Manuscript Details

Manuscript number	JEP_2019_4754_R1
Title	Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells
Article type	Research Paper

Abstract

Ethnopharmacological relevance: Despite advances in modern therapeutic strategies, cancer remains the second leading cause of death worldwide. Therefore, there is a constant need to develop more efficient anticancer targeting strategies. The anticancer therapeutic proprieties of medicinal plants and their bioactive compounds have been reported for several years, making natural extracts and/or compounds derived from these a promising source of novel anticancer agents. Sand dune plants are subjected to severe environmental stresses, leading to the development of adaptations, including the production of secondary metabolites with a wide range of bioactivities, such as: anti-inflammatory, analgesic, antiseptic, hypoglycaemic, hypotensive, antinociceptive, antioxidant and anticancer. Aim of the study: The anticancer potential of sand dune plants remains under-investigated, so this research describes the characterisation of the composition of bioactive EOs from sand-dune plants of Peniche (Portugal), and assessment of their activity in vitro and potential mechanism of action. Materials and Methods: EOs were extracted from six sand-dune species of plants from Peniche sand dunes: Crithmum maritimum L., Seseli tortuosum L., Artemisia campestris subsp. maritima (DC.) Arcang., Juniperus phoenicea var. turbinata (Guss.) Parl., Otanthus maritimus (L.) Hoffmanns. & Link, and Eryngium maritimum L.. EOs composition was fully characterised chemically using Gas Chromatography-Mass Spectrometry (GC-MS). The assessment of anticancer activity and mechanism of action was performed in vitro using breast and colorectal cancer 2D and 3D spheroid cell line models, through cell proliferation assay, western blotting analysis, and cell cycle analysis. Results: EOs from the majority of the species tested (S. tortuosum, A. campestris subsp. maritima, O. maritimus, and E. maritimum) were mainly composed by hydrocarbon compounds (sequisterpenes and monoterpenes), showing antiproliferative activity in both 2D and 3D models. EO extracted from S. tortuosum and O. maritimus were identified as having the lowest IC50 values for both cell lines when compared with the other species tested. Furthermore, this antiproliferative activity was associated with increased p21 expression and induction of apoptosis. Conclusions: The present study suggests that EOs extracted from S. tortuosum and O. maritimus present promising cytotoxic properties. Further evaluation of the extracts and their key components as potential anticancer agents should therefore be explored.

Keywords	Sand-dune plants; Essential oils; Natural products; Antitumoral properties
Taxonomy	Antitumor Activity, Medicinal Use of Plants, Natural Product Toxicology

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Submission Files Included in this PDF

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Cover letter

Dear Prof. Alvaro Viljoen

Editor-in-Chief

Journal of Ethnopharmacology

Please find in upload the manuscript "**Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells",** to be submitted to Journal of Ethnopharmacology.

This paper describes the characterization of the bioactive essential oils (EOs) from sand-dune plants, and the assessment of their antitumor activity and potential mechanism of action.

The main achievements of this work are:

- EOs from the majority of the species tested (*S. tortuosum*, *A. marítima*, *O. maritimus*, and *E. maritimum*) showed anti-proliferative activity both in 2D and 3D cancer cell models*;*
- EOs from *S. tortuosum* and *O. maritimus* were identified as having the lowest IC_{50} values for both cell lines when compared with the other species tested;
- These two EOs show a cytotoxic effect against cancer cells, through decreased survival signaling and induction of apoptosis;
- This study suggests that EOs extracted from *S. tortuosum* and *O. maritimus* present promising anti-cancer properties, and further exploration as potential anticancer agents should be addressed.

Yours sincerely,

Célia Cabral

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Re: Beeby et al. manuscript submission – responses to reviewers comments (reference JEP_2019_4754**)**

Dear Editor in Chief

We would like to submit for your consideration our revised manscript "**Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells**" by Ellie Beeby, Mariana Magalhães, Juliana Poças, Thomas Collins, Marco F.L. Lemos, Lillian Barros, Isabel C.F.R. Ferreira, and ourselves.

We are submitting this revised manuscript after extensive changes after the comments and modifications suggested to an earlier version of this manuscript (reference JEP_2019_4754). We are grateful for these comments, and the study is more powerful as a result. The response to reviewers comment and how they were addressed in this new version of the manuscript are itemised at the end of this letter.

The authors are confident this revised manuscript will be of interest to the readers of Journal of Ethnopharmacology and the field at large, and look forward to hearing from you.

Should you require any additional information, please do not hesitate to contact us.

Thank you for your consideration.

Yours sincerely,

Isabel Pires, PhD & Celia Cabral, PhD

Response to reviewers' comments (authors responses noted in *italic*)

We thank the reviewers for their time in reading our manuscript, and for their comments and suggestions. We are grateful for these comments, and the study is more powerful as a result. In the response to comments below we hope to have responded to all queries noted, as part of the revision process for the manuscript.

Comments from the editors and reviewers:

Reviewer 1

We thank the referee for their comments and valuable insight, and have responded to each comment below.

1. Why the authors chose the breast cancer cell line in this study rather than other human cancer cell lines? Their anticancer data on one more breast cancer cell line could make a plus for the study.

We have used two human cancer cell lines, MCF (breast cancer cell line) and RKO (colorectal cancer cell line) to perform our initial evaluation of these EOS. We agree that using more cell lines would be useful, and we intend to evaluate the efficacy of the top EO hits in further cell lines from varied tumour types as part of a subsequent study, however this is unfortunately outside the scope of the present work.

2. How about the relation to their folk uses on breast diseases?

As far as we know, there are no specific indications for the use of these plants in traditional medicine to breast cancer. However, there are indications regarding the anti-inflammatory and anticancer effects. We designed the study in order to improve the scientific knowledge of this group of species from sand dunes.

3. How about the cytotoxicities of those essential oils?

We have evaluated the cytotoxic effects of the EOs in both the cancer cells and a non-cancer cell line model HEK293T, as noted in Supplementary Figure 4.

4. The apoptotic rates of those essential oils on the breast cancer cells?

We appreciate this comment, but evaluation of apoptotic rates is outside the scope of this study. We have evaluated cell death by looking at subG1 populations via FACS (which not directly specific to apoptosis but can indicate presence of cell death and later-state apoptosis), and completed this with evaluating the cleavage of PARP, as well as looking at its total expression, as it is a substrate of effector caspases and therefore a very good marker for apoptosis induction. We hope to perform this evaluation at a later date as part of a subsequent study.

5. How about the protein expressions of mitochondria-dependant pathway？

We appreciate this suggestion, as the evaluation of PARP cleavage does not specify which type of apoptosis pathway was engaged upon EO treatment. Unfortunately, this is outside the scope of this study. We hope to perform the evaluation of any direct impact of the EOS on both the extrinsic and intrinsic apoptotic pathways specifically at a later date.t a later date as part of a subsequent study.

Reviewer 2

We thank the referee for their comments and valuable insight, and have responded to each comment below.

The present article "Secondary metabolites (essential oils) from sand-dune plants induce anticancer effects *in vitro*" is interesting and innovative; however, it does not demonstrate that the essential oils extracted from plants have anti-cancer properties, but only anti-proliferative and apoptotic effects in the cell lines. The term "cancer" in this paper is overused. I advise the authors to review the whole text and the title and limit the term "cancer" as much as possible.

We thank the reviewer for this point, which is a valid one as we did not analyse the effects in vivo or in patients. We have revised the title and text to reflect this.

Furthermore, I advise the authors to shorten the introduction and on the contrary, describe in detail the extraction of EOs and to clearly define in the Materials and Methods the concentration corresponding to the volumes used in the cell tests for example (1ul = mg/ml X?).

We appreciate the advice and we shorten the introduction. However, regarding the extraction of essential oils we used the standard method described in the European Phamacopeia and we have the citation of this reference. Regarding the use of volume instead of mass is because the essential oil is already a liquid and it is easier to dilute t to the experiments. The density of the essential oil is approximately 1 (slightly inferior) but for calculations usually is assumed 1. So, following the formula, 1 µL/mL = 1mg/mL.

I recommend that authors also perform a hemolysis test, at the maximum concentration used, using human erythrocytes.

We appreciate this suggestion, but unfortunately this is outside the scope of this study. We hope to perform these at a later date as part of a subsequent study.

Reviewer 3

The current study intended to explore the potential of sand dune plants on anticancer and the composition of bioactive EOs from sand-dune plants of Peniche (Portugal), as well as assess the potential mechanism of action.

We thank the referee for their comments and valuable insight, and have responded to each comment below.

Major comments:

1. The effect of EOs on cell survival and apoptosis should be determined by colony formation and AnnxinV staining instead of just detected expression of related protein.

We appreciate the comment regarding the suggestion to perform clonogenic survival assays. However, we have used a long-term 3D model assay (spheroid assays), which also assesses the impact of the oils on long term survival and viability. Importantly, we chose this rather than the clonogenic survival assay, as 3D models are more biologically relevant for the tumour microenvironment biology, and take into consideration oxygen and metabolite/catabolite gradients.

We also appreciate the suggestion to use Annexin V as another apoptosis induction assay. However, we have evaluated cell death by looking at subG1 populations via FACS (which not directly specific to apoptosis but can indicate presence of cell death and later-state apoptosis), and completed this with evaluating the cleavage of PARP, as well as looking at its total expression, as it is a substrate of effector caspases and therefore a very good marker for apoptosis induction.

2. The effects of EOs from the 6 dune plant species panel on number of spheroids should be analyzed.

The spheroid assay used looks at the impact on the impact of treatment on the volume of individual spheroids, not their number. The use of alterative assays such as the tumourspheres assay is unfortunately outside the scope of this study. We hope to perform these at a later date as part of a subsequent study.

3. The in vivo study should be performed to assess the anti-tumor effects of OEs in vivo.

We appreciate the suggestion to move our evaluation of these oils to in vivo models, but unfortunately this is outside the scope of this study. We hope to perform these at a later date as part of a subsequent study.

4. The table and image should be separated (Fig. 1C, Fig. 3B).

We have revised the Figures and manuscript text to address this comment, 1C and 3B are now included as separate tables.

5. MTS don't need to be dissolved by DMSO, why can author dissolved it using DMSO?

DMSO was used to solubilise the oils, not to dissolve the MTS. We have changed the wording in the text to clarify this.

6. The dilution of antibodies should be addressed in section of methods.

We have now included this in the supplementary material.

7. Why analyzed statistical significance using 2-way ANOVA instead of one-way ANOVA.

We used a 2-way ANOVA as we have two independent variables being evaluated in our study, normally EO treatment and time. Hope this clarifies this point.

8. The images for chemical composition of OEs should be provided.

We added the chromatograms of the 6 species as supplementary figures.

Reviewer 4

Although the work is interesting and worthy to investigate a new anticancer agent, however, it needs extensive revisions before considering further. The comments are given below.

We thank the referee for their comments and valuable insight, and have responded to each comment below.

1. Identification of essential oil constituents should be done by calculating their RI values.

The RI values are now available in the tables.

2. How this study is novel as the essential oils used in the study and their activity are already known.

The novelty of this study is based on the evaluation of a group of plants from the same region and environment, with specific adaptations to this environment (dune plants with adaptations to exposure to heat, wind, and sated water). Although some of the plants have been evaluated in the literature individually, not all have been evaluated, of the ones that have not all have been for anti-proliferative proprieties, and specifically, not as a group of plants adapted to the same environment. We hope this clarifies the novelty of the work submitted here, and have revised the introduction to highlight these points.

3. Essential oils are volatile in nature and due to their stability issue, developing a drug mainly for oral route is difficult. Although topical use is possible like for aromatherapy and some skin diseases. Now, how this study justify an anticancer drug development from an essential oil.

The present study in vitro is only designed to evaluate the activity of the essential oils in the target cells. Indeed essential oils are volatile, but other natural problems have the same problem of stability. So to undergo this issue, for future studies, e.g. in animals and in future to develop a drug, the development of a formulation to carry the drug is mandatory and we have already other essential oils being incorporated in pharmaceutical formulations for various pathologies.

4. Most of the constituents of studied essential oils are common and also found it a variety of oils, their anticancer activity cannot be considered as unique. Hence, this is again a novelty issue of the work.

We thank the reviewer for this comment. We have addressed the issue of study novelty regarding comment number 2.

5. Experiments conducted for in vitro anticancer activities are well analysed and appreciable.

We thank the referee for their positive comment regarding our in vitro analyses.

Secondary metabolites (essential oils) from sand-dune plants induce ~~antic~~ cytotoxic effects in cancer cells ~~*in vitro*~~

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Abstract

Ethnopharmacological relevance: Despite advances in modern therapeutic strategies, cancer remains the second leading cause of death worldwide. Therefore, there is a constant need to develop more efficient anticancer targeting strategies. The anticancer therapeutic proprieties of medicinal plants and their bioactive compounds have been reported for several years, making natural extracts and/or compounds derived from these a promising source of novel anticancer agents. Sand dune plants are subjected to severe environmental stresses, leading to the development of adaptations, including the production of secondary metabolites with a wide range of bioactivities, such as: anti-inflammatory, analgesic, antiseptic, hypoglycaemic, hypotensive, antinociceptive, antioxidant and anticancer.

Aim of the study: The anticancer potential of sand dune plants remains under-investigated, so this research describes the characterisation of the composition of bioactive EOs from sand-dune plants of Peniche (Portugal), and assessment of their ~~antitumor~~ activity in vitro and potential mechanism of action.

Materials and Methods: EOs were extracted from six sand-dune species of plants from Peniche sand dunes: *Crithmum maritimum* L., *Seseli tortuosum* L., *Artemisia campestris* subsp. *maritima* (DC.) Arcang., *Juniperus phoenicea* var. *turbinata* (Guss.) Parl., *Otanthus maritimus* (L.) Hoffmanns. & Link, and *Eryngium maritimum* L.. EOs composition was fully characterised chemically using Gas Chromatography-Mass Spectrometry (GC-MS). The assessment of anticancer activity and mechanism of action was performed *in vitro* using breast and colorectal cancer 2D and 3D spheroid cell line models, through cell proliferation assay, western blotting analysis, and cell cycle analysis.

Results: EOs from the majority of the species tested (*S. tortuosum*, *A. campestris* subsp. *maritima*, *O. maritimus*, and *E. maritimum*) were mainly composed by hydrocarbon compounds (sequisterpenes and monoterpenes), showing antiproliferative activity in both 2D and 3D models. EO extracted from *S. tortuosum* and *O. maritimus* were identified as having the lowest IC_{50} values for both cell lines when compared with the other species tested. Furthermore, this antiproliferative~~tumor~~ activity was associated with increased p21 expression and induction of apoptosis.

Conclusions: The present study suggests that EOs extracted from *S. tortuosum* and *O. maritimus* present promising ~~anti~~cytotoxic ~~cancer~~ properties. Further evaluation of the extracts and their key components as potential anticancer agents should therefore be explored.

KEYWORDS: Sand-dune plants; Essential oils; Natural products; Anticancer properties

Abbreviations

EO, essential oil; GC, gas chromatography; GC-MS, gas chromatography-mass spectroscopy; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DMSO, Dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; IC50, half maximal inhibitory concentration; PBS, phosphate buffered saline; PARP, poly (ADP-ribose) polymerase; AKT, protein kinase B; p53, tumor protein 53; p21, cyclin-dependent kinase inhibitor protein; FACS, fluorescence-activated cell sorting; ER, endoplasmic reticulum; mTOR, mechanistic target of rapamycin; NF??B, factor nuclear kappa B.

1. Introduction

Cancer remains the second leading cause of death worldwide, responsible for 8.7 million deaths per year, even though improvements in diagnosis and treatment strategies and an increased understanding of tumour biology (Fitzmaurice et al., 2017; Nagai and Kim, 2017). Cancer development and progression is driven by a series of genetic and epigenetic alterations that lead to a dysregulated cell proliferation, survival, and invasiveness, promoted by cell cycle progression deregulation, angiogenesis, metabolic switch, and resistance to apoptosis (Roos et al., 2016; Sever and Brugge, 2015). Conventional anticancer therapeutic strategies continue to mostly rely on either radiotherapy or conventional chemotherapy approaches (Miller et al., 2016). Interestingly, several of these chemotherapy compounds are either directly derived from plant extracts or chemically modified versions of specific phytoproducts, including conventional chemotherapy agent taxol/paclitaxel (Blowman et al., 2018). Plants and their bioactive compounds have been used in the treatment of disease since ancient times. An increasingly expanding body of research has demonstrated that plant-derived products have promising anti-inflammatory, anti-bacterial, anti-oxidant, and anticancer effects, with fewer side-effects than other compounds (Blowman et al., 2018; Cabral et al., 2017; Dhifi et al., 2016). EO (essential oil) phytoproducts comprise multifunctional chemical compounds extracted from aromatic plants and are responsible for the great majority of therapeutic activity attributed to these plants (Edris, 2007; Sharifi-Rad et al., 2017). EOs are secondary metabolites, mainly constituted by oxygenated compounds and hydrocarbons, in which the proportion and concentration of the complex mixture will determine the biological activity of the EO (Blowman et al., 2018; Gautam et al., 2014). Some of the main chemical constituents of EOs are terpenes, which are associated with various biological activities, including anticancer proprieties (Dhifi et al., 2016).

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, chosen for this research are described below.

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* (DC.) Arcang. grows in Coastal sands, usually in primary dunes (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).

Crithmum maritimum L., 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.~~ 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).

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* L., usually named ‘sea holly’ in England or ‘Panicaut desmers’ in France, grows wild on the sandy beaches of western Europe, the Mediterranean basin and the Black Sea (Küpeli *et al.* 2006a; 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). *E. maritimum* has also been reported to exhibit different therapeutic uses in folk medicine (Küpeli *et al.* 2006a).

The genus *Juniperus* L. (Cupressaceae) has~~is represented~~ approximately ~~by~~ 70 species in the Northern Hemisphere (Nakanishi *et al.* 2004; Seca & Silva 2005). *Juniperus phoenicea* var. *turbinata* (Guss.) Parl. 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 (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 extensive~~ly~~ 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~~ includinge 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).

