/mL (figure 3.2.3 D and 3.2.4 D) – treatment with Juniperus *turbinata* subsp. *turbinata* EO lead a decrease in cellular viability for both cell lines, as indicated by IC_{50} values demonstrated. However, Juniperus *turbinata* subsp. *turbinata* EO proved to be toxic to cancer cell lines tested.

At a final concentration 1 µL/mL (figure 3.2.6 D and table III), the IC_{50} values were 5.78E-02 µL/mL and 1.13E-01 µL/mL for RKO and MCF7, respectively. This values indicates that was necessary a lower maximal concentration to decrease viability in 50% of cell culture, *in vitro*, at starting final concentration 1 µL/mL.

Until present, to our knowledge there are no published works on the anticancer activity of *Juniperus turbinata* subsp. *turbinata.* However, this EO had in your chemical composition, as reported above, as major compounds α-pinene, β-phellandrene. These compounds have been reported as anticancer properties (Bowles 2003; Edris 2007; Djilani & Dicko 2010). Akrout and collaborators reported too that terpenes, compounds present in *Juniperus turbinata* subsp. *turbinata* EO from Peniche, could be responsible for all or part of the antitumor activity in diverse types of human cancer (Akrout *et* al. 2011).

4.3.5 *Otanthus maritimus* (L.) Hoffmans. & Link

Treatment with *Otanthus maritimus* EO led to a decrease in cellular viability in both RKO and MCF7 cell lines at starting final concentration 5 µL/mL (figure 3.2.1 E and 3.2.2 E). This concentration of *Otanthus maritimus* EO showed to be very toxic in cancer cell lines tested. For this reason EO was tested in RKO and MCF7 at different concentration, namely, 1 µL/mL (figure 3.2.3 E and 3.2.4 E).

IC_{50} values diversify between concentrations tested, namely for 1 µL/mL the IC_{50} values were 1.52E-02 and 1.23E-02 (figure 3.2.6 E and table III). The activity EO from *Otanthus maritimus* was investigated regarding several different biological properties, as reported (Agias *et al.* 2005; Basile *et al.* 2013). To our knowledge there are no published works on the anticancer activity of this oil. However, as reported by Akrout and collaborators, terpenes, compounds present above 10% (of total concentration) in *Otanthus maritimus* EO from Peniche, might be responsible for all or part of the antitumor activity in diverse types of human cancer (Akrout *et al.* 2011; Li *et al.* 2009; Darmanin *et al.* 2009; Crowell 1999).

4.3.6 *Eryngium maritimum* L.

Exposure to *Eryngium maritimum* EO treatment at a concentration of 5 µL/mL (figure 3.2.1 F and 3.2.2 F), mediated an increase in cellular viability, which indicating that cellular integrity was not compromised, was not expected. The most significant increase in cellular viability was shown by MCF7 cell line comparing to RKO cell line (figure 3.2.5 F and table II).

For a starting concentration of 1 µL/mL with a dilution of 1:10, *Eryngium maritimum* EO treatment, showed a decrease in cellular viability (figure 3.2.3 F and 3.2.4 F), as expected. This suggests this EO to have some anticancer properties but only at the lower concentrations tested. Comparing to a final concentration of 5 µL/mL with a dilution of 1:2, *Eryngium maritimum* EO treatment in the serial dilutions, at a final concentration 1 µL/mL had clearly lower IC_{50} values. The IC_{50} values determined for this dilution range were 8.59E-04 µL/mL and 2.72E-03 µL/mL for RKO and MCF7 cell lines, respectively (figure 3.2.6 F and table III). The difference between this concentrations tested are very high showing this EO was not properly indicated for a complement in cancer therapy. *Eryngium maritimum* EO showed to be a viscous liquid from the start of these studies and difficult to dilute in the chosen vehicle, DMSO, and medium. Due to these issues, further research with more inter-experimental replicas should be performed.

Studies regarding *Eryngium maritimum* EOs extraction are very scarce. However, *Eryngium maritimum* EOs have been investigated regarding its anti-inflammatory, antinociceptive antioxidant, antimicrobial, antimalarial and antileishmanial activity (Darriet *et al.* 2014; Meot-Duros *et al.* 2008; Küpeli *et al.* 2006; Fokialakis *et al.* 2006). This plant extract have been studied, and Yurdakök & Baydan investigated cytotoxic effect in two species from plant extracts belonging to *Eryngium* L. genus, among them, the species *Eryngium*

maritimum. The aerial and roots parts of the plant were harvested from Balikesir (Turkey) and lyophilized. Anticancer properties of the EOs extracted from this species were tested on human hepatocellular carcinoma (HepG2) and human laryngeal epidermoid carcinoma (Hep2) cell models. The range of concentrations tested were between 8.33 and 266.62 mg/mL. Yurdakök & Baydan in this study, used different cell viability techniques comparing to the present work, namely, they analysed the cytotoxic effect by lactate dehydrogenase (LDH) leakage and MTT cell viability assays. They reported that *Eryngium maritimum* exhibited cytotoxic effect in Hep2 cell line with IC_{50} values for root parts of 30.25 mg/mL and in HepG2 cell line for aerial and root parts of 32.42 and 35.01 mg/mL, respectively. They concluded the EOs extracted from this species had cytotoxic activity against cell lines described, and therefore, potential promising anticancer properties. However, further analysis is needed to confirm this activity (Yurdakök & Baydan 2013).