Otanthus maritimus (L.) Hoffmans. & Link belongs to the family Asteraceae. ~~and it grows on maritime sands along the coasts of South and West Europe, northwards to South-East Ireland.~~ This species 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 (Reutter 1923; Tsoukatou *et al.* 2000). In 2013, Cabral and collaborators evaluated the anti-inflammatory activity in vitro and comproved this bioactivity of the essential oil (Cabral *et al.* 2013).

Seseli tortuosum L. 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. In Turkish folk medicine, the fruit of *Seseli tortuosum* 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.* 2006b; Tosun *et al.* 2006) and antifungal activities (Gonçalves *et al.* 2012).

Bearing in mind that inflammation is a well established key hallmark of cancer and has a key role in promoting tumorigenesis (Hanahan & Weinberg, 2011), and taking into account that these sand-dune plants from Peniche: *A. campestris* subsp. *maritima*, *C. maritimum*, *E. maritimum*, *J. phoenicea* var. *turbinata*, *O. maritimus* and *S. tortuosum* have anti-inflammatory properties, this study aims to assess the anticancer properties of the essential oils (EOs) of these plants. EO extracts from these six species were tested as potential cytotoxic ~~anticancer~~ agents using 2D and 3D

in vitro models of cancer, as well as, in a non-cancer cell line to evaluate~~discard~~ off-target risks. Mechanisms of action of the EOs presenting the most pronounced cytotoxic effect were further evaluated regarding the impact on key signalling pathways involved in cell survival, cell cycle regulation, and cell death mechanisms.

2. Materials and Methods

2.1. Plant Materials

Plant samples were collected from the sand dunes of Consolação beach, Peniche, Portugal. The aerial parts of the six species were collected during the flowering stage (July). The voucher specimens were identified by a plant taxonomist (Célia Cabral) and deposited in the Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra, under the numbers: J. Poças 02013 (*A. campestris* subsp. *maritima*), J. Poças 06013 (*C. maritimum*), J. Poças 02013 (*E. maritimum*), J. Poças 05013 (*J. phoenicea* var. *turbinata*), J. Poças 01013 (*O. maritimus*), and J. Poças 04013 (*S. tortuosum*).

2.2. Extraction of essential oils

EOs were isolated by hydrodistillation for 3 hours using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). Extracts were stored in glass vials at 4ºC in the dark.

2.3. Chemical characterisation of essential oils using Gas Chromatography-Mass Spectrometry (GC-MS)

EOs were analysed by gas chromatography (GC) for quantification of extract components, and GC coupled with mass spectrometry (GC-MS) for identification of EOs components, as previously described (Falcão et al., 2018). In brief, the GC-MS unit consisted on a Perkin Elmer system with a Clarus® 580 GC module and a Clarus® SQ 8 S MS module, equipped with DB-5MS fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W Scientific, Inc.). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C. The transfer line temperature was 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization

energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The software Turbomass (software version 6.1.0, Perkin Elmer, Shelton, CT, USA) for Windows was used for data acquisition. The identity of the components was assigned by comparison of their retention indices, relative to C7-C40 n-alkane indices and GC-MS spectra from a commercial MS database. Compounds were quantified as area percentages of total volatiles using the relative values directly obtained from peak total ion current (TIC). Analyses were performed in triplicate.

2.4. Cell line culture and treatment

HEK293-T cell line (human embryonic kidney), RKO cell line (colorectal cancer), and MCF7 cell line (breast cancer) were purchased from ATCC and ECCAC, respectively. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biowest) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% sodium pyruvate (Biowest). Cells were maintained at 37˚C and 5% CO_2, in a humidified incubator. All cell lines were routinely tested as negative for mycoplasma. For the various experimental setups, cells (2D or 3D models) were treated with a range of concentrations of EOs or the cytotoxic agent Doxorubicin hydrochloride (Sigma Aldrich). DMSO (Dimethyl sulfoxide) was used as a vehicle control when relevant.

2.5. Spheroid generation, treatment, and imaging

Spheroids were grown using MCF7 cells, seeded at a density of $2x10^4$ cells per well in ultra-low adherence round-bottomed 96-well plates (Corning). At least 12 spheroids were generated per condition. After aggregation, spheroids were treated and media was replaced every 2 days for a total duration of 14-day treatment. Spheroids were imaged using the GelCount instrument (Oxford Optronix), and spheroid size was determined using the ImageJ software (NIH), as previously reported (Pires et al., 2012; Schneider et al., 2012).

2.6. Cell viability and growth inhibition evaluation

Cell viability was evaluated through 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96® Aqueous One solution Cell proliferation assay (Promega) as per manufacturer's instructions. In brief, cells were seeded at a density $2x10^4$ (HEK293-T), $1x10^4$ (RKO), and $5x10^3$ (MCF7) cells per well in a 96-well plate. After 24 hours, cells were treated with either vehicle (DMSO) diluted in

media, or a serial dilution of EOs in media or with the cytotoxic agent Doxorubicin. EOs were initially diluted in DMSO in a 1:4 ratio (EO/DMSO). This was further diluted in media in order to obtain a 1 µL/ml concentration, and further diluted 1:10 in order to obtain a range of EO concentrations (1 µL/ml - 10^{-5} µL/ml). 48 hours post-treatment, media was removed and replaced with fresh culture medium containing MTS reagent solution. Cells were further incubated at 37ºC and 5% CO_2. After 4 hours, the absorbance of the plate was read at 490 nm in a microplate reader (Bioteck ELx800). Growth inhibition was determined as the percentage of viable cells in relation to untreated cells. IC_{50} (half maximal inhibitory concentration) values were calculated using GraphPad Prism Software (GraphPad Software).

2.7. Fluorescence-activated cell sorting (FACS) cell cycle analysis

Cells (adherent and floating) were harvested, the cell pellet was resuspended in 1X PBS, and the cell suspension fixed in 70% Ethanol in 1X PBS. Cells were washed in 1X PBS and incubated in 1X PBS with 10 µg/ml of propidium iodide (Sigma) and 100 µg/ml of RNAse (Sigma). FACS analysis was performed previously reported (Pires et al., 2010), using a FACS Calibur analyzer (BD Biosciences). Data were analysed using the ModFIT software (Verity Software House).

2.8. Cell lysis and Western Blot analysis

Whole cell lysates were prepared as previously described (Poujade et al., 2018). Briefly, cells were washed with PBS 1X and detached mechanically. Detached cells were also collected. Cell pellets were resuspended with UTB lysis buffer (9 M Urea, 75 mM Tris-HCl pH 7.5 and 0.15 M β-mercaptoethanol). Lysates were sonicated and clarified, and protein concentration was determined using a NanoDrop spectrophotometer (ND-1000 version 3.5.2). 50 µg were loaded in SDS-PAGE gels and processed for western blotting. The antibodies used for western blot analysis were anti-p53 DO1 (Santa Cruz Biotechnology), anti-p21, anti-PARP, anti-pAKT, anti-total AKT (Cell Signaling Technology). Anti β-actin (Santa Cruz Biotechnology) was used as loading control. Further antibody details are present in Supplementary Table 1. Membranes were developed using the Fluorescent Imager ChemiDoc system and the Imager Lab software (Biorad). Densitometric analysis of band intensity relative to the β-actin loading control bands and the vehicle only control samples was performed using ImageJ software (NIH) (Schneider et al., 2012).

2.9. Statistical analysis

All the experiments were performed using triplicates, being representative of at least three independent experiments (replication is noted in Figure legends). Results are expressed as mean ± SD, unless otherwise noted. Statistical significance was determined by Student's *t*-test (one variable) or 2-way ANOVA with Tukey post hoc multiple comparison test using GraphPad Prism (GraphPad Software). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

3. Results

3.1. Essential oils composition

Yield and main compounds for each species are summarized in Table 1. The extracted EOs had variable yields for the different species, ranging between 0.08% (*E. maritimum*) and 0.66% (*S. tortuosum*). The highest yields were obtained for *S. tortuosum* and *A. campestris* subsp. *maritima* with values of 0.66% and 0.47%, respectively. Additionally, the descriptive analysis of EOs composition showed that *S. tortuosum* and *J. turbinata* EOs are mainly composed by monoterpenes (Table S2~~1~~C and S2~~1~~F; Figure S1C and S1F). The same is verified for *C. maritimum* and *O. maritimus* EOs, which are also composed by monoterpenes (Table S2~~1~~B and S2~~1~~E; Figure S1B and S1E). Meanwhile, *E. maritimum* EO is mainly constituted by sesquiterpenes, contrary to *A. campestris* subsp. *maritima* EO which presents a composition based on a mixture of monoterpenes and sesquiterpenes (Table S2~~1~~D and S2~~1~~A; Figure S1D and S1A).

3.2. Evaluation of the cytotoxic effect of EOs extracts from the six dune plant species panel

The potential antitumor activity of the EO extracts from the six species was initially evaluated using the MTS proliferation/viability assay. MCF7 (breast) and RKO (colorectal) cancer cells were treated for 72 hours with a serial dilution range of concentrations of extracted EOs, starting at 1 µL/ml. Cell viability was determined in relation to control samples (cells treated with DMSO). EOs extracted from *O. maritimus*, *S. tortuosum*, *A. campestris* subsp. *maritima*, and *E. maritimum* exhibited a pronounced antiproliferative/anti-viability effect in both cell lines (Figure 1A-B, Table 2), as compared with the known chemotherapy agent Doxorubicin (IC_{50} of 0.08 µM, Figure S2~~1~~-S3~~2~~). Interestingly, EOs extracted from *C. maritimum* and *J. turbinata* did not exhibit a significant cytotoxic effect in either cell lines, as relative proliferative capacity did not drop below 70% of control. IC_{50} (inhibitory concentration needed

to reduce proliferation/viability by 50% compared to control) values for each EO extract were also determined (~~Figure~~ Table 2~~1C~~). *S. tortuosum* and *O. maritimus* EOs presented the lowest IC_{50} concentration values for the RKO cell line, 0.034 and 0.34 µL/mL respectively. The outcome was similar for MCF7 cells, with EOs obtained from *S. tortuosum*, *O. maritimus*, and *E. maritimum* having the lowest IC_{50} concentrations (0.0086, 0.21, and 0.15 µL/mL, respectively). Additionally, all extracts had a reduced cytotoxic effect in non-cancer cells HEK-293T relatively to the observed in cancer cell lines, indicating a potential therapeutic window between cancer and non-cancer cell treatment with the EO extracts (Figure S4~~3~~). These data indicate that EOs extracted from *O. maritimus*, *S. tortuosum*, *A. campestris* subsp. *maritima*, and *E. maritimum* induced a robust antiproliferative/anti-viability activity in cancer cell lines *in vitro*.

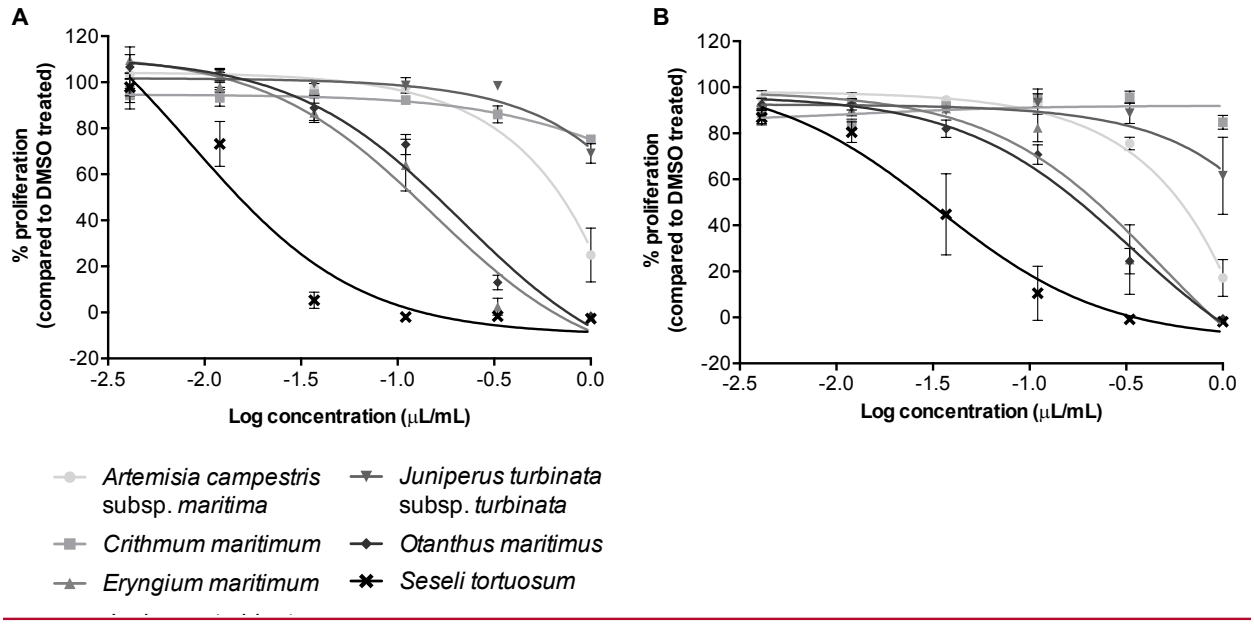


Figure 1. Cytotoxic effect of EOs extracts from the 6 dune plant species panel

MCF7 **(A)** and RKO **(B)** cells were seeded at a density of $1x10^4$ and $5x10^3$ cells per well in a 96-well plate, respectively. Triplicate wells were seeded per condition. Cells were treated with a range of EO concentrations (1:10 serial dilution 1 µL/ml - 10^{-5} µL/ml). Vehicle-only controls were prepared by diluting DMSO in media at 1 µL/ml. Cells were exposed to EO treatment for 72 hours and an MTS assay was subsequently performed. Scatter plots **(A, B)** represent cell viability expressed as percentage survival of control (n=3 independent experiments). ~~**(C)**~~ IC_{50} values for both cell lines (n=3 independent experiments) are noted in Table 2.~~.~~

3.3. Impact of treatment with EOs from the six dune plant species panel on in vitro 3D cancer models

As four species were identified as having potential ~~anticance~~anti-proliferative~~r~~ activity using the MTS assay, it was important to determine the efficacy of all EO extracts in tumour microenvironmental-relevant models. For this, multicellular spheroid models, derived from cancer cell lines, are well established as *in vitro* 3D models to be used to test the therapeutic efficacy of novel agents (Zanoni et al., 2016). MCF7 spheroids were treated with the different EO extracts for 15 days (with treatment replenished every 2-3 days), during which spheroid size was determined (Figure 2). As it can be observed, a decrease in spheroid volume in relation to the vehicle control (DMSO) was observed for all species, with the exception of *C. maritimum* EO extracts (Figure 2B). The impact on spheroid volume varied between species, with the most pronounced effects observed for *O. maritimus, E. maritimum, S. tortuosum*, and *J. turbinata* EOs, with a reduction of spheroid volume of more than 50% by 14 days (Figure 2C-G). Furthermore, *O. maritimus* and *E. maritimum* EO extract treatment led to a clear reduction in spheroid volume and integrity even after 6 days of treatment (Figure 2C, F, and G). These data show that *O. maritimus*, *E. maritimum*, and *S. tortuosum* EO extracts also have an antiproliferative/anti-viability effect in more complex 3D *in vitro* models.

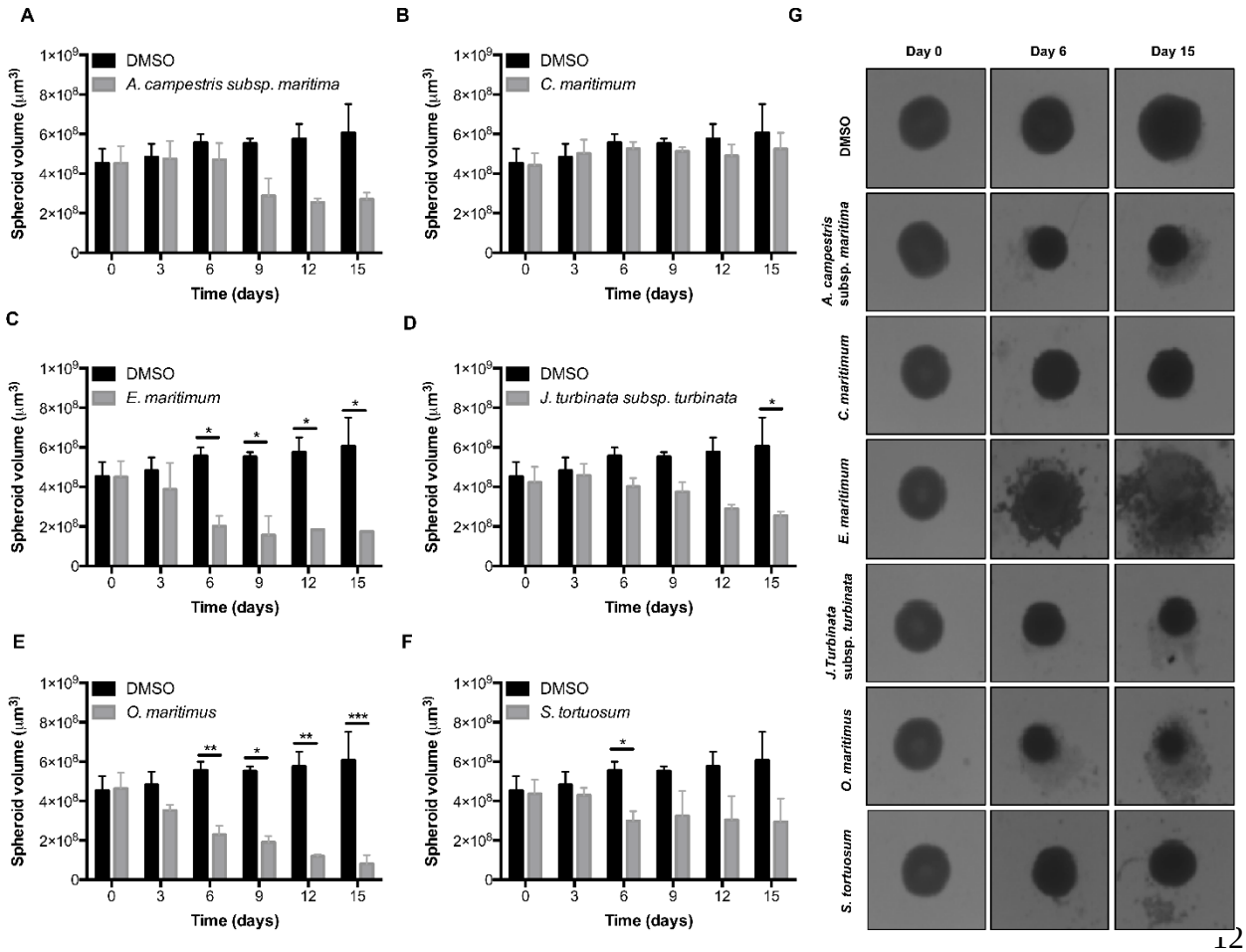


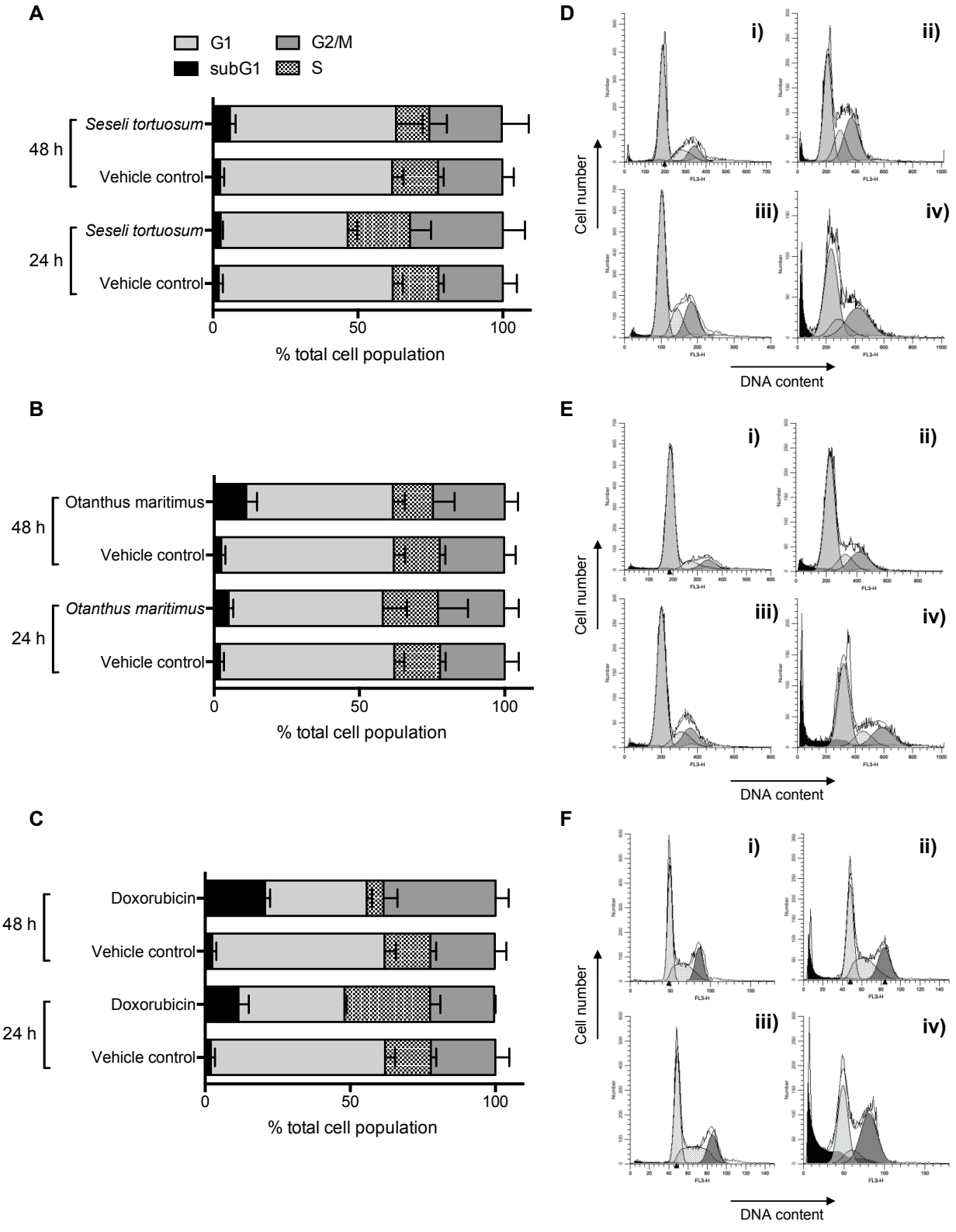
Figure 2. Impact of treatment with EOs from the 6 dune plant species panel on *in vitro* 3D cancer models

MCF7 3D spheroids were established from $2.5x10^4$ cells/well. 12 spheroids were established per condition. Spheroids were treated with either vehicle-only control (1 µL/ml DMSO in media) or 1 µL/ml EO extracts from the 6 dune plants in media. Treatment was maintained for 15 days, with medial refreshed regularly. Spheroids were imaged every 3 days and spheroid volume was determined. (**A-F)** Histograms representing the mean spheroid volume (n=3 independent experiments): **(A)** *A. campestris* subsp. *maritima,* **(B)** *C. maritimum*, **(C)** *E. maritimum*, **(D)** *J. phoenicia* var. *turbinata*, **(E)** *O. maritimus*, and **(F)** *S. tortuosum*. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ **(G)** Representative images of treated spheroids at days 0, 6, and 15.

3.4. Evaluation of the mechanism of action of S. tortuosum and O. maritimum EO extracts

S. tortuosum and *O. maritimus* EO products were selected as the best leads ~~as anticancer agents~~ for further evaluation from our initial six species plant panel. This was due to the EO extracts from these two species having the highest extraction yields and lowest IC_{50} values. In order to evaluate the mechanism of action of these extracts, two approaches were undertaken. Firstly, the impact of treatment with these extracts on cell cycle regulation was evaluated using flow cytometry (Figure 3 and Table 3). Secondly, the impact of EO treatment on survival, cell cycle, and apoptosis signalling pathways were analysed using western blotting (Figure 4 and Figure S5). For both these approaches, MCF7 cells were treated with 1 µL/mL EOs from *S. tortuosum* and *O. maritimus* for 24 and 48 hours. Treatment with 2 µM Doxorubicin was used as a positive control, as this is a well-established conventional chemotherapy agent. The results in Figure 3 indicate there was no clear trend in changes in cell cycle distribution after EO extract treatment, albeit a significant decrease (***$p<0.005$ and *$p<0.05$) in the percentage of cells in the G1 phase after treatment with *S. tortuosum* (24 h) and *O. maritimus* (48 h) was observed. Interestingly, although there are no observable alterations of p53 levels, p21 protein expression is significantly upregulated in EO-treated samples (Figure 4A and Figure S54A-B). This contrasted with treatment with Doxorubicin, which, as expected, had a clear impact on cell cycle distribution, including a decrease of cells in G1/S phases and increase in G2/M phase, p53 stabilization and increase in p21 expression, denoting a clear G2/M cell cycle arrest (Figure 3-4, Table 3, and Figure S54C). Importantly, an increase in the sub-G1 population (which can be associated with decreased viability through apoptosis induction) for EO treated samples was also observed, particularly for *O. maritimum* (Figure 3 and Table 3). In order to clarify if this is occurring via increased apoptosis, PARP cleavage, a marker of apoptosis downstream of caspase activation, was evaluated for all conditions using western blotting (Figure 4 and Figure S54). A significant decrease in total PARP (PARP) levels and a significant increase in cleaved PARP (cPARP) levels was observed for all samples (Figure 4A-B, Figure S54A-B). These data indicate that loss of viability is potentially occurring via induction of apoptosis. Finally, as there was no clear stabilization of p53 in EO-treated cells, the impact of EO extract treatment on other survival signalling pathways was also evaluated (Figure 4). For this, AKT and phospho-AKT levels were analysed using western blotting, as AKT signalling is associated with both pro-survival and anti-apoptosis regulation (Kalimuthu and Se-Kwon, 2013). It was observed that, although there were no observable alterations in total AKT levels after treatment, there is a significant decrease in total phospho-ATK levels (Figure 4A).

In summary, these data indicate that treatment with EOs extracted from both *S. tortuosum* and *O. maritimus* did not induce a significant cell cycle arrest response, albeit leading to increased p21 levels. However, EO extract treatment increased pro-apoptotic signalling, concomitant with decrease in pro-survival AKT expression. This indicates that the antiproliferative/anti-viability effect observed after treatment with these extracts can be underpinned by an increase in apoptotic cell death via AKT.


A
G1
G2/M
subG1
S
48 h
Seseli tortuosum
Vehicle control
24 h
Seseli tortuosum
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50
100
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B
48 h
Otanthus maritimus
Vehicle control
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Otanthus maritimus
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Figure 3. Impact of *O. maritimus* and *S. tortuosum* EO extract treatment on cell cycle progression

MCF7 cells were treated for 24 and 48 hours with either vehicle-only control (1 µL/ml DMSO in media), 1 µL/ml *S. tortuosum*, 1 µL/ml *O. maritimum* EO extracts, or 2 µM Doxorubicin. Samples were analysed by flow cytometry for DNA content. **(A-C)** Proportion of cells in the different cell cycle phases (G1, S, G2/M), as well as subG1 cell debris content (SubG1) is presented as percentages of total cell population. Stacked bar graphs represent means for n=3 independent experiments. ~~**(B)**~~ Statistical significance of differences between means of vehicle control vs EO for both species, per timepoint and cell cycle phase is noted on Table 2. **(D~~C~~-F~~E~~)** Representative FACS analysis histograms from n=3 independent experiments of *S. tortuosum* **(D~~C~~)**, *O. maritimus* **(E~~D~~)**, and Doxorubicin **(F~~E~~)** treatments. Key: i) Vehicle control 24 hours; ii) EO/Doxorubicin 24 hours; iii) Vehicle control 48 hours; ii) EO/Doxorubicin 48 hours.

Beeby et al, **Figure 4**

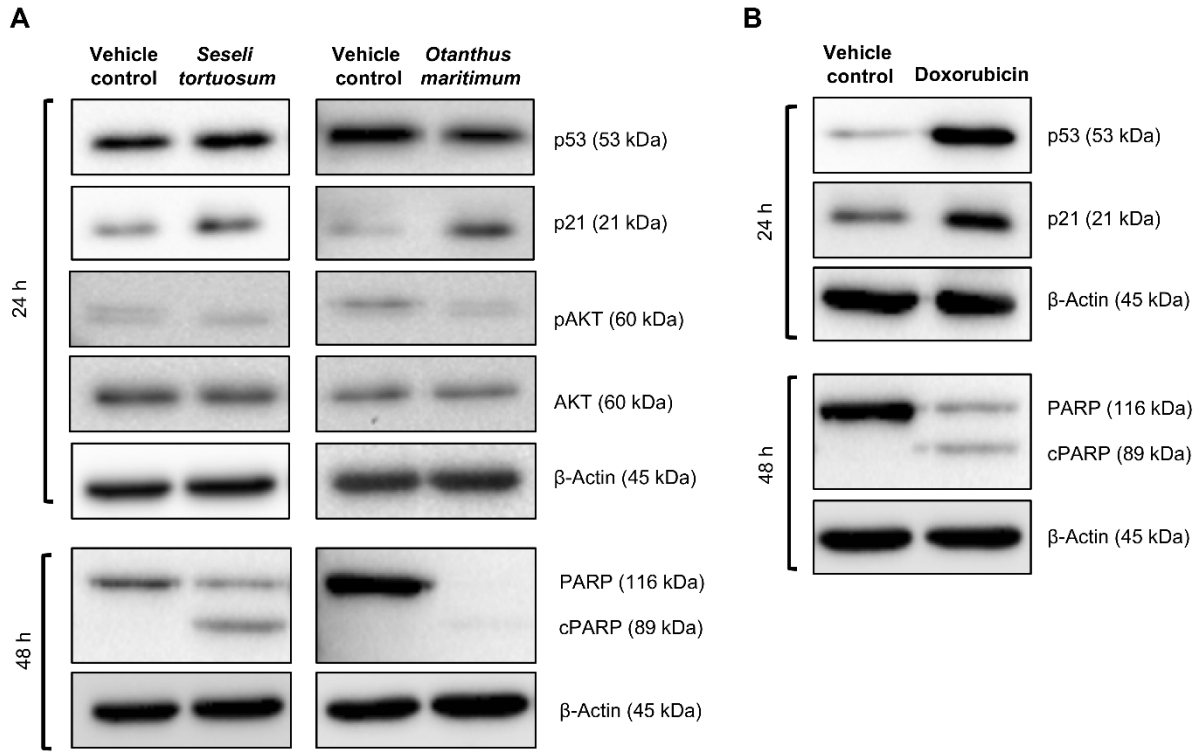

Figure 4. Impact of *O. maritimus* and *S. tortuosum* treatment on cell survival and cell death signalling.

MCF7 cells were treated with either vehicle-only control (1 µL/ml DMSO in media), 1 µL/ml *S. tortuosum* or *O. maritimum* EO extracts **(A)** or vehicle control/2 µM Doxorubicin **(B)** for 24 and 48 hours. p53, p21, phospho-AKT, total AKT, and PARP levels were analyzed by Western blotting. β-actin was used as loading control. Representative blots of n=3 independent experiments are shown.

4. Discussion

Since ancient times plants have been used in medicine to treat different pathologies. Consequently, this traditional use conducted to an increasing interest in the study of plants bioactive compounds as potential therapeutic agents (Asadi-Samani et al., 2016). Moreover, phytoproducts, such as EOs, containing active chemical compounds have been one of the main target focus for the design of novel anticancer therapies (Blowman et al., 2018; Dhifi et al., 2016; Gautam et al., 2014).

Based on that, this work aimed to assess the anticancer activity of EOs extracted from six species of sand-dune plants, containing in their chemical composition several compounds that have known therapeutic activity. The present study has shown that EOs from *S. tortuosum*, *O. maritimus*, and *E. maritimum* induced a significant decrease in cell viability and/or proliferation, both in 2D and 3D *in vitro* cancer models, and presented the lowest IC_{50} values of the panel of species. This ~~anticancer~~ cytotoxic effect exhibited a profile similar to that with the conventional chemotherapy agent Doxorubicin. These results were somehow expected, as these EOs mixtures have in their composition compounds with previously described anticancer effects and present relative low cytotoxicity effect in non-cancer cells treated with the same concentrations of EOs.

All compounds identified in the EOs of the species studied are either predominantly monoterpenes (*C. maritimum*; *J. turbinata*; *O. maritimus*; and *S. tortuosum*), sesquiterpenes (*E. maritimum*), or a mix of both these hydrocarbon compounds (*A. campestris*) (Table 1). Previous studies have reported anticancer activity induced by sesquiterpenes and monoterpenes, via apoptosis and promotion of antiproliferative effect in cancer cell lines (Asadi-Samani et al., 2016; Bhalla et al., 2013). Furthermore, Sylvestre and colleagues analysed the chemical composition of *Myrica gale* EOs collected into two fractions during extraction (30 and 60 minutes) and its anticancer effect. The higher percentage of compounds detected were monoterpenes (30 minutes fraction) and sesquiterpenes (60 minutes fraction), with the 60 minutes fraction showing a higher anticancer activity associated with decreased cell viability (Sylvestre et al., 2005). A study by Lone and colleagues showed that EO extracted from the leaf of *Senecio graciliflorus* was able to induce a strong therapeutic effect against human lung cancer cell lines, with this activity attributed to the main components of the EO, α-pinene (a monoterpene also present in *S. tortuosum* in the present study) and ocimene (Lone et al., 2014). In another study, Ramadan and coworkers showed that Egyptian juniper oil, containing 26.19% of α-pinene, was associated with a more pronounced anticancer effect against HepG2, MCF7, and A549 cancer cell lines when compared with the commonly used chemotherapeutic drug, Doxorubicin (Ramadan et al., 2015). *E. maritimum*

EO was shown to be composed predominantly by sesquiterpenes, including germacrene D. A recent study investigating the activity of EOs from *Magnolia grandiflora* flowers, containing in majority monoterpenes and sesquiterpenes, including germacrene D (sesquiterpene present in *E. maritimum* EO) and β-pinene (monoterpene present in *A. campestris* and *S. tortuosum* EOs) induced a cytotoxic effect against various cancer cell lines (Morshedloo et al., 2017). Furthermore, several studies have indicated that monoterpenes identified in *O. maritimus* EOs, such as chrysanthenone, are also components of extracts from other species reported to have anticancer activities, such as *Pulicaria incisa* and *Artemisia herba-alba* (Shahat et al., 2017; Tilaoui et al., 2015). All the described works support the potential anticancer activity attributed to the main compounds observed in the extracted EOs. However, it was not possible, at this stage, to evaluate whether specific compounds, or mixture of components, are responsible for the observed antiproliferative effects.

S. tortuosum and *O. maritimus* were further investigated to elucidate if the effect was cytostatic (solely impact on cell proliferation) or cytotoxic (increased cell death). EOs from another species, *E. maritimum*, had a similar impact on cell proliferation relative to the other two species, although the EO extraction had a much lower yield, so was not further investigated. Our data showed that the effect of the treatment with *O. maritimum* and *S. tortuosum* were mostly associated with a cytotoxic effect underpinned by decreased survival signalling and increased apoptosis. Previous studies support these results, once they reported that the monoterpene components α-pinene, β-pinene, and chrysanthenone have cytotoxic effect in cancer cells through pro-apoptotic proprieties and the impact in the regulation of AKT pathway (Suhail et al., 2011; Zhou et al., 2007). No significant impact on cell cycle progression was observed for both *O. maritimum* and *S. tortuosum* EOs, even though there was an increase of p21 levels, which presented a slight discrepancy with the literature, as α- and β-pinene, some of the main compounds of *S. tortuosum* EO, are known to be involved in cell cycle arrest (Suhail et al., 2011). Thus, the results showed that *O. maritimum* and *S. tortuosum* EOs most probably predominantly induced loss of viability via induction of apoptosis. This antitumoral mechanism of action differs from the positive control Doxorubicin, which induces apoptosis subsequent to DNA-damage induced double-strand breaks and cell cycle arrest (Tacar et al., 2013). EOs from other species previously reported to possess anticancer properties to induce loss of viability and/or antiproliferative mechanisms through a series of mechanisms, including direct impact on mitochondrial potential activating the intrinsic apoptotic pathway, ER-stress signalling activation, inhibition of survival pathways such as mTOR or NF??B signalling, or increased oxidative stress (Girola et al., 2015; Hassan et al., 2010; Seal et al., 2012). Therefore, future work will focus on further exploring the

mechanism of action of *S. tortuosum* and *O. maritimus* regarding their ~~anticancer~~ cytotoxic and pro-apoptotic activity. Of particular interest are the components of *O. maritimus*, none of which have been reported in the literature to have anticancer ~~-~~effects. Future work will also focus on evaluating the role of specific components of EOs vs. the original complex extract mixtures, and any potential impact of these in combination with more conventional therapeutic approaches, such as conventional chemotherapy and radiotherapy, both *in vitro* and *in vivo*.