Comparing the activity of the EOs extracted for all the species tested in this study, the EOs from *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* mediated the highest efficacy in decreasing cellular viability (by MTS assay), as noted by the lower IC_{50} values. Other EOs – *Crithmum maritimum*, *Juniperus turbinata* subsp. *turbinata* and *Eryngium maritimum* – also showed a decrease in cellular viability in both concentrations tested - except *Eryngium maritimum* (just in 1 µL/mL) - however, the IC_{50} values were higher. The EO from *Eryngium maritimum* plant was the only that showed a substantial increase in cellular viability, when tested at final concentration 5 µL/mL.

As cellular viability decreased, for the EOs and concentrations already reported, this could indicate that cellular integrity may have been compromised by different mechanisms. In this situation, the plasma membrane integrity could be damaged, the structure of nucleus could change and chromatin could have been condensed, and to prove this, it would be interesting to study the cellular structure and the chromatin condensation, in order to realize if the mechanism responsible for the decrease of cellular viability is apoptosis, necrosis, or autophagy.

The previous suggest the EOs extracted from *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* EOs extracted from these species, by presenting a clear decrease in cellular viability, could be used in future complements of health applications, as medicaments for example. However, further research is necessary to

confirm this. More repeats should be done, since the inter-experimental replicas in this study were variable for each EOs tested. In dose-response parameter and IC_{50} values it was observed that the variability of the data were high. Increasing the inter-experimental replicas for each EOs mixture would be expected to reduce the variability of the data associated in each EO for both cancer cell lines.

4.4 Viability determination methodologies in experimental cancer biology

Viability cell-based assays are often used for screening collections of compounds to determine if the tested molecules have effects on cell proliferation (Riss *et al.* 2004). Cell-based assays that estimate the number of viable eukaryotic cells are also widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function (Riss *et al.* 2004). *In vitro* cultured cells exist as a heterogeneous population. When populations of cells are exposed to test compounds they do not all respond simultaneously (Riss *et al.* 2003). Cells exposed to drug/compound may respond over the course of several hours, depending on many factors including the mechanism of cell death, the concentration of the drug/compound, and the time of exposure (Riss *et al.* 2003). As a result of culture heterogeneity, the data from most plate-based assay formats represent an average of the signal from the population of cells (Riss *et al.* 2003).

The MTS assay was used to determine cell viability in MCF7 and RKO cell lines. The major advantages of MTS assay are the ability to measure cell number without having to disturb the cells by the removal of the experimental culture medium, and, comparing to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, MTS assay does not require prior formazan solubilisation (Riss *et al.* 2004). However, the MTS assay has some disadvantages, as requiring a time of incubation (between 1 and 4 hours), after 4/5 hours becomes toxic to cells (not so toxic than MTT assay), have interference by reducing compounds, limited sensitivity, and the kits are expensive (Riss *et al.* 2004).

Huang and collaborators determined that MTS assay, in culture medium, had a variety of interacting factors when absorbance is read (Huang *et al.* 2004). They tested the influence of culture medium, the presence of FBS and the concentration of albumin (present in serum). In this study, Huang and collaborators determined: (1) RPMI medium contributed either to the activity/viability of the cells during the assay or the amount of formazan produced; (2) 2% FBS reduced the RPMI response (as opposed to RPMI without FBS com-

plementary) suggesting mitochondrial activity in DPBS was significantly enhanced by 2% serum and (3) albumin present in 10% serum can reduce the assay absorbance by 50% (doubling of cell number can be obscured) depending on the concentration of fatty acids.

Other cell-based assays, as cell-based assays for detecting cytotoxicity in cells *in vitro* could be used instead cell viability assays (Fotakis & Timbrell 2006). Cytotoxicity activity leads to a reduction in cell viability and initiates cell death through necrosis and/or apoptosis (Fotakis & Timbrell 2006). However, with cytotoxic assays it was only possible detect the cytotoxic potential of a test substance (Fotakis & Timbrell 2006). In case the aim was differentiate between necrotic and apoptotic processes, further assays should be used to follow possible membrane damage, for example, in cytosolic enzymes (lactate dehydrogenase – LDH - or dead-cell protease) (Fotakis & Timbrell 2006).

4.5 Study the mode of action of EOs

Hanahan and Weinberg argued that the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation (Hanahan & Weinberg 2011). Normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth-and-division cycle, thereby ensuring a homeostasis of cell number and thus maintenance of normal tissue architecture and function. In order for the cell to follow the pathway to become cancerous, it must acquire six unique traits (figure 4.5.1) as a result of altered cell physiology (Hanahan & Weinberg 2011).

These defining traits of cancer cells are: (1) the ability to generate their own growth signals or respond to weak growth signals that are ignored by healthy cells; (2) insensitivity to anti-proliferative signals; (3) resistance to cellular suicide mechanisms that normally cause aberrant cells to die by apoptosis; (4) the capacity for limitless replication; (5) the ability to stimulate new blood vessel development in order to allow for tumour growth; and (6) the capacity to invade tissues, at first locally, and later to spread or metastasise throughout the body (Hanahan & Weinberg 2011).