To our knowledge, this is the first study proposing this class of phytoproducts, i.e. EOs, from *S. tortuosum* and *O. maritimus*, as potential and promising ~~anticancer~~ agents against cancer cells *in vitro*. These two EO mixtures show a pronounced cytotoxic effect against cancer cells, through decreased survival signalling and induction of apoptosis.

5. Conclusion

Our study is the first to propose EO phytoproducts from *S. tortuosum* and *O. maritimus* as potential and promising anti-cancer agents. These two EOs show a cytotoxic effect against cancer cells, through decreased survival signalling and induction of apoptosis.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest regarding the publication of this paper.

List of the authors and respective contributions

Experiments were carried out by EB, JP, LB, and CC. Data analysis was carried out by TC, IF, CC, and IMP. IMP and CC designed the experiments, with contribution from JP. MM, CC, and IMP wrote the paper with contributions and editing by all other authors. Funding was secured by ML, CC, and IMP.

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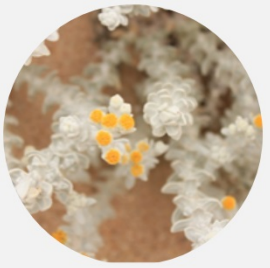
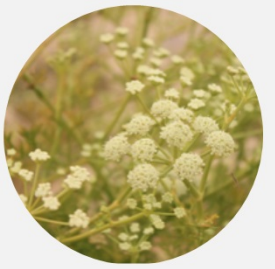
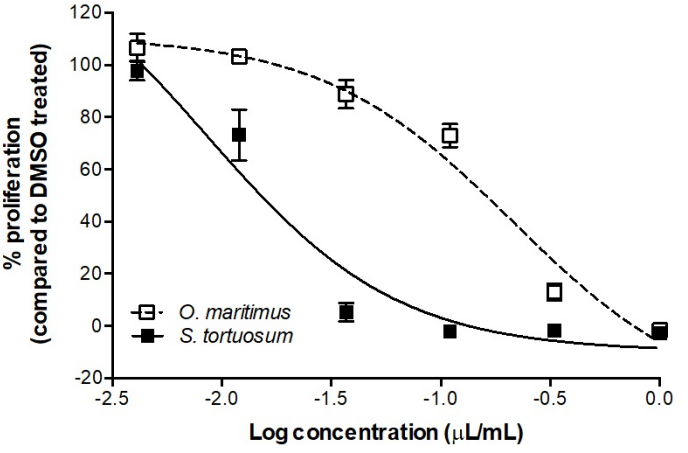


Otanthus maritimus

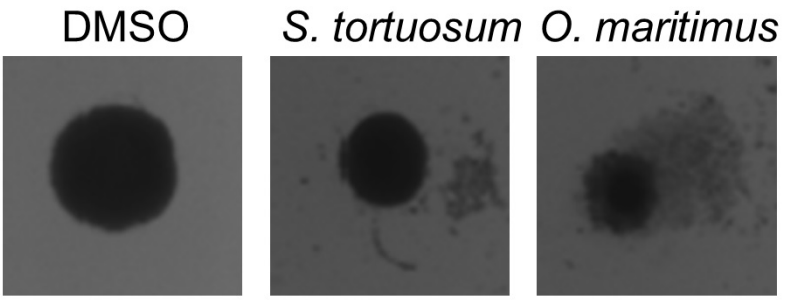


Seseli tortuosum

↓ Viability/
Proliferation (2D)



↓ Spheroid
volume (3D)



↑ Apoptosis

Secondary metabolites (essential oils) from sand-dune plants induce cytotoxic effects in cancer cells

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Abstract

Ethnopharmacological relevance: Despite advances in modern therapeutic strategies, cancer remains the second leading cause of death worldwide. Therefore, there is a constant need to develop more efficient anticancer targeting strategies. The anticancer therapeutic proprieties of medicinal plants and their bioactive compounds have been reported for several years, making natural extracts and/or compounds derived from these a promising source of novel anticancer agents. Sand dune plants are subjected to severe environmental stresses, leading to the development of adaptations, including the production of secondary metabolites with a wide range of bioactivities, such as: anti-inflammatory, analgesic, antiseptic, hypoglycaemic, hypotensive, antinociceptive, antioxidant and anticancer.

Aim of the study: The anticancer potential of sand dune plants remains under-investigated, so this research describes the characterisation of the composition of bioactive EOs from sand-dune plants of Peniche (Portugal), and assessment of their activity in vitro and potential mechanism of action.

Materials and Methods: EOs were extracted from six sand-dune species of plants from Peniche sand dunes: *Crithmum maritimum* L., *Seseli tortuosum* L., *Artemisia campestris* subsp. *maritima* (DC.) Arcang., *Juniperus phoenicea* var. *turbinata* (Guss.) Parl., *Otanthus maritimus* (L.) Hoffmanns. & Link, and *Eryngium maritimum* L.. EOs composition was fully characterised chemically using Gas Chromatography-Mass Spectrometry (GC-MS). The assessment of anticancer activity and mechanism of action was performed *in vitro* using breast and colorectal cancer 2D and 3D spheroid cell line models, through cell proliferation assay, western blotting analysis, and cell cycle analysis.

Results: EOs from the majority of the species tested (*S. tortuosum*, *A. campestris* subsp. *maritima*, *O. maritimus*, and *E. maritimum*) were mainly composed by hydrocarbon compounds (sequisterpenes and monoterpenes), showing antiproliferative activity in both 2D and 3D models. EO extracted from *S. tortuosum* and *O. maritimus* were identified as having the lowest IC_{50} values for both cell lines when compared with the other species tested. Furthermore, this antiproliferative activity was associated with increased p21 expression and induction of apoptosis.

Conclusions: The present study suggests that EOs extracted from *S. tortuosum* and *O. maritimus* present promising cytotoxic properties. Further evaluation of the extracts and their key components as potential anticancer agents should therefore be explored.

KEYWORDS: Sand-dune plants; Essential oils; Natural products; Antitumoral properties

Abbreviations

EO, essential oil; GC, gas chromatography; GC-MS, gas chromatography-mass spectroscopy; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DMSO, Dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; IC50, half maximal inhibitory concentration; PBS, phosphate buffered saline; PARP, poly (ADP-ribose) polymerase; AKT, protein kinase B; p53, tumor protein 53; p21, cyclin-dependent kinase inhibitor protein; FACS, fluorescence-activated cell sorting; ER, endoplasmic reticulum; mTOR, mechanistic target of rapamycin; NF??B, factor nuclear kappa B.

1. Introduction

Cancer remains the second leading cause of death worldwide, responsible for 8.7 million deaths per year, even though improvements in diagnosis and treatment strategies and an increased understanding of tumour biology (Fitzmaurice et al., 2017; Nagai and Kim, 2017). Cancer development and progression is driven by a series of genetic and epigenetic alterations that lead to a dysregulated cell proliferation, survival, and invasiveness, promoted by cell cycle progression deregulation, angiogenesis, metabolic switch, and resistance to apoptosis (Roos et al., 2016; Sever and Brugge, 2015). Conventional anticancer therapeutic strategies continue to mostly rely on either radiotherapy or conventional chemotherapy approaches (Miller et al., 2016). Interestingly, several of these chemotherapy compounds are either directly derived from plant extracts or chemically modified versions of specific phytoproducts, including conventional chemotherapy agent taxol/paclitaxel (Blowman et al., 2018). Plants and their bioactive compounds have been used in the treatment of disease since ancient times. An increasingly expanding body of research has demonstrated that plant-derived products have promising anti-inflammatory, anti-bacterial, anti-oxidant, and anticancer effects, with fewer side-effects than other compounds (Blowman et al., 2018; Cabral et al., 2017; Dhifi et al., 2016). EO (essential oil) phytoproducts comprise multifunctional chemical compounds extracted from aromatic plants and are responsible for the great majority of therapeutic activity attributed to these plants (Edris, 2007; Sharifi-Rad et al., 2017). EOs are secondary metabolites, mainly constituted by oxygenated compounds and hydrocarbons, in which the proportion and concentration of the complex mixture will determine the biological activity of the EO (Blowman et al., 2018; Gautam et al., 2014). Some of the main chemical constituents of EOs are terpenes, which are associated with various biological activities, including anticancer proprieties (Dhifi et al., 2016).

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, chosen for this research are described below.

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* (DC.) Arcang. grows in Coastal sands, usually in primary dunes (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).

Crithmum maritimum L., commonly known as sea fennel or rock samphire, is a perennial member of the Apiaceae family (Meot-Duros *et al.* 2010). 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).

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* L., usually named ‘sea holly’ in England or ‘Panicaut desmers’ in France, grows wild on the sandy beaches of western Europe, the Mediterranean basin and the Black Sea (Küpeli *et al.* 2006a; 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). *E. maritimum* has also been reported to exhibit different therapeutic uses in folk medicine (Küpeli *et al.* 2006a).

The genus *Juniperus* L. (Cupressaceae) has approximately 70 species in the Northern Hemisphere (Nakanishi *et al.* 2004; Seca & Silva 2005). *Juniperus phoenicea* var. *turbinata* (Guss.) Parl. 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 (Seca & Silva 2005). 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 extensive 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. including 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).

Otanthus maritimus (L.) Hoffmans. & Link belongs to the family Asteraceae. This species 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 (Reutter 1923; Tsoukatou *et al.* 2000). In 2013, Cabral and collaborators evaluated the anti-inflammatory activity in vitro and comproved this bioactivity of the essential oil (Cabral *et al.* 2013).

Seseli tortuosum L. 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). Several *Seseli* species are reported in ancient literature for various healing effects, namely herbal remedy for human inflammation, swelling, rheumatism, pain and common cold. In Turkish folk medicine, the fruit of *Seseli tortuosum* 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.* 2006b; Tosun *et al.* 2006) and antifungal activities (Gonçalves *et al.* 2012).

Bearing in mind that inflammation is a well established key hallmark of cancer and has a key role in promoting tumorigenesis (Hanahan & Weinberg, 2011), and taking into account that these sand-dune plants from Peniche: *A. campestris* subsp. *maritima*, *C. maritimum*, *E. maritimum*, *J. phoenicea* var. *turbinata*, *O. maritimus* and *S. tortuosum* have anti-inflammatory properties, this study aims to assess the anticancer properties of the essential oils (EOs) of these plants. EO extracts from these six species were tested as potential cytotoxic agents using 2D and 3D *in vitro* models of cancer, as well as, in a non-cancer cell line to evaluate off-target risks. Mechanisms of action of the EOs presenting the most pronounced cytotoxic effect were further evaluated regarding the impact on key signalling pathways involved in cell survival, cell cycle regulation, and cell death mechanisms.

2. Materials and Methods

2.1. Plant Materials

Plant samples were collected from the sand dunes of Consolação beach, Peniche, Portugal. The aerial parts of the six species were collected during the flowering stage (July). The voucher specimens were identified by a plant taxonomist (Célia Cabral) and deposited in the Herbarium of Medicinal Plants, Faculty of Pharmacy, University of Coimbra, under the numbers: J. Poças 02013 (*A. campestris* subsp. *maritima*), J. Poças 06013 (*C. maritimum*), J. Poças 02013 (*E. maritimum*), J. Poças 05013 (*J. phoenicea* var. *turbinata*), J. Poças 01013 (*O. maritimus*), and J. Poças 04013 (*S. tortuosum*).

2.2. Extraction of essential oils

EOs were isolated by hydrodistillation for 3 hours using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). Extracts were stored in glass vials at 4ºC in the dark.

2.3. Chemical characterisation of essential oils using Gas Chromatography-Mass Spectrometry (GC-MS)

EOs were analysed by gas chromatography (GC) for quantification of extract components, and GC coupled with mass spectrometry (GC-MS) for identification of EOs components, as previously described (Falcão et al., 2018). In brief, the GC-MS unit consisted on a Perkin Elmer system with a Clarus® 580 GC module and a Clarus® SQ 8 S MS module, equipped with DB-5MS fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific, Inc.). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector temperatures, 280°C. The transfer line temperature was 280°C; ion source temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40-300 u; scan time, 1 s. The software Turbomass (software version 6.1.0, Perkin Elmer, Shelton, CT, USA) for Windows was used for data acquisition. The identity of the components was assigned by comparison of their retention indices, relative to C7-C40 n-alkane indices and GC-MS spectra from a commercial MS database. Compounds were quantified as area percentages of total volatiles using the relative values directly obtained from peak total ion current (TIC). Analyses were performed in triplicate.

2.4. Cell line culture and treatment

HEK293-T cell line (human embryonic kidney), RKO cell line (colorectal cancer), and MCF7 cell line (breast cancer) were purchased from ATCC and ECCAC, respectively. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Biowest) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% sodium pyruvate (Biowest). Cells were maintained at 37˚C and 5% CO_2, in a humidified incubator. All cell lines were routinely tested as negative for mycoplasma. For the various experimental setups, cells (2D or 3D models) were treated with a range of concentrations of EOs or the cytotoxic agent Doxorubicin hydrochloride (Sigma Aldrich). DMSO (Dimethyl sulfoxide) was used as a vehicle control when relevant.

2.5. Spheroid generation, treatment, and imaging

Spheroids were grown using MCF7 cells, seeded at a density of $2x10^4$ cells per well in ultra-low adherence round-bottomed 96-well plates (Corning). At least 12 spheroids were generated per condition. After aggregation, spheroids were treated and media was replaced every 2 days for a total duration of 14-day treatment. Spheroids were imaged using the GelCount instrument (Oxford Optronix), and spheroid size was determined using the ImageJ software (NIH), as previously reported (Pires et al., 2012; Schneider et al., 2012).

2.6. Cell viability and growth inhibition evaluation

Cell viability was evaluated through 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96® Aqueous One solution Cell proliferation assay (Promega) as per manufacturer's instructions. In brief, cells were seeded at a density $2x10^4$ (HEK293-T), $1x10^4$ (RKO), and $5x10^3$ (MCF7) cells per well in a 96-well plate. After 24 hours, cells were treated with either vehicle (DMSO) diluted in media, or a serial dilution of EOs in media or with the cytotoxic agent Doxorubicin. EOs were initially diluted in DMSO in a 1:4 ratio (EO/DMSO). This was further diluted in media in order to obtain a 1 µL/ml concentration, and further diluted 1:10 in order to obtain a range of EO concentrations (1 µL/ml - 10^{-5} µL/ml). 48 hours post-treatment, media was removed and replaced with fresh culture medium containing MTS reagent solution. Cells were further incubated at 37ºC and 5% CO_2. After 4 hours, the absorbance of the plate was read at 490 nm in a microplate reader

(Bioteck ELx800). Growth inhibition was determined as the percentage of viable cells in relation to untreated cells. IC_{50} (half maximal inhibitory concentration) values were calculated using GraphPad Prism Software (GraphPad Software).

2.7. Fluorescence-activated cell sorting (FACS) cell cycle analysis

Cells (adherent and floating) were harvested, the cell pellet was resuspended in 1X PBS, and the cell suspension fixed in 70% Ethanol in 1X PBS. Cells were washed in 1X PBS and incubated in 1X PBS with 10 μg/ml of propidium iodide (Sigma) and 100 μg/ml of RNAse (Sigma). FACS analysis was performed previously reported (Pires et al., 2010), using a FACS Calibur analyzer (BD Biosciences). Data were analysed using the ModFIT software (Verity Software House).

2.8. Cell lysis and Western Blot analysis

Whole cell lysates were prepared as previously described (Poujade et al., 2018). Briefly, cells were washed with PBS 1X and detached mechanically. Detached cells were also collected. Cell pellets were resuspended with UTB lysis buffer (9 M Urea, 75 mM Tris-HCl pH 7.5 and 0.15 M β-mercaptoethanol). Lysates were sonicated and clarified, and protein concentration was determined using a NanoDrop spectrophotometer (ND-1000 version 3.5.2). 50 μg were loaded in SDS-PAGE gels and processed for western blotting. The antibodies used for western blot analysis were anti-p53 DO1 (Santa Cruz Biotechnology), anti-p21, anti-PARP, anti-pAKT, anti-total AKT (Cell Signaling Technology). Anti β-actin (Santa Cruz Biotechnology) was used as loading control. Further antibody details are present in Supplementary Table 1. Membranes were developed using the Fluorescent Imager ChemiDoc system and the Imager Lab software (Biorad). Densitometric analysis of band intensity relative to the β-actin loading control bands and the vehicle only control samples was performed using ImageJ software (NIH) (Schneider et al., 2012).

2.9. Statistical analysis

All the experiments were performed using triplicates, being representative of at least three independent experiments (replication is noted in Figure legends). Results are expressed as mean ± SD, unless otherwise noted. Statistical

significance was determined by Student's *t*-test (one variable) or 2-way ANOVA with Tukey post hoc multiple comparison test using GraphPad Prism (GraphPad Software). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$

3. Results

3.1. Essential oils composition

Yield and main compounds for each species are summarized in Table 1. The extracted EOs had variable yields for the different species, ranging between 0.08% (*E. maritimum*) and 0.66% (*S. tortuosum*). The highest yields were obtained for *S. tortuosum* and *A. campestris* subsp. *maritima* with values of 0.66% and 0.47%, respectively. Additionally, the descriptive analysis of EOs composition showed that *S. tortuosum* and *J. turbinata* EOs are mainly composed by monoterpenes (Table S2C and S2F; Figure S1C and S1F). The same is verified for *C. maritimum* and *O. maritimus* EOs, which are also composed by monoterpenes (Table S2B and S2E; Figure S1B and S1E). Meanwhile, *E. maritimum* EO is mainly constituted by sesquiterpenes, contrary to *A. campestris* subsp. *maritima* EO which presents a composition based on a mixture of monoterpenes and sesquiterpenes (Table S2D and S2A; Figure S1D and S1A).

3.2. Evaluation of the cytotoxic effect of EOs extracts from the six dune plant species panel

The potential antitumor activity of the EO extracts from the six species was initially evaluated using the MTS proliferation/viability assay. MCF7 (breast) and RKO (colorectal) cancer cells were treated for 72 hours with a serial dilution range of concentrations of extracted EOs, starting at 1 µL/ml. Cell viability was determined in relation to control samples (cells treated with DMSO). EOs extracted from *O. maritimus*, *S. tortuosum*, *A. campestris* subsp. *maritima*, and *E. maritimum* exhibited a pronounced antiproliferative/anti-viability effect in both cell lines (Figure 1A-B, Table 2), as compared with the known chemotherapy agent Doxorubicin (IC_{50} of 0.08 µM, Figure S2-S3). Interestingly, EOs extracted from *C. maritimum* and *J. turbinata* did not exhibit a significant cytotoxic effect in either cell lines, as relative proliferative capacity did not drop below 70% of control. IC_{50} (inhibitory concentration needed to reduce proliferation/viability by 50% compared to control) values for each EO extract were also determined (Table 2). *S. tortuosum* and *O. maritimus* EOs presented the lowest IC_{50} concentration values for the RKO cell line, 0.034 and 0.34 µL/mL respectively. The outcome was similar for MCF7 cells, with EOs obtained from *S. tortuosum*, *O. maritimus*, and *E. maritimum* having the lowest IC_{50} concentrations (0.0086, 0.21, and 0.15 µL/mL, respectively).

Additionally, all extracts had a reduced cytotoxic effect in non-cancer cells HEK-293T relatively to the observed in cancer cell lines, indicating a potential therapeutic window between cancer and non-cancer cell treatment with the EO extracts (Figure S4). These data indicate that EOs extracted from *O. maritimus*, *S. tortuosum*, *A. campestris* subsp. *maritima*, and *E. maritimum* induced a robust antiproliferative/anti-viability activity in cancer cell lines *in vitro*.

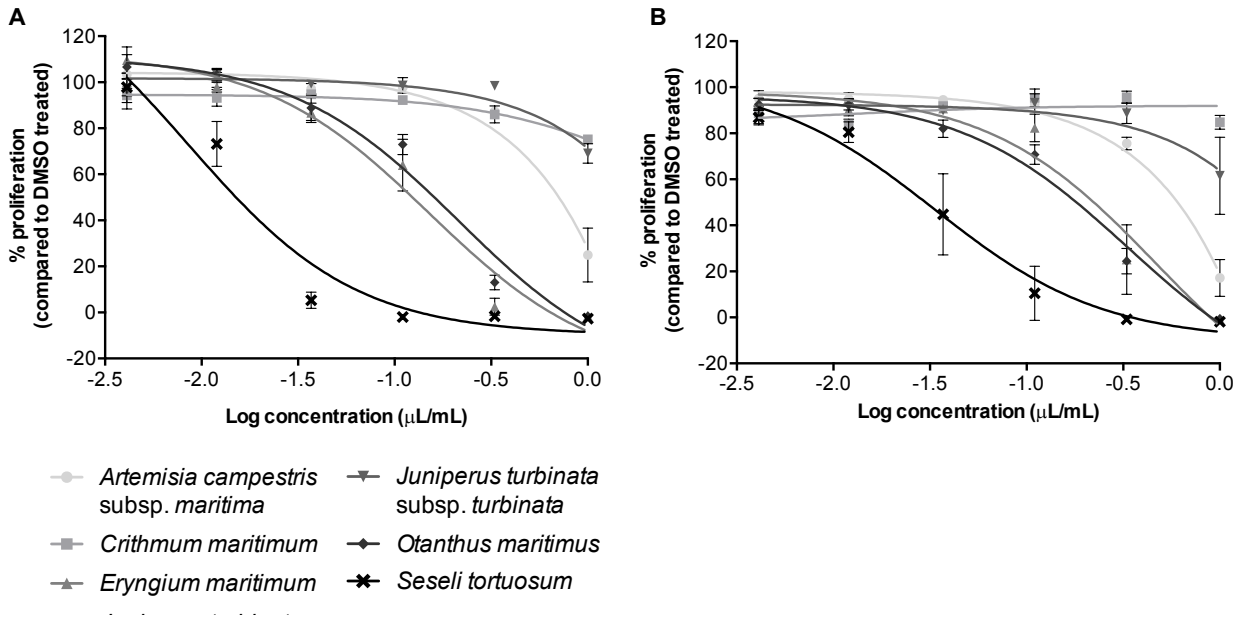

Figure 1. Cytotoxic effect of EOs extracts from the 6 dune plant species panel

MCF7 **(A)** and RKO **(B)** cells were seeded at a density of $1x10^4$ and $5x10^3$ cells per well in a 96-well plate, respectively. Triplicate wells were seeded per condition. Cells were treated with a range of EO concentrations (1:10 serial dilution 1 µL/ml - 10^{-5} µL/ml). Vehicle-only controls were prepared by diluting DMSO in media at 1 µL/ml. Cells were exposed to EO treatment for 72 hours and an MTS assay was subsequently performed. Scatter plots **(A, B)** represent cell viability expressed as percentage survival of control (n=3 independent experiments). IC_{50} values for both cell lines (n=3 independent experiments) are noted in Table 2.

3.3. Impact of treatment with EOs from the six dune plant species panel on in vitro 3D cancer models

As four species were identified as having potential anti-proliferative activity using the MTS assay, it was important to determine the efficacy of all EO extracts in tumour microenvironmental-relevant models. For this, multicellular spheroid models, derived from cancer cell lines, are well established as *in vitro* 3D models to be used to test the therapeutic efficacy of novel agents (Zanoni et al., 2016). MCF7 spheroids were treated with the different EO extracts for 15 days (with treatment replenished every 2-3 days), during which spheroid size was determined (Figure 2). As it can be observed, a decrease in spheroid volume in relation to the vehicle control (DMSO) was observed for all species, with the exception of *C. maritimum* EO extracts (Figure 2B). The impact on spheroid volume varied between species, with the most pronounced effects observed for *O. maritimus, E. maritimum, S. tortuosum*, and *J. turbinata* EOs, with a reduction of spheroid volume of more than 50% by 14 days (Figure 2C-G). Furthermore, *O. maritimus* and *E. maritimum* EO extract treatment led to a clear reduction in spheroid volume and integrity even after 6 days of treatment (Figure 2C, F, and G). These data show that *O. maritimus*, *E. maritimum*, and *S. tortuosum* EO extracts also have an antiproliferative/anti-viability effect in more complex 3D *in vitro* models.

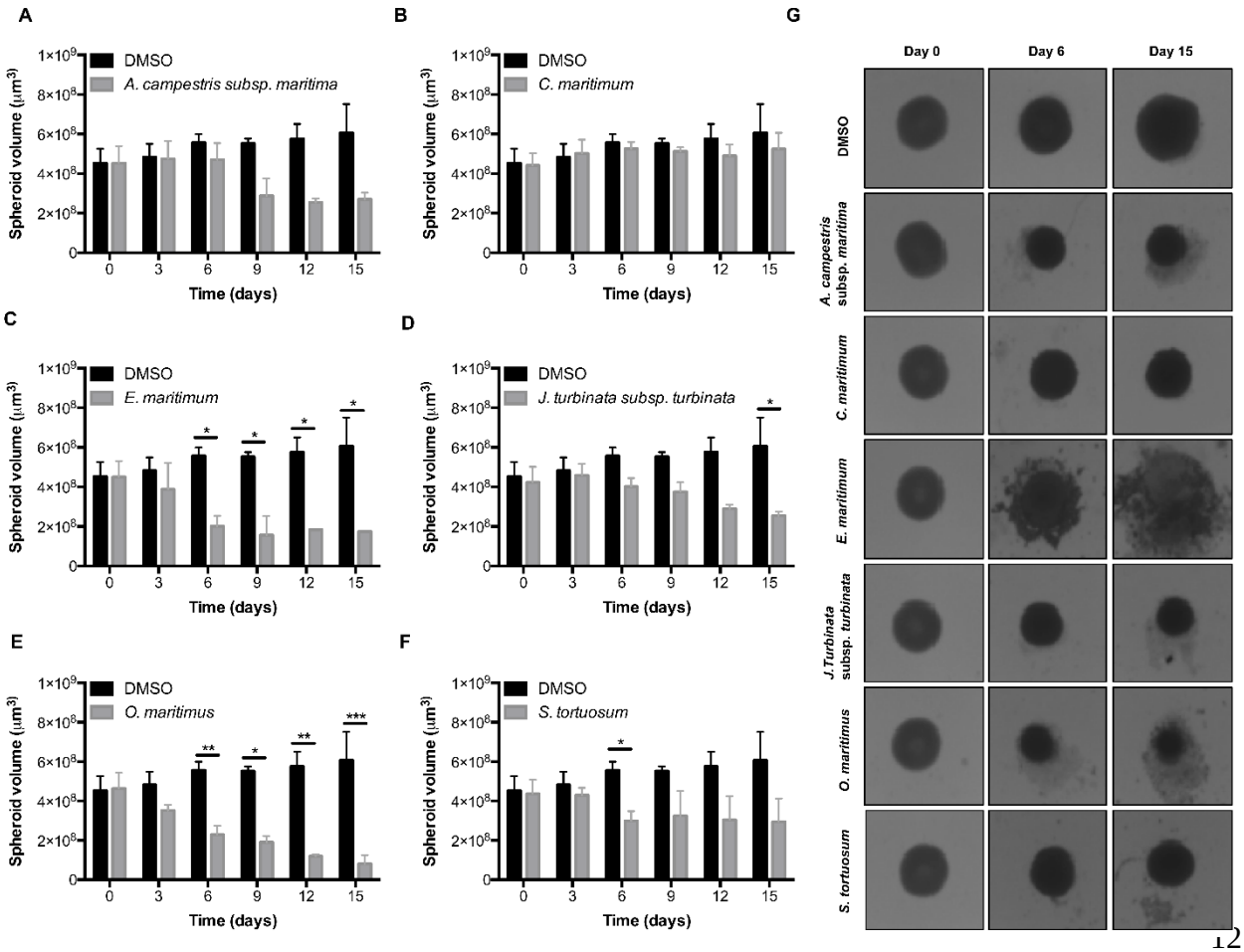

Figure 2. Impact of treatment with EOs from the 6 dune plant species panel on *in vitro* 3D cancer models

MCF7 3D spheroids were established from $2.5x10^4$ cells/well. 12 spheroids were established per condition. Spheroids were treated with either vehicle-only control (1 µL/ml DMSO in media) or 1 µL/ml EO extracts from the 6 dune plants in media. Treatment was maintained for 15 days, with medial refreshed regularly. Spheroids were imaged every 3 days and spheroid volume was determined. (**A-F)** Histograms representing the mean spheroid volume (n=3 independent experiments): **(A)** *A. campestris* subsp. *maritima,* **(B)** *C. maritimum*, **(C)** *E. maritimum*, **(D)** *J. phoenicia* var. *turbinata*, **(E)** *O. maritimus*, and **(F)** *S. tortuosum*. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ **(G)** Representative images of treated spheroids at days 0, 6, and 15.

3.4. Evaluation of the mechanism of action of S. tortuosum and O. maritimum EO extracts

S. tortuosum and *O. maritimus* EO products were selected as the best leads for further evaluation from our initial six species plant panel. This was due to the EO extracts from these two species having the highest extraction yields and lowest IC_{50} values. In order to evaluate the mechanism of action of these extracts, two approaches were undertaken. Firstly, the impact of treatment with these extracts on cell cycle regulation was evaluated using flow cytometry (Figure 3 and Table 3). Secondly, the impact of EO treatment on survival, cell cycle, and apoptosis signalling pathways were analysed using western blotting (Figure 4 and Figure S5). For both these approaches, MCF7 cells were treated with 1 µL/mL EOs from *S. tortuosum* and *O. maritimus* for 24 and 48 hours. Treatment with 2 µM Doxorubicin was used as a positive control, as this is a well-established conventional chemotherapy agent. The results in Figure 3 indicate there was no clear trend in changes in cell cycle distribution after EO extract treatment, albeit a significant decrease (***$p<0.005$ and *$p<0.05$) in the percentage of cells in the G1 phase after treatment with *S. tortuosum* (24 h) and *O. maritimus* (48 h) was observed. Interestingly, although there are no observable alterations of p53 levels, p21 protein expression is significantly upregulated in EO-treated samples (Figure 4A and Figure S5A-B). This contrasted with treatment with Doxorubicin, which, as expected, had a clear impact on cell cycle distribution, including a decrease of cells in G1/S phases and increase in G2/M phase, p53 stabilization and increase in p21 expression, denoting a clear G2/M cell cycle arrest (Figure 3-4, Table 3, and Figure S5C). Importantly, an increase in the sub-G1 population (which can be associated with decreased viability through apoptosis induction) for EO treated samples was also observed, particularly for *O. maritimum* (Figure 3 and Table 3). In order to clarify if this is occurring via increased apoptosis, PARP cleavage, a marker of apoptosis downstream of caspase activation, was evaluated for all conditions using western blotting (Figure 4 and Figure S5). A significant decrease in total PARP (PARP) levels and a significant increase in cleaved PARP (cPARP) levels was observed for all samples (Figure 4A-B, Figure S5A-B). These data indicate that loss of viability is potentially occurring via induction of apoptosis. Finally, as there was no clear stabilization of p53 in EO-treated cells, the impact of EO extract treatment on other survival signalling pathways was also evaluated (Figure 4). For this, AKT and phospho-AKT levels were analysed using western blotting, as AKT signalling is associated with both pro-survival and anti-apoptosis regulation (Kalimuthu and Se-Kwon, 2013). It was observed that, although there were no observable alterations in total AKT levels after treatment, there is a significant decrease in total phospho-ATK levels (Figure 4A).

In summary, these data indicate that treatment with EOs extracted from both *S. tortuosum* and *O. maritimus* did not induce a significant cell cycle arrest response, albeit leading to increased p21 levels. However, EO extract treatment increased pro-apoptotic signalling, concomitant with decrease in pro-survival AKT expression. This indicates that the antiproliferative/anti-viability effect observed after treatment with these extracts can be underpinned by an increase in apoptotic cell death via AKT.

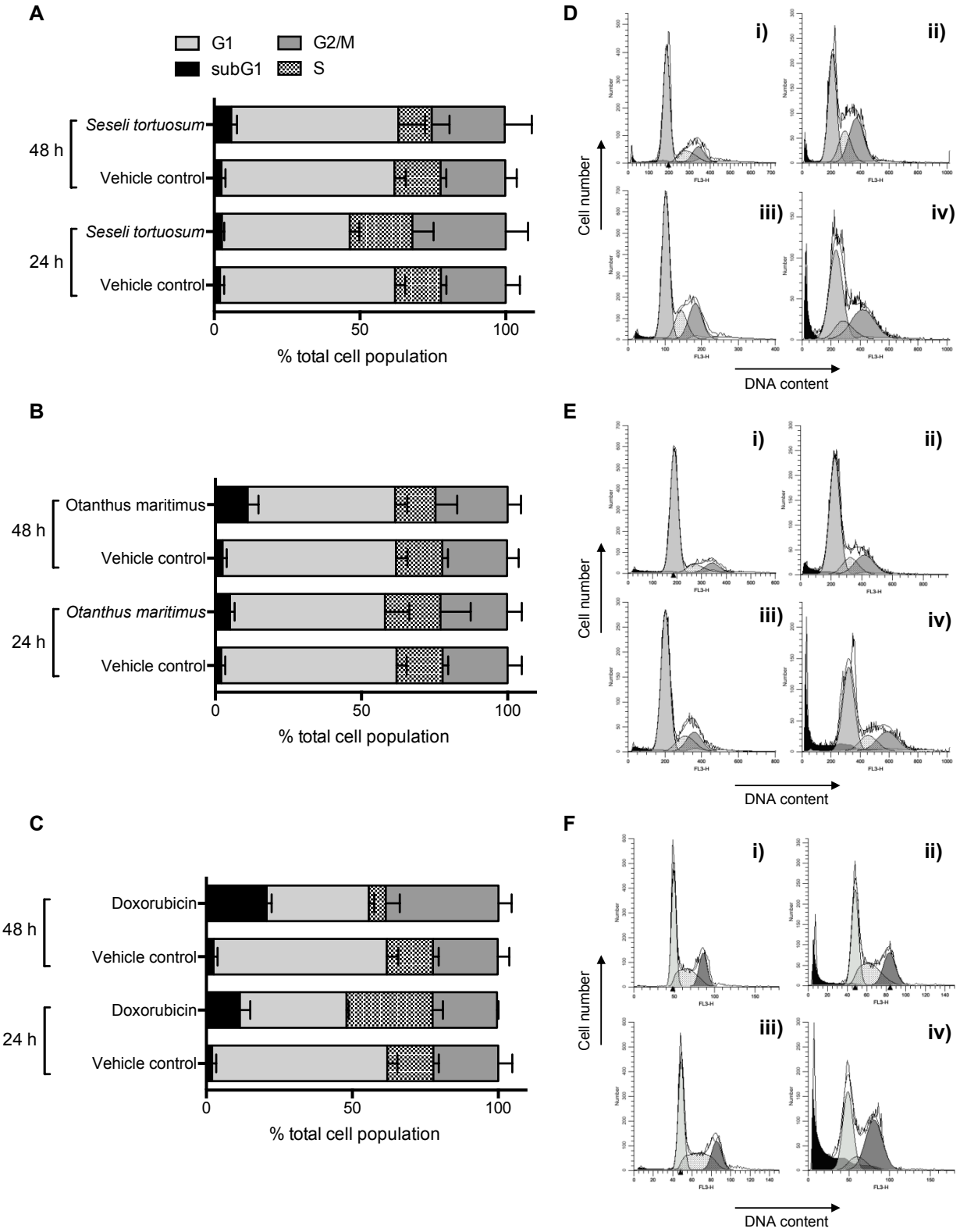

Figure 3. Impact of *O. maritimus* and *S. tortuosum* EO extract treatment on cell cycle progression

MCF7 cells were treated for 24 and 48 hours with either vehicle-only control (1 µL/ml DMSO in media), 1 µL/ml *S. tortuosum*, 1 µL/ml *O. maritimum* EO extracts, or 2 µM Doxorubicin. Samples were analysed by flow cytometry for DNA content. **(A-C)** Proportion of cells in the different cell cycle phases (G1, S, G2/M), as well as subG1 cell debris content (SubG1) is presented as percentages of total cell population. Stacked bar graphs represent means for n=3 independent experiments. Statistical significance of differences between means of vehicle control vs EO for both species, per timepoint and cell cycle phase is noted on Table 2. **(D-F)** Representative FACS analysis histograms from n=3 independent experiments of *S. tortuosum* **(D)**, *O. maritimus* **(E)**, and Doxorubicin **(F)** treatments. Key: i) Vehicle control 24 hours; ii) EO/Doxorubicin 24 hours; iii) Vehicle control 48 hours; ii) EO/Doxorubicin 48 hours.