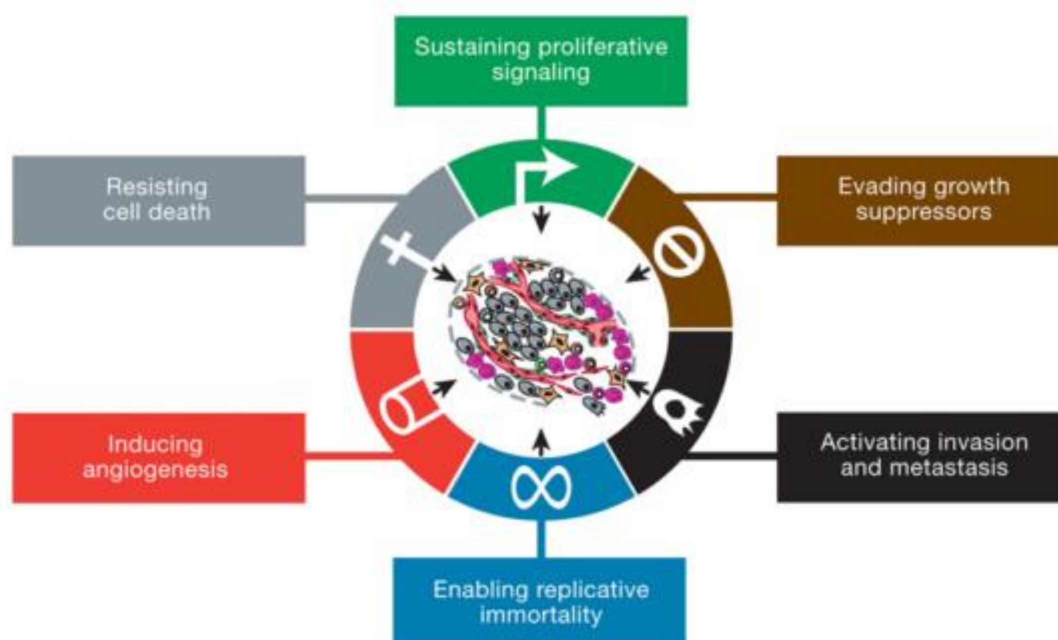

Figure 4.5.1 – The six hallmarks of cancer proposed by Hanahan and Weinberg (Hanahan & Weinberg 2011).

So far, there is no study that can give a clear idea regarding the mode of action of the EOs field. Given the complexity of their chemical composition, it is suggested that this mode of action is complex, and it is difficult to identify the molecular pathway of action. Because of the scarce of studies in this field, this work will help to understand the different pathways that EOs could use to decrease cellular viability/proliferation.

4.5.1 Mechanism of action of *Seseli tortuosum* and *Otanthus maritimus* EOs

Stresses, both intrinsic and extrinsic to the cell, can act upon the p53 pathway (Harris & Levine 2005). Among the signals that activate the p53 transcription factor is damage to the integrity of the DNA (Harris & Levine 2005). Once p53 is stabilised and activated, it initiates a transcriptional program that reflects the nature of the stress signal, such as cell cycle arrest, cellular senescence or apoptosis (Harris & Levine 2005) (figure 4.5.1.1).

A major player in the p53 mediated G_1 arrest is the p21 gene product that inhibits cyclin E-cdk2 (Harris & Levine 2005). This cyclin-dependent kinase acts upon the Rb protein to

derepress the E2F1 activity that promotes the transcription of genes involved in preparing a cell to progress from G_1 to S phase in the cell cycle (Harris & Levine 2005). The p53 induced G_2 arrest is mediated in part by the synthesis of 14-3-3σ, a protein that binds to Cdc25C and keeps it in the cell cytoplasm. Cdc25C is a phosphatase that acts upon cyclin B-Cdc2, a kinase that is essential for the G_2 to M phase transition. Keeping Cdc25C in the cytoplasm prevents it from activating cyclin B-Cdc2 in the nucleus and these cells are blocked in the G_2/M phase of the cell cycle (Harris & Levine 2005).

After verifying that the EOs extracted from the dune plants from Peniche showed a decrease in cellular viability, two species that demonstrated the clearest potential anticancer activity were chosen for the study of their mechanism of action. Initially, Western blot technique was used to investigate the effects of the EOs on known changes in specific proteins that are associated in biological processes. Specifically, changes in caspase 3 cleavage (for programmed cell death/apoptosis), and p21 and p53 expression (associated with cell cycle arrest and the induction of the DNA damage response) were investigated.

Caspases (cysteinyl aspartate-specific proteases) are a family of important signalling molecules with various tasks depending on the subtype and organ involved (Green 2000). Although the precise role in the initiation and progression of apoptosis is not known for all caspases, their involvement as an indicator alone and as a potential leverage point for drug research makes them widely researched molecules (Green 2000; Dudai *et al.* 2005). This protease has been implicated as an “effector” Caspase associated with the initiation of the “death cascade” and is therefore an important marker of the cells entry point into the apoptotic signalling pathway (Green 2000; Dudai *et al.* 2005). When apoptosis is activated, caspases cleave multiple protein substrates “en masse”, which leads to the loss of cellular structure and function, and ultimately results in cell death (Green 2000; Dudai *et al.* 2005). In particular, caspases -8, -9, and -3 have been implicated in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in the Fas/CD95 pathway, and caspase-3 more downstream, activated by multiple pathways (Green 2000). As cleaved caspase 3 was not observed in both cell lines tested (figure 3.3.1 A and B), the effector of the apoptosis (in this study) was not exhibited by other caspases and/or other proteins, concluding that apoptosis was not induced by EOs treatments.