Beeby et al, **Figure 4**

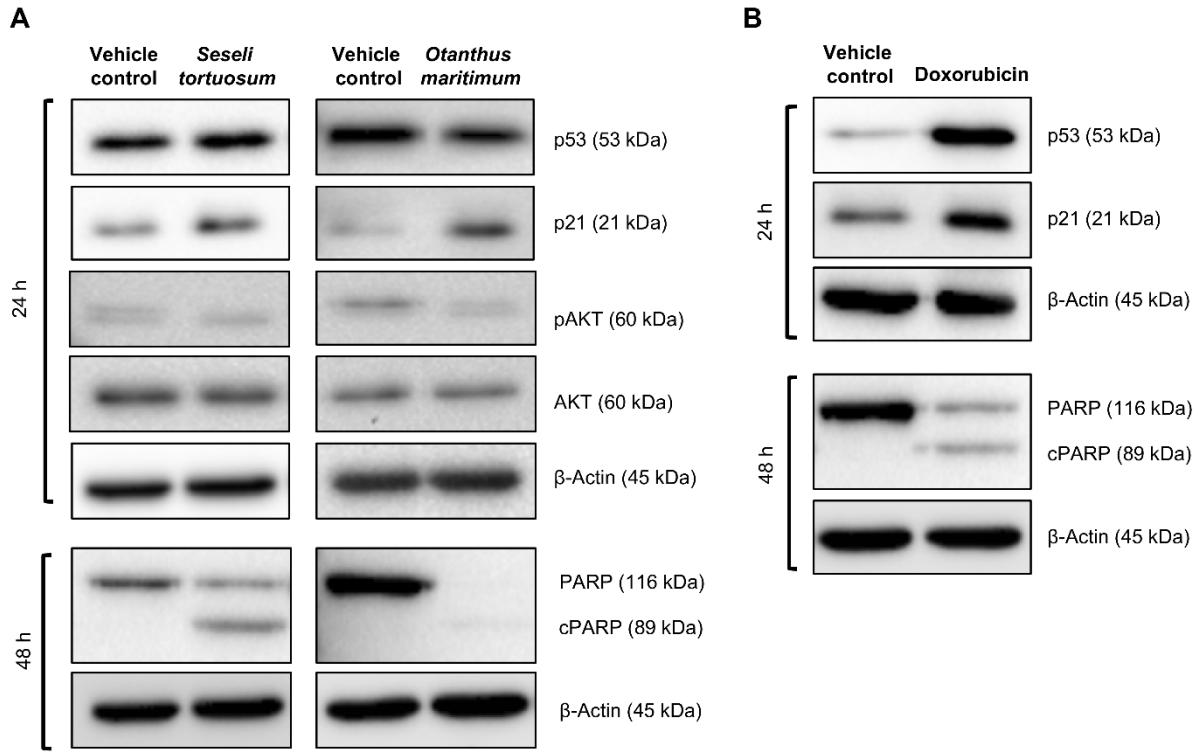

Figure 4. Impact of *O. maritimus* and *S. tortuosum* treatment on cell survival and cell death signalling.

MCF7 cells were treated with either vehicle-only control (1 µL/ml DMSO in media), 1 µL/ml *S. tortuosum* or *O. maritimum* EO extracts **(A)** or vehicle control/2 µM Doxorubicin **(B)** for 24 and 48 hours. p53, p21, phospho-AKT, total AKT, and PARP levels were analyzed by Western blotting. β-actin was used as loading control. Representative blots of n=3 independent experiments are shown.

4. Discussion

Since ancient times plants have been used in medicine to treat different pathologies. Consequently, this traditional use conducted to an increasing interest in the study of plants bioactive compounds as potential therapeutic agents (Asadi-Samani et al., 2016). Moreover, phytoproducts, such as EOs, containing active chemical compounds have been one of the main target focus for the design of novel anticancer therapies (Blowman et al., 2018; Dhifi et al., 2016; Gautam et al., 2014).

Based on that, this work aimed to assess the anticancer activity of EOs extracted from six species of sand-dune plants, containing in their chemical composition several compounds that have known therapeutic activity. The present study has shown that EOs from *S. tortuosum*, *O. maritimus*, and *E. maritimum* induced a significant decrease in cell viability and/or proliferation, both in 2D and 3D *in vitro* cancer models, and presented the lowest IC_{50} values of the panel of species. This cytotoxic effect exhibited a profile similar to that with the conventional chemotherapy agent Doxorubicin. These results were somehow expected, as these EOs mixtures have in their composition compounds with previously described anticancer effects and present relative low cytotoxicity effect in non-cancer cells treated with the same concentrations of EOs.

All compounds identified in the EOs of the species studied are either predominantly monoterpenes (*C. maritimum*; *J. turbinata*; *O. maritimus*; and *S. tortuosum*), sesquiterpenes (*E. maritimum*), or a mix of both these hydrocarbon compounds (*A. campestris*) (Table 1). Previous studies have reported anticancer activity induced by sesquiterpenes and monoterpenes, via apoptosis and promotion of antiproliferative effect in cancer cell lines (Asadi-Samani et al., 2016; Bhalla et al., 2013). Furthermore, Sylvestre and colleagues analysed the chemical composition of *Myrica gale* EOs collected into two fractions during extraction (30 and 60 minutes) and its anticancer effect. The higher percentage of compounds detected were monoterpenes (30 minutes fraction) and sesquiterpenes (60 minutes fraction), with the 60 minutes fraction showing a higher anticancer activity associated with decreased cell viability (Sylvestre et al., 2005). A study by Lone and colleagues showed that EO extracted from the leaf of *Senecio graciliflorus* was able to induce a strong therapeutic effect against human lung cancer cell lines, with this activity attributed to the main components of the EO, α-pinene (a monoterpene also present in *S. tortuosum* in the present study) and ocimene (Lone et al., 2014). In another study, Ramadan and coworkers showed that Egyptian juniper oil, containing 26.19% of α-pinene, was associated with a more pronounced anticancer effect against HepG2, MCF7, and A549 cancer cell lines when compared with the commonly used chemotherapeutic drug, Doxorubicin (Ramadan et al., 2015). *E. maritimum*

EO was shown to be composed predominantly by sesquiterpenes, including germacrene D. A recent study investigating the activity of EOs from *Magnolia grandiflora* flowers, containing in majority monoterpenes and sesquiterpenes, including germacrene D (sesquiterpene present in *E. maritimum* EO) and β-pinene (monoterpene present in *A. campestris* and *S. tortuosum* EOs) induced a cytotoxic effect against various cancer cell lines (Morshedloo et al., 2017). Furthermore, several studies have indicated that monoterpenes identified in *O. maritimus* EOs, such as chrysanthenone, are also components of extracts from other species reported to have anticancer activities, such as *Pulicaria incisa* and *Artemisia herba-alba* (Shahat et al., 2017; Tilaoui et al., 2015). All the described works support the potential anticancer activity attributed to the main compounds observed in the extracted EOs. However, it was not possible, at this stage, to evaluate whether specific compounds, or mixture of components, are responsible for the observed antiproliferative effects.

S. tortuosum and *O. maritimus* were further investigated to elucidate if the effect was cytostatic (solely impact on cell proliferation) or cytotoxic (increased cell death). EOs from another species, *E. maritimum*, had a similar impact on cell proliferation relative to the other two species, although the EO extraction had a much lower yield, so was not further investigated. Our data showed that the effect of the treatment with *O. maritimum* and *S. tortuosum* were mostly associated with a cytotoxic effect underpinned by decreased survival signalling and increased apoptosis. Previous studies support these results, once they reported that the monoterpene components α-pinene, β-pinene, and chrysanthenone have cytotoxic effect in cancer cells through pro-apoptotic proprieties and the impact in the regulation of AKT pathway (Suhail et al., 2011; Zhou et al., 2007). No significant impact on cell cycle progression was observed for both *O. maritimum* and *S. tortuosum* EOs, even though there was an increase of p21 levels, which presented a slight discrepancy with the literature, as α- and β-pinene, some of the main compounds of *S. tortuosum* EO, are known to be involved in cell cycle arrest (Suhail et al., 2011). Thus, the results showed that *O. maritimum* and *S. tortuosum* EOs most probably predominantly induced loss of viability via induction of apoptosis. This antitumoral mechanism of action differs from the positive control Doxorubicin, which induces apoptosis subsequent to DNA-damage induced double-strand breaks and cell cycle arrest (Tacar et al., 2013). EOs from other species previously reported to possess anticancer properties to induce loss of viability and/or antiproliferative mechanisms through a series of mechanisms, including direct impact on mitochondrial potential activating the intrinsic apoptotic pathway, ER-stress signalling activation, inhibition of survival pathways such as mTOR or NF??B signalling, or increased oxidative stress (Girola et al., 2015; Hassan et al., 2010; Seal et al., 2012). Therefore, future work will focus on further exploring the

mechanism of action of *S. tortuosum* and *O. maritimus* regarding their cytotoxic and pro-apoptotic activity. Of particular interest are the components of *O. maritimus*, none of which have been reported in the literature to have anticancer effects. Future work will also focus on evaluating the role of specific components of EOs vs. the original complex extract mixtures, and any potential impact of these in combination with more conventional therapeutic approaches, such as conventional chemotherapy and radiotherapy, both *in vitro* and *in vivo*.

To our knowledge, this is the first study proposing this class of phytoproducts, i.e. EOs, from *S. tortuosum* and *O. maritimus*, as potential and promising agents against cancer cells *in vitro*. These two EO mixtures show a pronounced cytotoxic effect against cancer cells, through decreased survival signalling and induction of apoptosis.

5. Conclusion

Our study is the first to propose EO phytoproducts from *S. tortuosum* and *O. maritimus* as potential and promising anti-cancer agents. These two EOs show a cytotoxic effect against cancer cells, through decreased survival signalling and induction of apoptosis.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest regarding the publication of this paper.

List of the authors and respective contributions

Experiments were carried out by EB, JP, LB, and CC. Data analysis was carried out by TC, IF, CC, and IMP. IMP and CC designed the experiments, with contribution from JP. MM, CC, and IMP wrote the paper with contributions and editing by all other authors. Funding was secured by ML, CC, and IMP.

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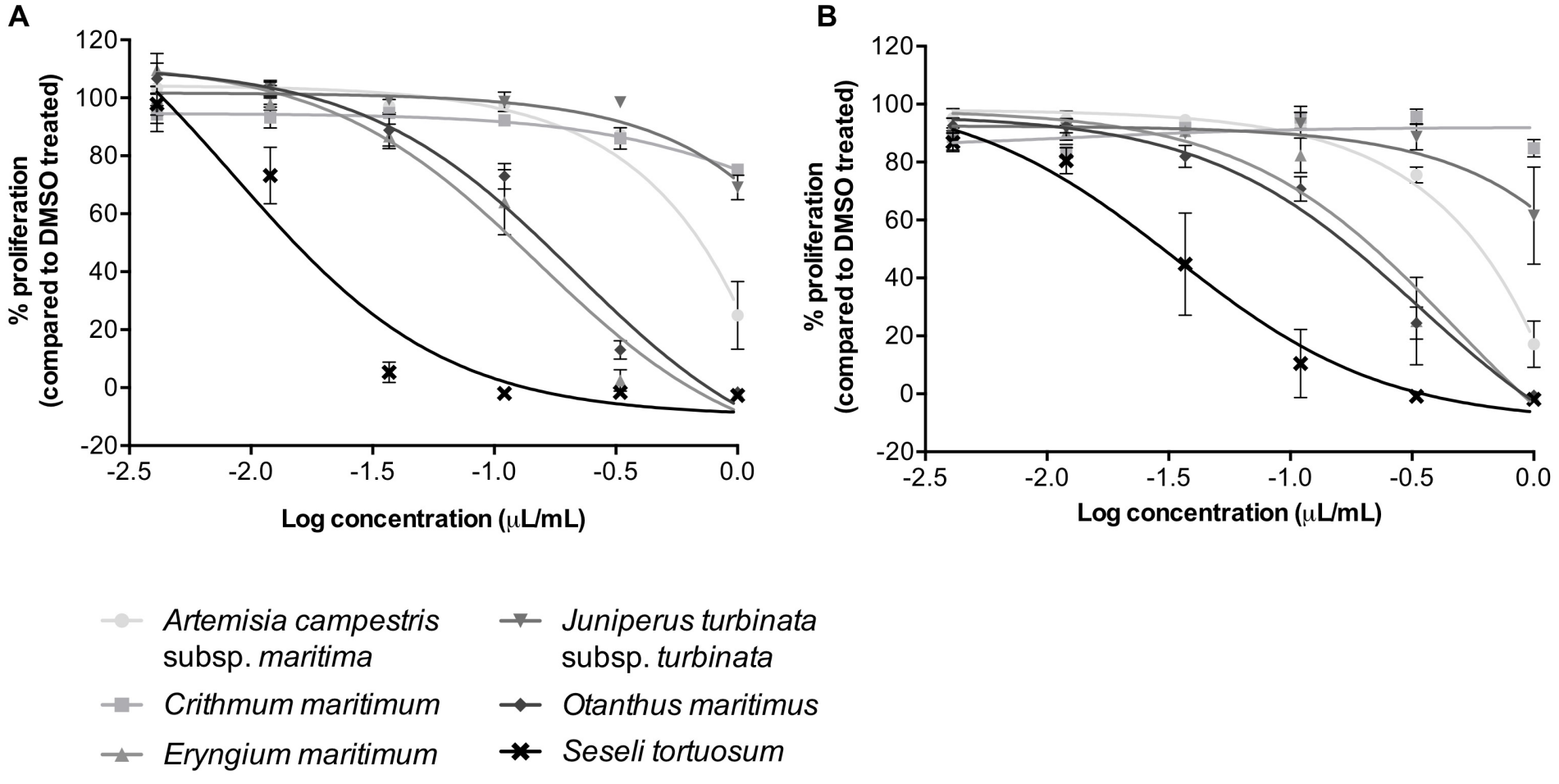
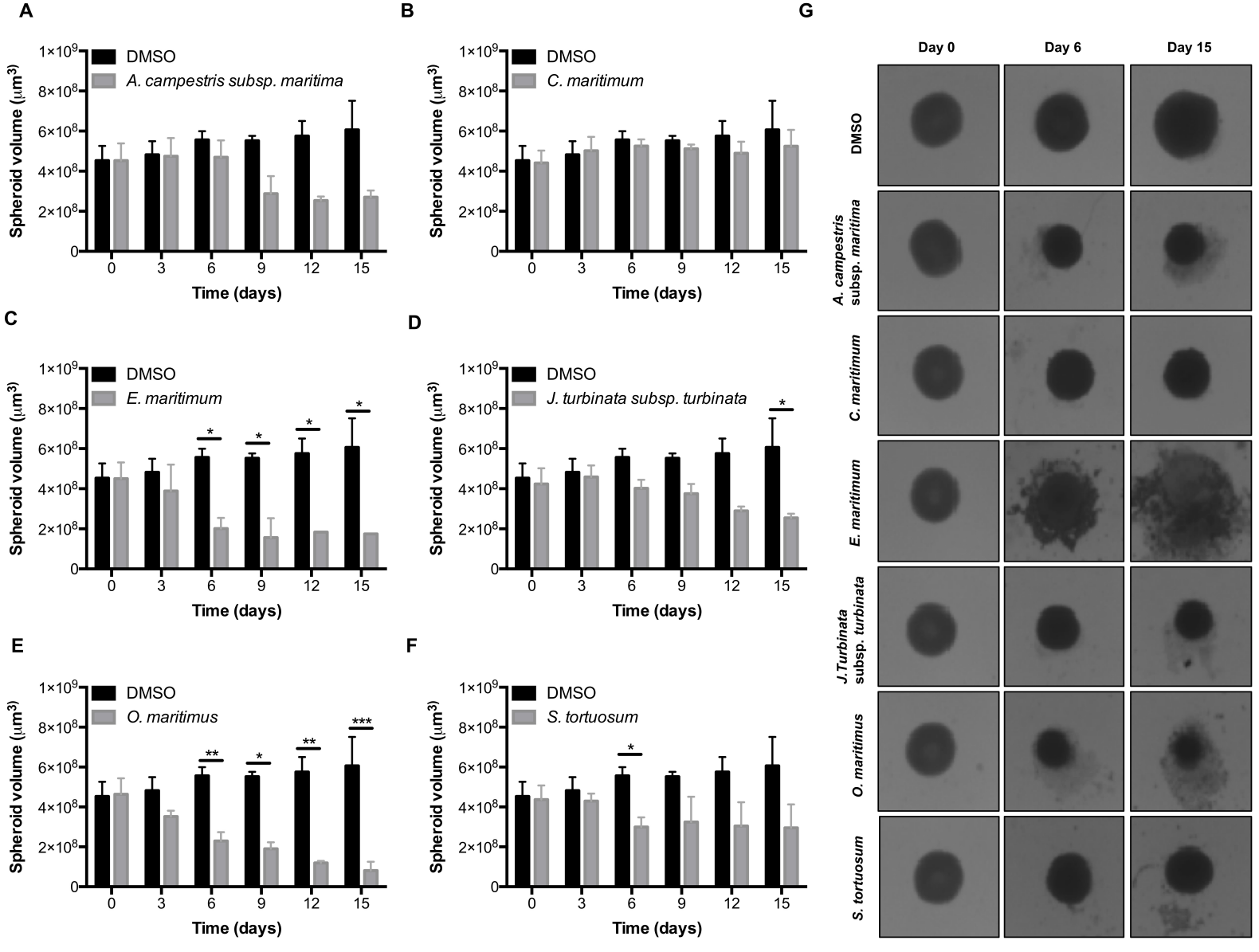
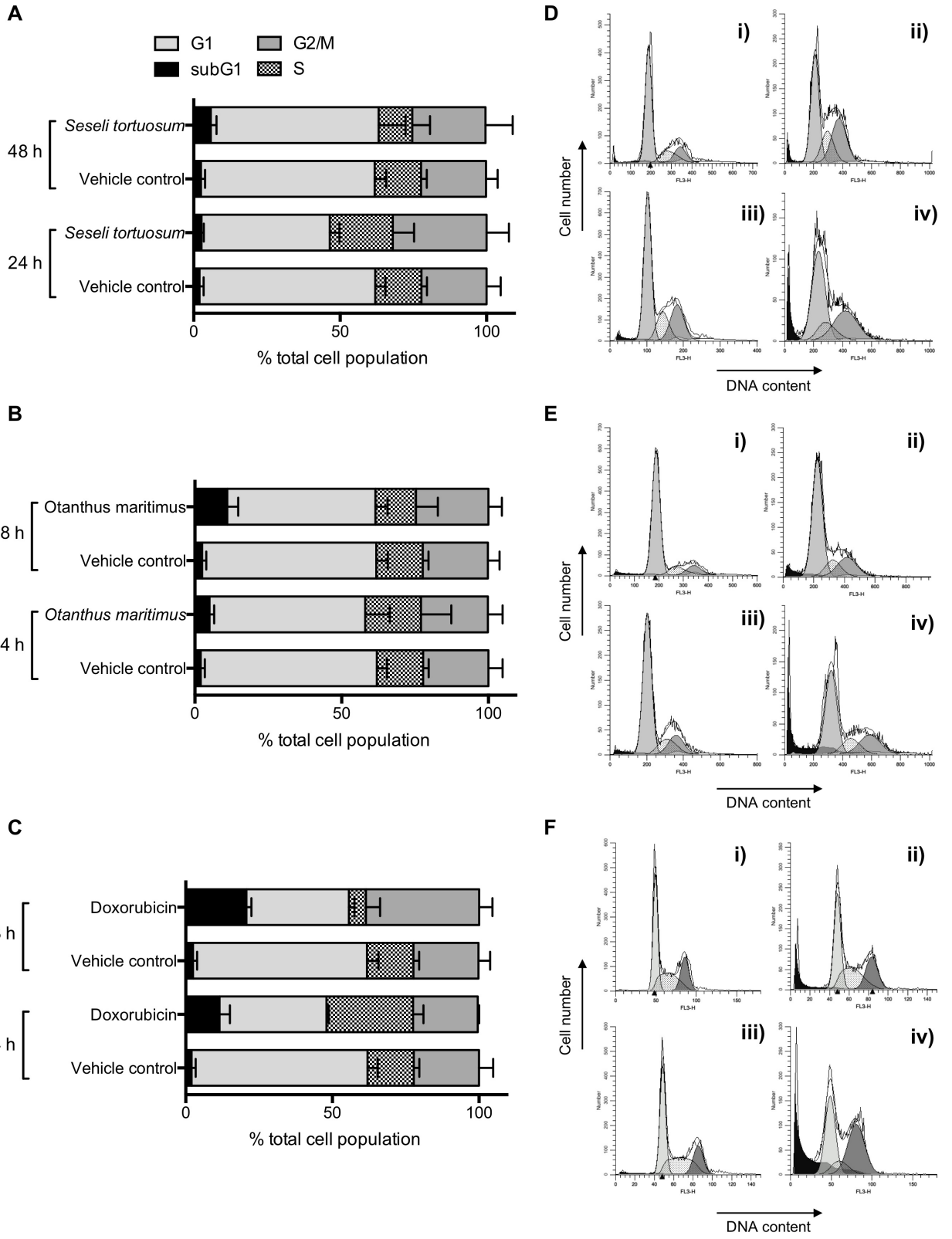
Beeby et al, **Figure 1**




A
B
C
D
E
F
G
DMSO
A. campestris subsp. maritima
C. maritimum
E. maritimum
J. turbinata subsp. turbinata
O. maritimus
S. tortuosum
Spheroid volume (μm3)
Time (days)
Day 0
Day 6
Day 15
J.Turbinata subsp. turbinata

Beeby et al, **Figure 3**



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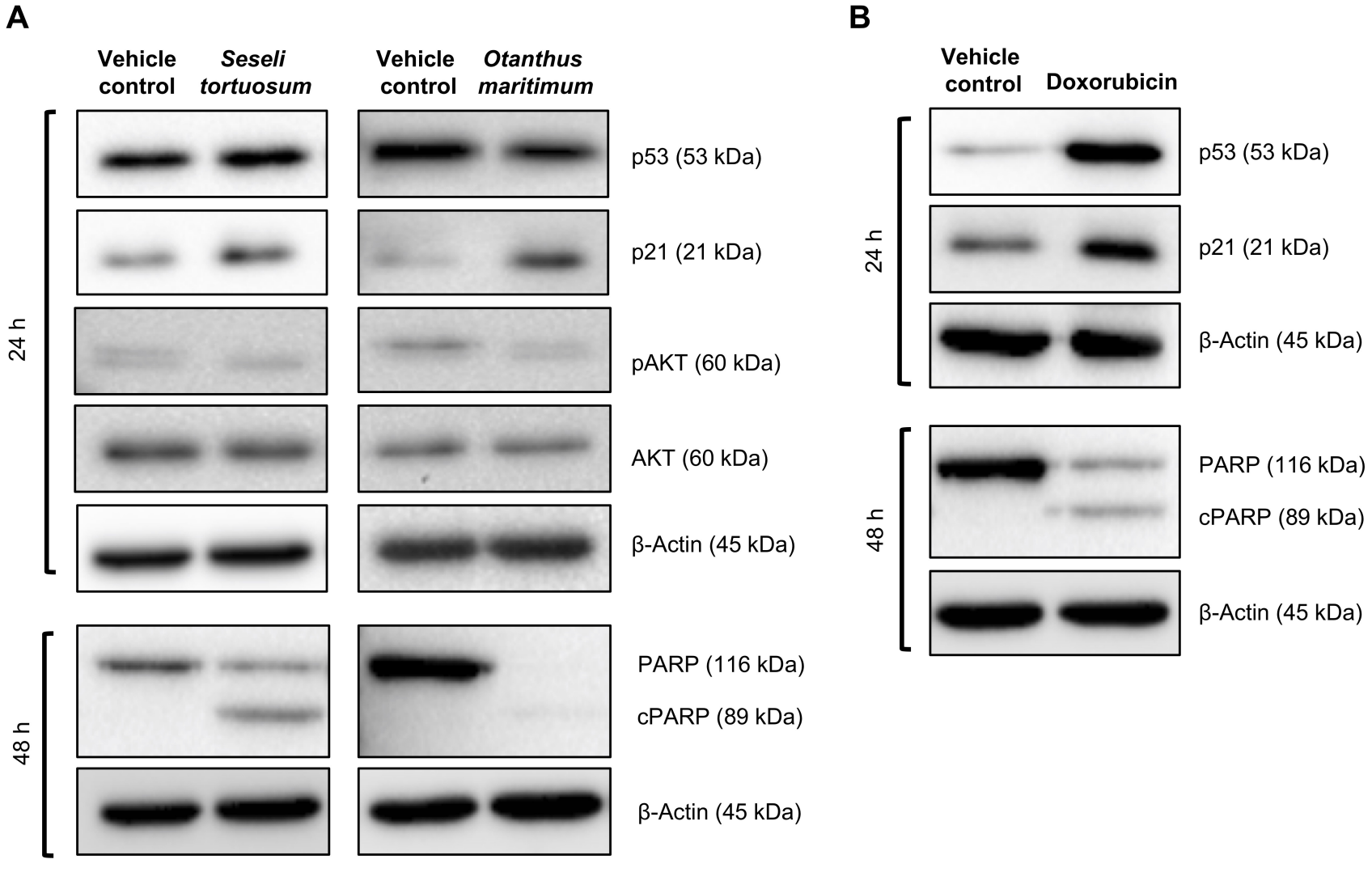


Supplementary Table Captions

Supplementary Table S1A. Detailed chemical composition of *Artemisia campestris* subsp. *maritima* essential oil.

Supplementary Table S1B. Detailed chemical composition of *Crithmum maritimum* essential oil.

Supplementary Table S1C. Detailed chemical composition of *Eryngium maritimum* essential oil.

Supplementary Table S1D. Detailed chemical composition of *Juniperus turbinata* subsp. *turbinata* essential oil.

Supplementary Table S1E. Detailed chemical composition of *Otanthus maritimus* essential oil.

Supplementary Table S1F. Detailed chemical composition of *Seseli tortuosum* essential oil.

Table 1 – Main components of EO extracts

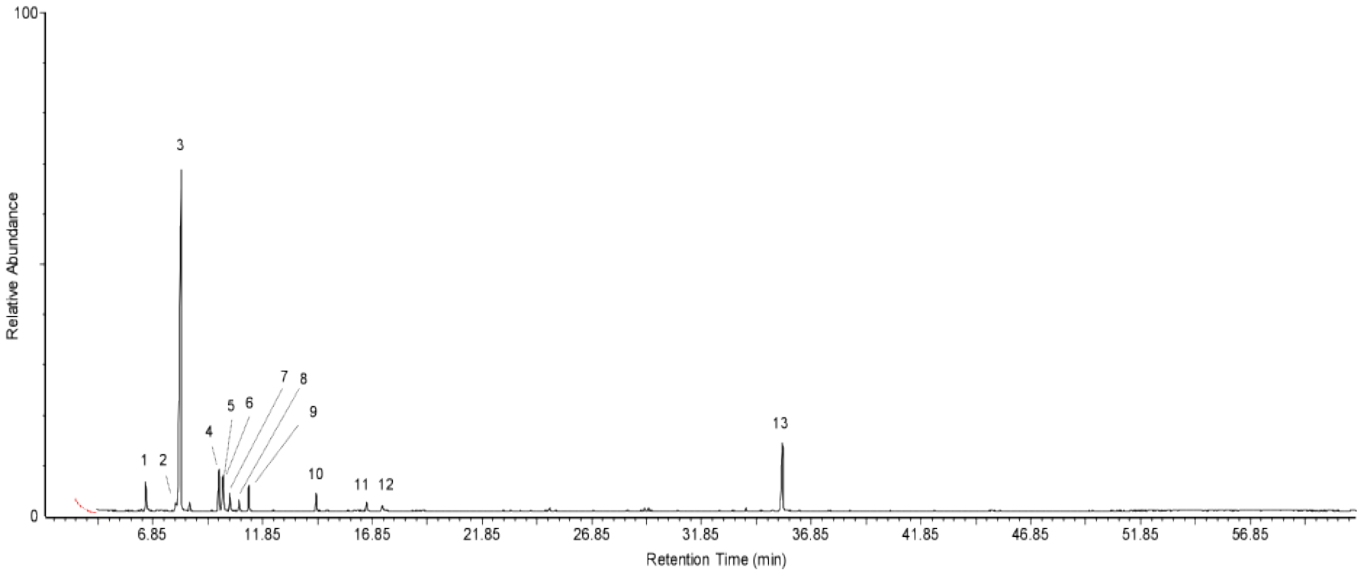
Species	Yield (%)	Main compounds (above 10%)
Artemisia campestris* subsp. *maritima	0.47	β-pinene isomer 1, γ-Muurolene
Crithmum maritimum	0.36	γ-terpinene, thymol methyl ether isomer 2, o-Cymene isomer 1, β-Phellandrene isomer 1
Eryngium maritimum	0.08	7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl-, D-germacrene
Juniperus turbinata* subsp. *turbinata	0.29	α-pinene, β-phellandrene isomer 2
Otanthus maritimus	0.12	Chrysanthenone isomer 1, Verbenyl acetate
Seseli tortuosum	0.66	α-pinene, β-pinene isomer 1

Table 2 – Cytotoxic effect of EOs extracts from the 6 dune plant species panel: IC_{50} values for both cell lines

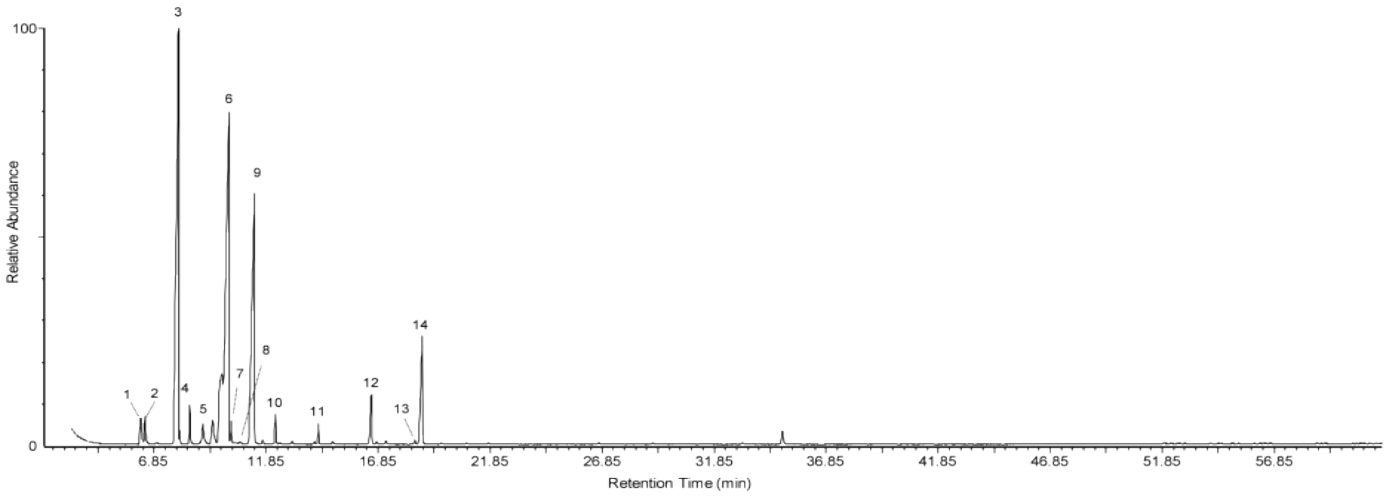
Species	IC_{50} (µL/mL)	
	MCF7	**RKO**
Artemisia campestris* subsp. *maritima	0.32	0.35
Crithmum maritimum	ND	ND
Eryngium maritimum	0.15	0.47
Juniperus turbinate* subsp. *turbinata	ND	ND
Otanthus maritimus	0.21	0.34
Seseli tortuosum	0.0086	0.034

Table 3 – Impact of *O. maritimus* and *S. tortuosum* EO extract treatment on cell cycle progression. Statistical significance of differences between means of vehicle control vs EO for both species, per timepoint and cell cycle phase.