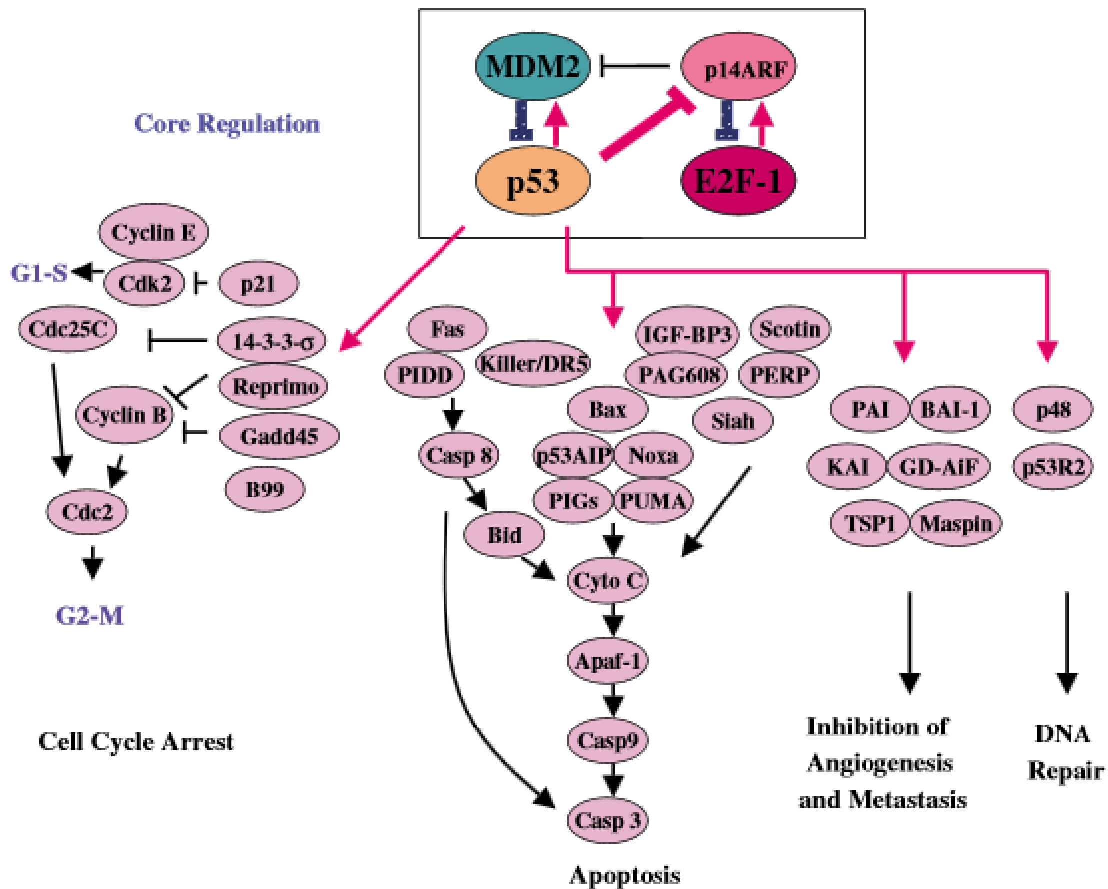

Figure 4.5.1.1 - Downstream targets of the p53 transcription factor mediate its different biological outcome. The genes in p53 activated network initiate one of three programs that result in cell cycle arrest (G_1 or G_2 blocks are observed), cellular senescence or apoptosis. (Harris & Levine, 2005).

In section 3.3, *Seseli tortuosum* EO treatment was tested by western blot carrying p53 - a tumor suppressor protein - and cleaved caspase 3 - responsible for programmed cell death/apoptosis signaling pathway.

According to the results observed in section 3.3, *Seseli tortuosum* EO treatments of the MCF7 cell line lead to a stabilisation in p53 protein expression (figure 3.3.1 A and C). This suggests that p53 was activated by stress in cells, associated to engagement of apoptosis, cell cycle arrest or cellular senescence. Once cleaved caspase 3 (an indicator of apoptosis induction) was not observed in present results, apoptosis does not seem to be induced by treatments with *Seseli tortuosum* EO.

Although caspase 3 is a precise effector of apoptosis, other methodologies that are used as apoptosis triggers could be used to confirm these results. These methodologies include

investigating any plasma membrane alterations (detecting early apoptosis markers), mito-chondrial changes (in apoptosis mitochondrial membrane suffers permeability and soluble proteins such as cytochrome c and pro-caspases are release) or DNA fragmentation (last phase in apoptosis resulting from the activation of endonucleases during the apoptotic process).

In the same western blot, *Seseli tortuosum* EO treatment was tested carrying p21 protein, which is relevant for study of cell cycle arrest (figure 3.3.1).

The p21 expression was observed in Western blot analysis, in all times tested, except at 48h in DMSO treatment. At 48h in EO treatment the level of p21 was very low. In 8h EO treatment and 72h DMSO treatment the level of area density were very high. The p21 was reported above (section 1.3.2) as a protein that interferes with the cell cycle, blocking it. When the transcription of the gene encoding p21 is stimulated by the transcription factor p53, p21 arrest the G_1/S phase-CDK/cyclin and S phase-CDK/cyclin complexes and inhibits their activity, blocking the entry to the next stage of the cell cycle (figure 1.3.2.1).

Treatment of RKO cells with *Seseli tortuosum* EO also led to a time-dependent increase in the protein levels of p53. These levels were more substantial than levels observed for MCF7 cells (figure 3.3.1 B and D).

In its turn, in RKO cell line, decreased p21 protein levels were observed in treatment 2 (EO at 8 hours), 3 (DMSO at 24h) and 7 (DMSO at 72 hours). Although an increase was shown for both treatments – DMSO and EO at 48 hours. Through this results, it is possible to deduce that a cell cycle arrest by stimulation of p21 was induced, since p53 was activated. Protein p53 were accumulated at high levels and transcription of genes, including gene that encodes p21 protein, was stimulated (Figure 1.3.2.1).

Otanthus maritimus EO treatment showed an increase of p53 protein expression levels (figure 3.3.2 A and C). The EOs treatments suggests that the protein amount had highest levels than DMSO treatments, except in 24 hours and 48 hours, which showed a slight decrease of these levels.

A clear increase of p53 protein expression levels was displayed by treatments at 72 hours, which was expected, as at 72 hours of incubation the confluence was very high before harvesting, comparing to the 8 hours of incubation. This indicates cellular stress in MCF7 cell line was induced, activating p53 protein.

An increase of p21 levels, as shown in figure 3.3.2 C, was observed until 72 hours - at this time, p21 was not observed. The levels of p21 amount suggests the EOs treatments were higher than DMSO treatments. This is what we expected because the confluence of MCF7 cells had an increase along times of incubation, before harvesting and preparation of cells for western blot. This suggests that a stress signal was transmitted to the p53 protein by post-translational modifications, and by activating p21 these cells were induced to cell cycle arresting.

In RKO cells (figure 3.3.1 B and D), it was observed that p53 had highest protein expression levels in all EO treatments, comparing to DMSO treatments. For the treatment 2 (EO at 8 hours), 6 (EO at 48 hours) and 8 (EO at 72 hours) the levels of protein expression were very similar, ranging an area density from 1.85 and 2.10. DMSO treatments levels were included between 0.35 and 1. According to the results obtained, the EO treatments in all time points tested had a higher number of cells that transmitted stress signals, activating the tumour suppressor p53 protein.

In the same cell line, p21 showed highest protein amount in *Otanthus maritimus* EO comparing to DMSO treatments, in every time point tested. The protein amount in EO at 48 hours had a significant increase of p21. The increase of this protein is according to what was expected, as the confluence of the cells increased with the progress of time of incubation. Merely, the protein amount of p21 in EO at 72 hours should be higher. It can be stated, according to results in figure 3.3.2 D, that an induction of cellular stress has occurred, due to cell cycle arrested by stimulation of protein p21.

4.5.2 Cell cycle analysis of *Seseli tortuosum* EO

The presence of increased p21 protein expression levels was detected after all treatments performed. A cell cycle progression study was performed with the objective to determine any potential effect on *Seseli tortuosum* EO on cell cycle, by Fluorescence-activated cell sorting (FACS). This technique was selected because of numerous advantages, namely, allowing very high purity (95-100%) of the desired population, the target cell population expresses very low level of the identified marker, allows separation based on differential marker density, is the only available purification technique that isolate cells based in internal staining or intracellular protein expression, and allows the purification of individual cells based on size, granularity and fluorescence (Basu *et al.* 2010).

All cells in G_1 phase have a uniform DNA content, as do cells in G_2/M; the latter have twice as much DNA as G_1 cells (figure 1.3.3.1). Under ideal conditions of DNA staining, the fluorescence intensities of all G_1 or G_2/M cells are expected to be uniform, and after analog-to-digital conversion of the electronic signal from the photomultiplier (representing their fluorescence intensity), to have uniform numerical values, respectively (Pozarowski & Darzynkiewicz 2004). In practice, however, the G_1 and G_2 cell populations are represented on frequency histograms by peaks of various widths (Pozarowski & Darzynkiewicz 2004).

In RKO cell line samples treated with *Seseli tortuosum* EO, the DNA content histograms are shown in figure 3.3.3 and percentage of cells per stage of the cell cycle are represented in table V. According to results, the amount of cells in G_1 phase (represented by M1) increased by the time of treatment. At 72 hours of EO treatment almost 58% of the cells were at G_1 phase, instead, at 8 hours incubation with EO, 32% of the cells were at G_1 phase indicating a potential cell cycle arrest in G_1 phase.

The successive increase by the time of incubation of the percentage of cells in S phase (represented by M2), which was not expected, may indicate that cells had some issue in intra-S checkpoint or DNA replication, possibly induced by of spontaneous chemical reactions in DNA, errors in DNA replication, or exposure to radiation or exogenous chemicals. At 48 hours of treatment the percentage of cells in S phase started to decrease indicating that cells had a possible cell cycle arrest in G_1 phase. For 48 hours and 72 hours treatment time points the results was what we expected because p21 was stimulated by p53 expression blocking the cell cycle arrest, possibly in G_1 phase. However, at 24 hours of treatment the percentage of cells, in the same cell cycle phase (S phase), had an increase, which was not expected because at this time point, applying the principle, that cell cycle arrest occur in G_1 phase, the percentage of cells in S phase should had a successive decrease.

The number of cells in G_2 phase (represented by M3), by the time of EO treatments, had a decrease of percentage of cells, what was expected, as the cell cycle arrest occurs in G_1 phase. This way, the percentage of cells in S and G_2 phases should have had a decrease comparing to G_1 phase. In *Seseli tortuosum* EO the decrease of percentage of cells was expected, indicating that cells in this phase were more sensitive, for example, to radiation, as preparation for synthesis of DNA, replication of DNA and cell growth were blocked. In DMSO treatments, the values (table V) of percentage of cells was has expected because if in G_1 phase the cell cycle had an arrest, the other two phases (S and G2 phases) should had a decrease in their percentage of cells. The percentage of cells at 72 hours in DMSO

was not expected since had an increase in this treatment and this value should be lower than DMSO at 72 hours.

In MCF7 samples with *Seseli tortuosum* EO treatment, the DNA content histograms are shown in figure 3.3.4 and percentage of cells per stage of the cell cycle are represented in table VI.

According to results, the control treatment (figure 3.3.4 A and table VI) was not quantifiable. One possible explanation for this happening, was the presence of doublets. One problem that must be overcome when obtaining results for DNA analysis, by FACS, is the exclusion of aggregates of cells (Nunez 2001). On a flow cytometer, two cells together may register as a single event, known as a doublet. If each of those two cells is diploid (2N), seen as one event, they have 4N DNA (Nunez 2001). In other words, the cells appear to have the same amount of DNA as a single tetraploid cell (G_0/G_1), or the same amount as a cell that is about to enter mitosis (Nunez 2001). The doublet problem can be resolved by employing a doublet discrimination gate based on the characteristics of fluorescence height (FL2-H), fluorescence area (FL2-A) and signal width (FL2-W) (Wang *et al.* 2011). Fluorescence height is the maximum fluorescence given out by each cell as it travels through the laser beam; fluorescence area is the total amount of fluorescence emitted during the same journey; and signal width is the time a cell takes to pass through the laser beam (Wang *et al.* 2011; Nunez 2001). These characteristics are different for a cell that is about to enter mitosis when compared to two cells that are together. In mitosis the cell does not double its membrane and cytoplasmic size and, because of that, passes through the laser more quickly than two cells together (Nunez 2001; Wang *et al.* 2011). Also, all of the DNA in the dividing cell is grouped together in one nucleus emitting a higher intensity of emitted fluorescence, compared with the DNA in two cells that are together (Nunez 2001; Wang *et al.* 2011). Thus, a doublet, which has two nuclei separated by cytoplasm, emits a lower intensity signal over a longer period. This appears as a higher signal width, lower signal height and the same area. These differences can be seen on histograms of FL2-Area vs. FL2-Width or FL2-Area vs FL2 (Nunez 2001; Wang *et al.* 2011; Ochatt 2006).

In the same cell line, MCF7, the G_1 phase had an increase, by the time, of percentage of cells, which indicates that the cell cycle could be arrested in this phase, as expected. The stimulation of p21 was the protein responsible for the cell cycle block. This event will carry to the block of the DNA replication (that occurs in S phase of the cell cycle).

The decrease by the time of incubation of percentage of cells in S phase (represented by M2) was expected because p21 was stimulated by p53 expression blocking the cell cycle arrest, possibly in G_1 phase. If the block of the cell cycle occurs in this phase, the percentage of cells in S and G2 phases will suffer a decrease (the DNA replication was compromised). In these results it is possible to observe that *Seseli tortuosum* EO treatment at 72 hours had an increase in the percentage of cells, which was not expected. As explained above, by the times points tested, EO treatment should have had a strong effect in cell cycle and, at this time point the percentage of cells in S phase, a decrease as DNA replication was been compromised. In this stage of the cell cycle, at 48 hours and 72 hours for DMSO, the percentage of cells was very low, which may indicate the presence of doublets.

In G_2 phase, by the time points tested of EO treatments the percentage of cells had a decreased in both treatments (DMSO and *Seseli tortuosum* EO), as predicted. At 72 hours, the percentage of cells was lower indicating the cells in this stage were more sensitive. This happened because DNA replication was compromised blocking the replication of cells in G_1 phase of the cell cycle.

Some mechanisms of action of EO field as anticancer agent have been suggested. Bhalla and colleagues suggested that various mechanisms such as antioxidant, antimutagenic and antiproliferative, enhancement of immune function and surveillance, enzyme induction and enhancing detoxification, modulation of multidrug resistance and synergistic mechanism of volatile constituents are responsible for their chemopreventive properties (Bahlla *et al.* 2013).

Gautam and collaborators (Gautam *et al.* 2014) suggested that EOs, in general, with therapeutic potential can act by two ways - chemoprevention and cancer suppression. The various mechanisms proposed in cancer treatment (figure 4.5.2.1) are activation of detoxification enzymes, modulation of DNA repair signalling, antimetastasis, and antiangiogenesis. Multiple pathways are involved in the antiproliferative activity demonstrated by the EOs in the cancer cells and EOs are even effective in the reduction of tumours in animal models. This makes EOs potential suitable anticancer agents being displayed on the normal cells.

One mechanism of action of EOs proposed by Gautam and colleagues was related with the expression of p21 and other negative regulators of cell cycle progression, blocking the cell cycle in different phases (Gautam *et al.* 2014). Treatment with patchouli alcohol,

which is an important component of *Pogostemon cablin* EO, has been reported to lead to an upregulation p21 expression and suppression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) expression in colorectal cancer cells with increase in dose (Gautam *et al.* 2014).

As p21 is a negative regulator of G_1 phase transition, increased expression of this protein by the action of patchouli alcohol is indicative of cell cycle inhibition (Gautam *et al.* 2014). Similar arrest of the G_1 transition has also been reported in different types of cancer in response to various other EOs (Gautam *et al.* 2014).

Treatment with EOs extracted from *Curcuma wenyujin* inhibits S/G_2 phase transition, leading to cancer cell death (Gautam *et al.* 2014). G_2/M phase transition arrest has been reported on the treatment of liver tumour (J-5) cells with diallyl trisulfide, garlic EO constituents (Gautam *et al.* 2014). Various constituents like geraniol, thymol, and carvacrol of EOs inhibit different phases of cell cycle (Gautam *et al.* 2014). Monoterpenes act by altering the expression of cell cycle. Genes such *DDIT3* (DNA-damage-inducible transcript 3), *IL8* (Interleukin 8), and *CDKNIA* (Cyclin-dependent kinase inhibitor 1A gene), which can modulate cell cycle arrest, have been reported to be upregulated by frankincense oil (Gautam *et al.* 2014). Therefore, EOs and their constituents can serve as effective anticancer substances by targeting cell cycle progression in cancer cells

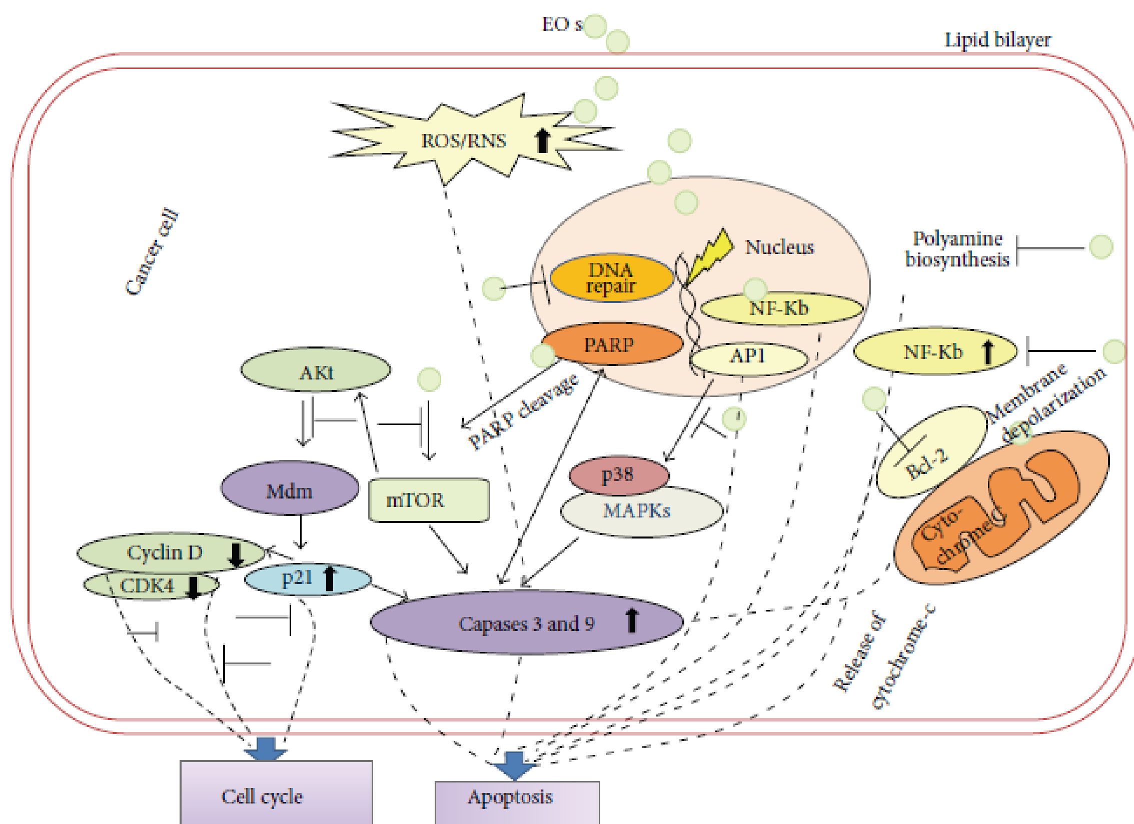

Figure 4.5.2.1 - EOs and their constituents target multiple pathways in cancer cells. EOs by virtue have cell membrane permeability and act on different cellular targets involved in various pathways. EOs increase intracellular ROS/RNS levels which results in apoptosis in cancer cells. Inhibition of Akt, mTOR, and MAPK pathways at different steps by EOs leads to corresponding up-/down regulation of various key biomolecules (and corresponding genes which are not shown in the figure). Alteration in expression of NF-κB by EOs and further binding of NF-κB to DNA result in apoptosis in cancer cells. Dephosphorylation of Akt by the action of EOs results in overexpression of p21, which either induces apoptosis by increasing caspases level or results in cell cycle arrest by binding to cyclins. In addition, EOs-induced mitochondrial stress leads to activation of Bcl-2 and membrane depolarisation resulting in enhanced release of cytochrome-C to the cytoplasm which induces apoptotic cell death in cancer cells. EOs also modulate DNA repair mechanisms by acting as DNA polymerase inhibitors and led to PARP cleavage which also results in apoptosis in cancer cells (Gautam *et al.* 2014).

Rezaei and colleagues reported that the pericarp methanolic extract of *Pistacia atlantica sub kurdica* (with local name of *Baneh*), as a rich source of active biological components with high antioxidant and radical scavenging activities, has ability to cease proliferation and induce apoptosis in T47D human breast cancer cells. This study aimed to clarify whether *Baneh* extract was able to alter cell cycle progression of T47D cells, and concluded that *Baneh* extract induced G_0/G_1 cell cycle arrest in conjunction with a marked decrease in expression of cyclin D1 and Cdk4 that was strongly dependent on time of exposure (Rezaei *et al.* 2012).

5. Conclusion and Future Work

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In nature, EOs play an important role in the protection of the plants as antibacterial, antiviral, antifungal, insecticides and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour pollination and seed dispersion EOs are as well very useful in the treatment of different diseases and their medicinal application has become very popular.

In this study, every EOs tested showed a decrease in cellular viability/proliferation. When comparing the activity of the EOs extracted from the species selected for the present study, it is possible to conclude that *Seseli tortuosum*, *Otanthus maritimus* and *Artemisia campestris* subsp. *maritima* EOs revealed the highest efficacy in decreasing cellular viability, in MTS assay and noted by the low IC_{50} values, deserving further research to comprove this results and possibly, using this EOs in health applications.The other EOs – *Crithmum maritimum*, *Juniperus turbinata* subsp. *turbinata* and *Eryngium maritimum* – also showed a decrease in cellular viability, however, the IC_{50} values were higher. *Eryngium maritimum* EO was the only oil mix that induced a substantial increase in cellular viability, when tested at a starting concentration 5 μL/mL. The starting concentration 5 μL/mL revealed to be very toxic to the cell lines tested.

A preliminary study of the mechanism of biological effect of the EOs, showed an increase of p53 protein in cells treated with *Seseli tortuosum* and *Otanthus maritimus* EO, pointing towards that the cellular stress was induced in the cells tested. Cleaved caspase 3 was not observed, indicating that apoptosis does not seem to be the cause of the decrease of cellular viability/proliferation. Other methods to test apoptosis induction should be used, for example, mitochondrial changes or DNA fragmentation.

The expression of p21 had an increase after treatment with the described EOs, leading to the conclusion that EO treatment is associated with cell cycle arrest. *Seseli tortuosum* EO treatment, with FACS technique, showed the possibility of the cell cycle arrest could be happening in the G_1 phase.

It is important to continue this study to give further evidences to the present results. More inter-replicas, principally in MTS assay should be done, in order to reduce the variability of the data verified in IC_{50} values and the number of inter-replicas in EOs should be equals in order to a best comparison possible between every EOs.

These results demonstrate the antiproliferation activity potential of the tested EOs from sand dune plants, and may consequently provide insight into the development of cancer research, and highlighting the importance for the preservation of dune systems.

The chemical profile of the tested EOs differ not only in the number of molecules but also in the relative amount of each compound. The chemical composition of the EOs can vary according to climate, soil composition, plant organ, age and vegetative cycle stage. So in order to obtain EOs of similar composition, they have to be extracted under the same conditions: same organ of the plant which has been growing on the same soil, under the same climate and has been collected in the same season. In this sense, it would be interesting to test the variability of effects from different locations of the Portuguese coast, in flowering stage. It would be interesting to use different extraction methods of the EOs and compare the different percentages of the chemical compounds in species of the present study.

In recent decades, EOs have been in increasing demand by manufacturers of foods, cosmetics and pharmaceuticals, hence the importance of conducting studies on EOs lies not only in their chemical characterization, but also in the possibility of linking the chemical contents with particular functional properties. In this regard, it is advisable to use methods for the assessment of biological activities that not only highlight aromatic or preservative activities, but also correlate with functional properties that might be potentially useful for pharmaceuticals, nutraceuticals and cosmetic applications.

EOs have been shown to affect positively the immune system on a chemical level despite their direct effect on tumour cells. Possibly, the activity of the main components of the EOs is modulated by compounds in low amount. Moreover, it is probable that several components have a significant role in the fragrance, density, texture, colour and above all, cell penetration, lipophilic or hydrophilic attraction and fixation on cell walls and membranes, and cellular distribution. For this reason, separate molecules should also require some studies in order to evaluate their biological properties. For biological purposes, it is very important to evaluate the entire EO rather than some of its components, because usually the different compounds have a synergistic effect.

Conventional chemotherapeutic agents used in cancer treatment are very toxic, therefore, the development of a new class of anticancer drugs that lack the toxicity of conventional chemotherapeutic agents and that are unaffected by common mechanisms of chemo resistance would be a major advance in cancer treatment. Bearing in mind this reality and the promising results here shown, further studies in order to better understand the mechanism of action of EOs in the decrease of cellular viability/proliferation should be addressed as it is of major concern to find novel potential drugs for cancer treatment.

6. References

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