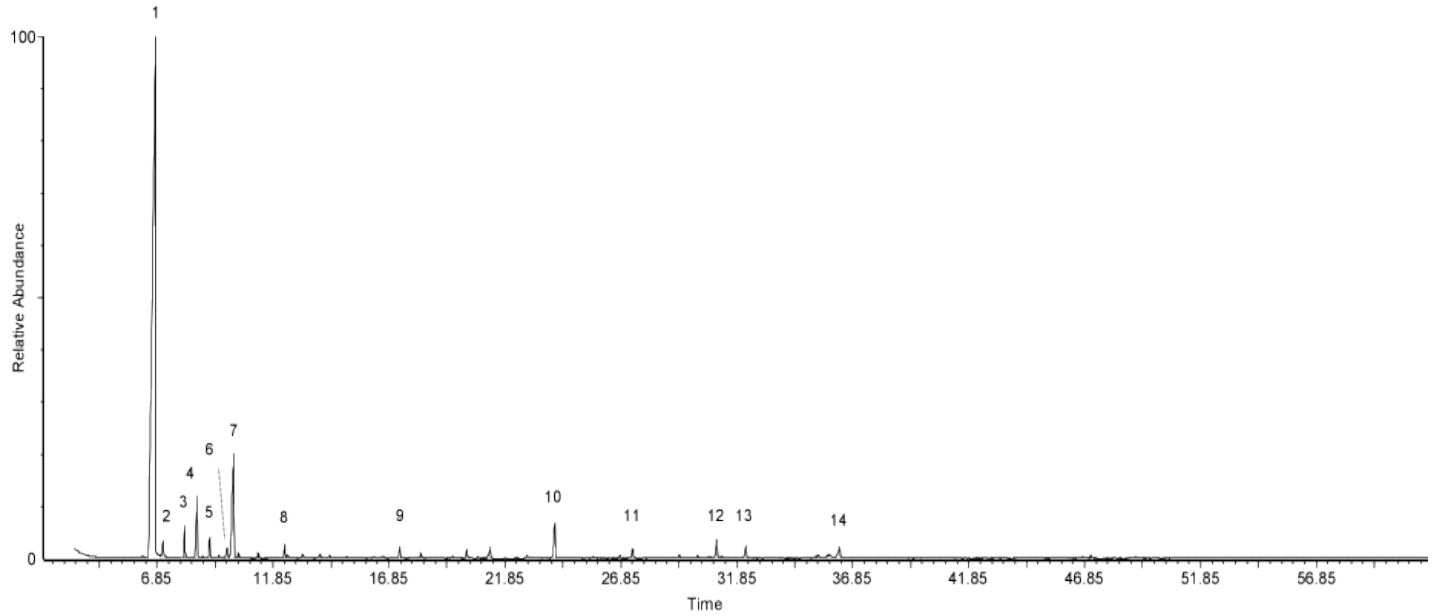
		subG1	G1	S	G2/M
Vehicle only *vs Seseli tortuosum*	**24 h**	p>0.05	*** p<0.05**	p>0.05	p>0.05
Vehicle only *vs Seseli tortuosum*	**48 h**	p>0.05	p>0.05	p>0.05	p>0.05
Vehicle only *vs Othantus maritimus*	**24 h**	p>0.05	p>0.05	p>0.05	p>0.05
Vehicle only *vs Othantus maritimus*	**48 h**	**** p<0.01**	*** p<0.05**	p>0.05	p>0.05
Vehicle only *vs* Doxorubicin	**24 h**	*** p<0.05**	****** p<0.0001**	**** p<0.01**	p>0.05
Vehicle only vs Doxorubicin	**48 h**	****** p<0.0001**	****** p<0.0001**	*** p<0.05**	****** p<0.0001**



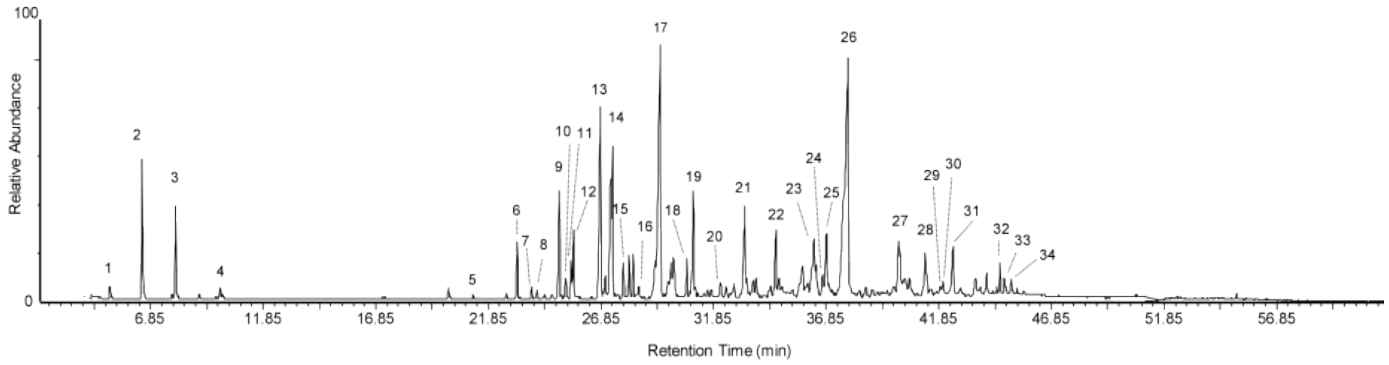
Supplementary Figure S1A. Chromatogram of *Artemisia campestris* subsp. *maritima* (DC.) Arcang essential oil composition.



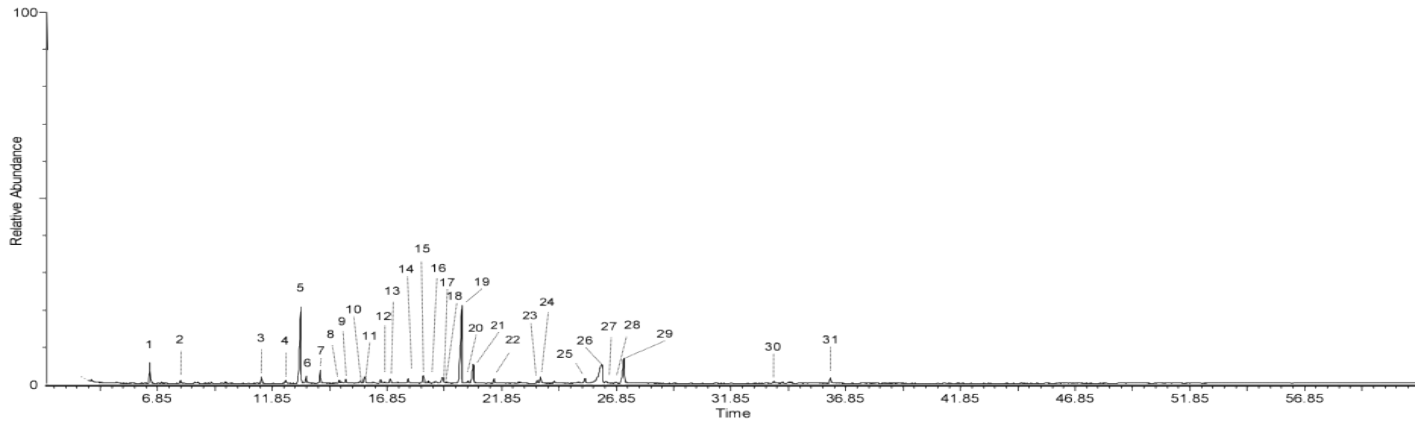
Supplementary Figure S1B. Chromatogram of *Crithmum maritimum* L. essential oil composition.



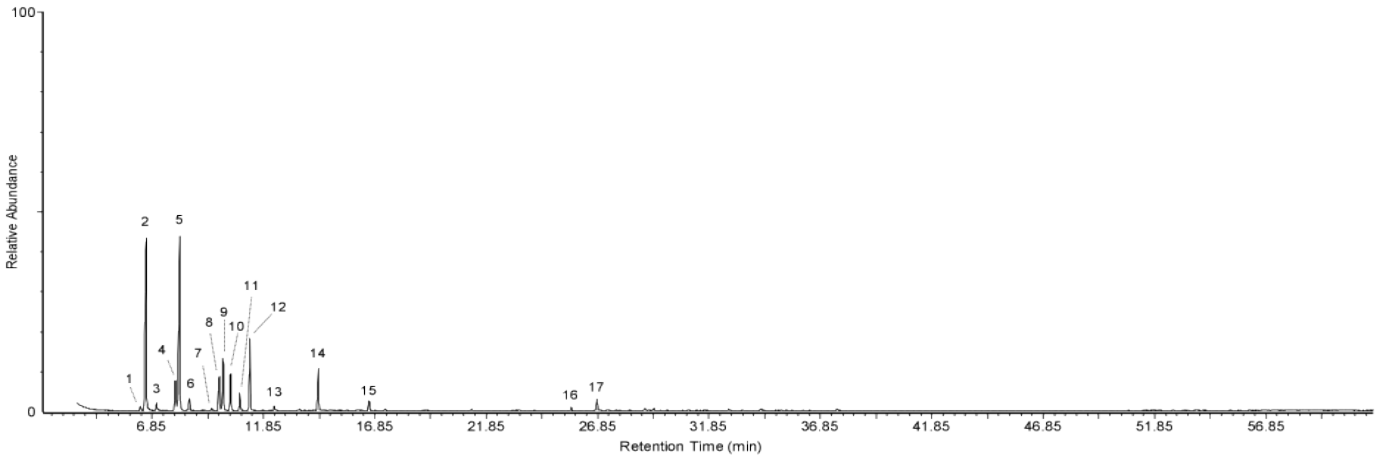
Supplementary Figure S1C. Chromatogram of *Juniperus phoenicea* var. *turbinata* (Guss.) Parl essential oil composition.



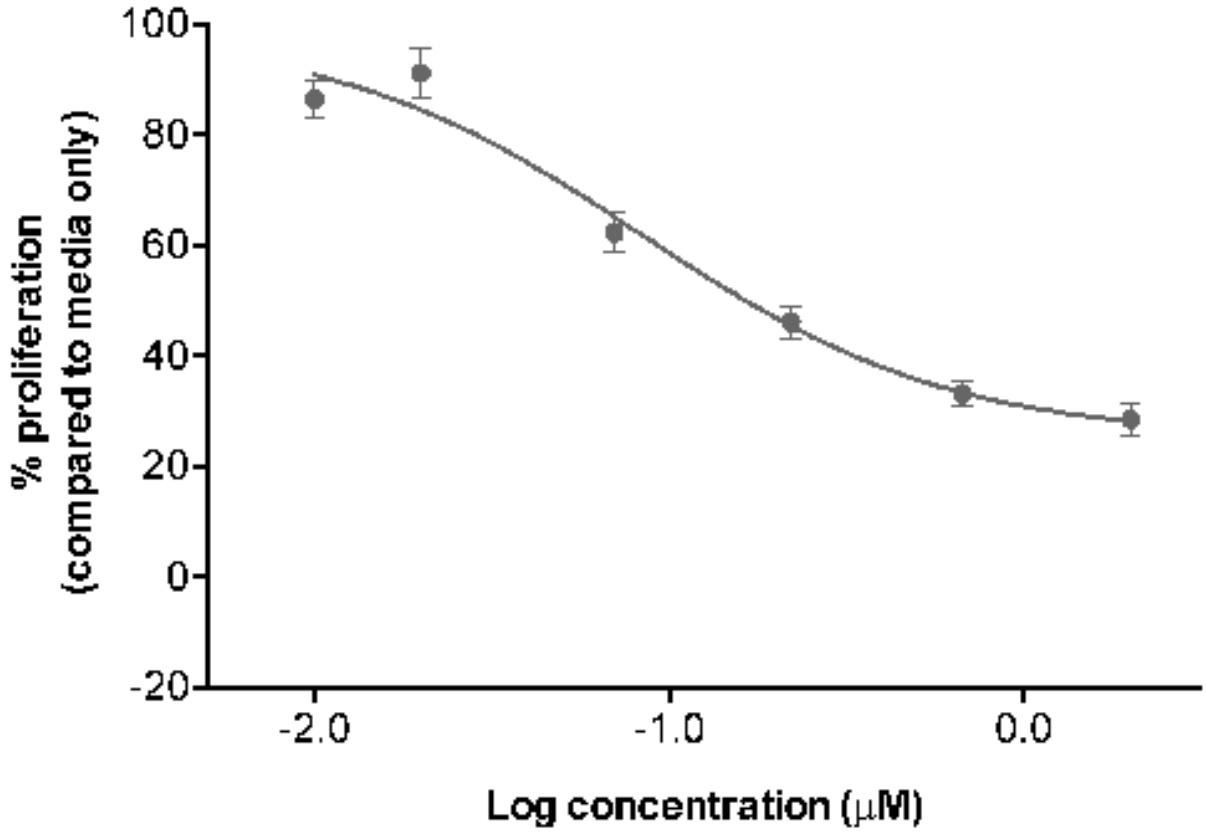
Supplementary Figure S1D. Chromatogram of *Eryngium maritimum* L. essential oil composition.



Supplementary Figure S1E. Chromatogram of *Otanthus maritimus* (L.) Hoffmanns. & Link essential oil composition.

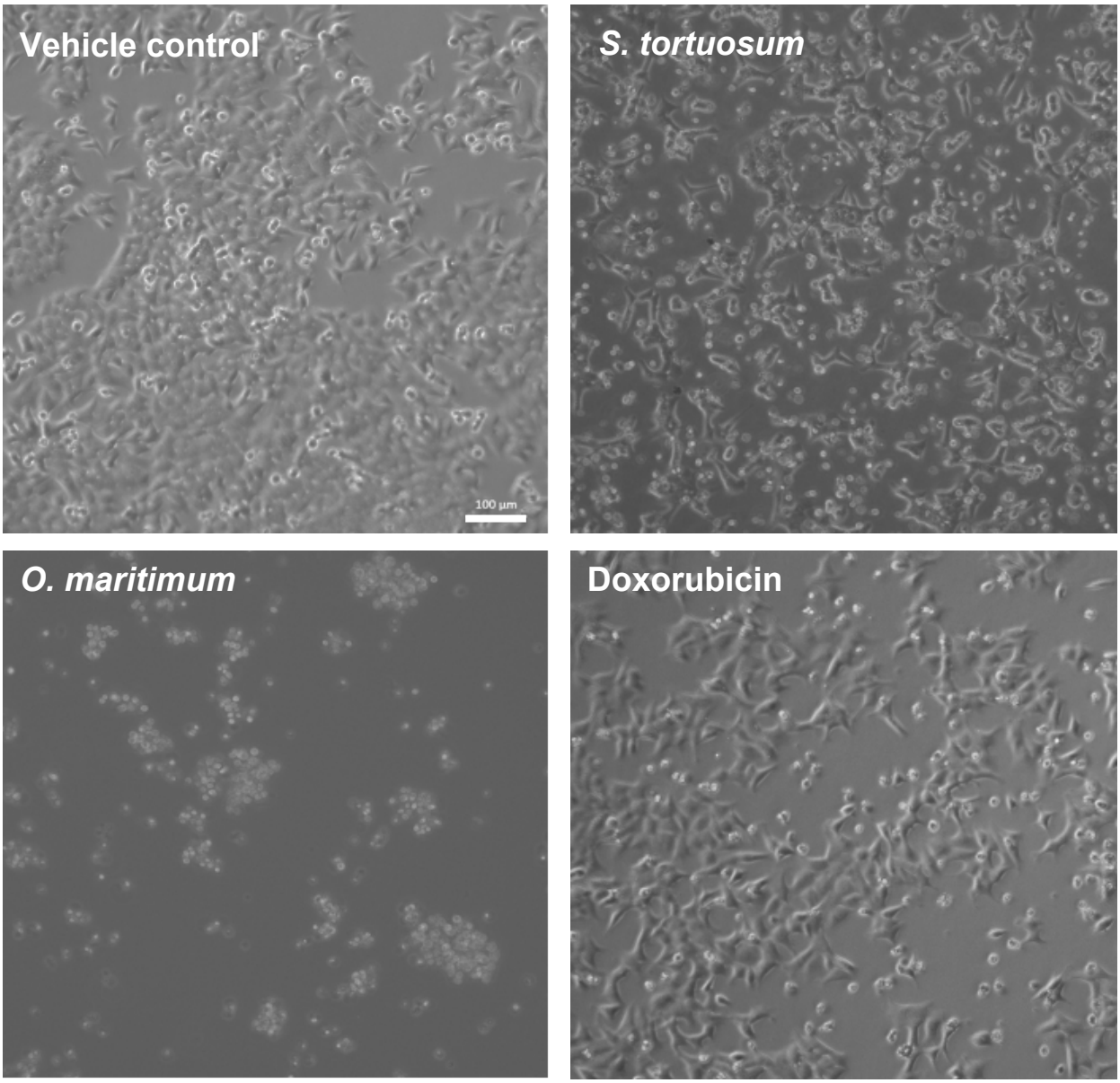


Supplementary Figure S1F. Chromatogram of *Seseli tortuosum* L. essential oil composition.



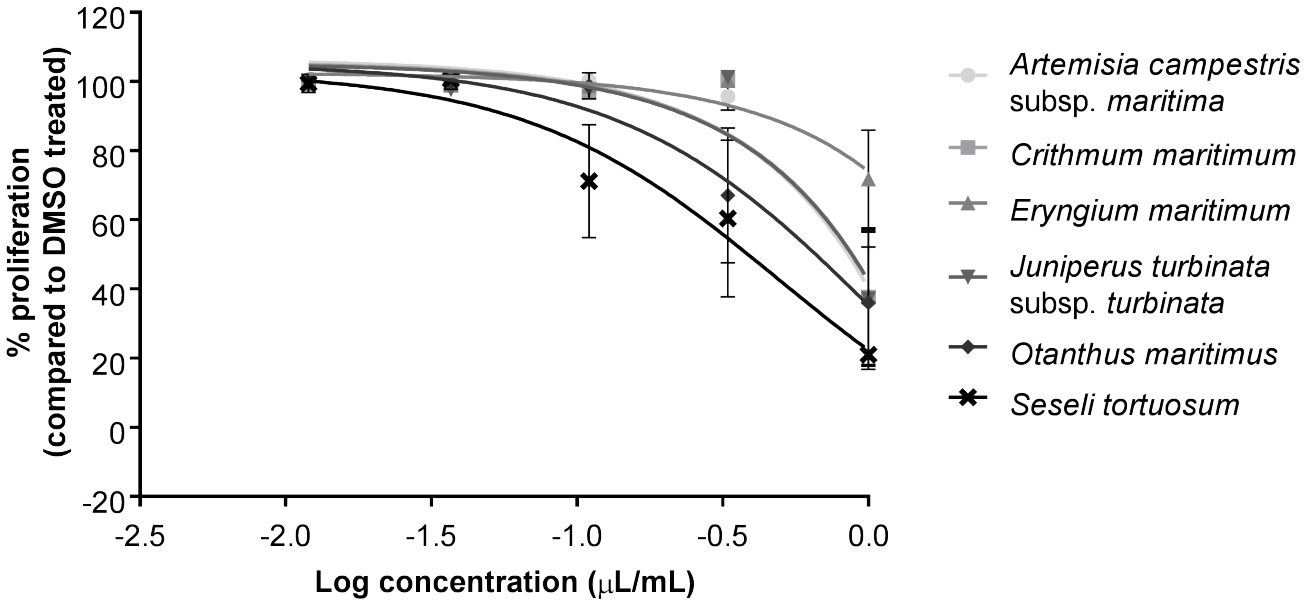
Supplementary Figure S2. Cytotoxic effect of Doxorubicin.

MCF7 cells seeded at a density of 1x104 cells per well in a 96-well plate in triplicates per condition. Cells were treated with a range of Doxorubicin concentrations (1:3 serial dilution 2 μM – 0.01 μM). Cells were exposed to treatment for 72 hours and an MTS assay was subsequently performed. Scatter plot represents cell viability expressed as percentage survival of control (n=3 independent experiments). IC50 value represents the average of n=3 independent experiments.



Supplementary Figure S3. Morphology of MCF7 cells treated with Otanthus maritimus and Seseli tortuosum EO extracts and Doxorubicin.

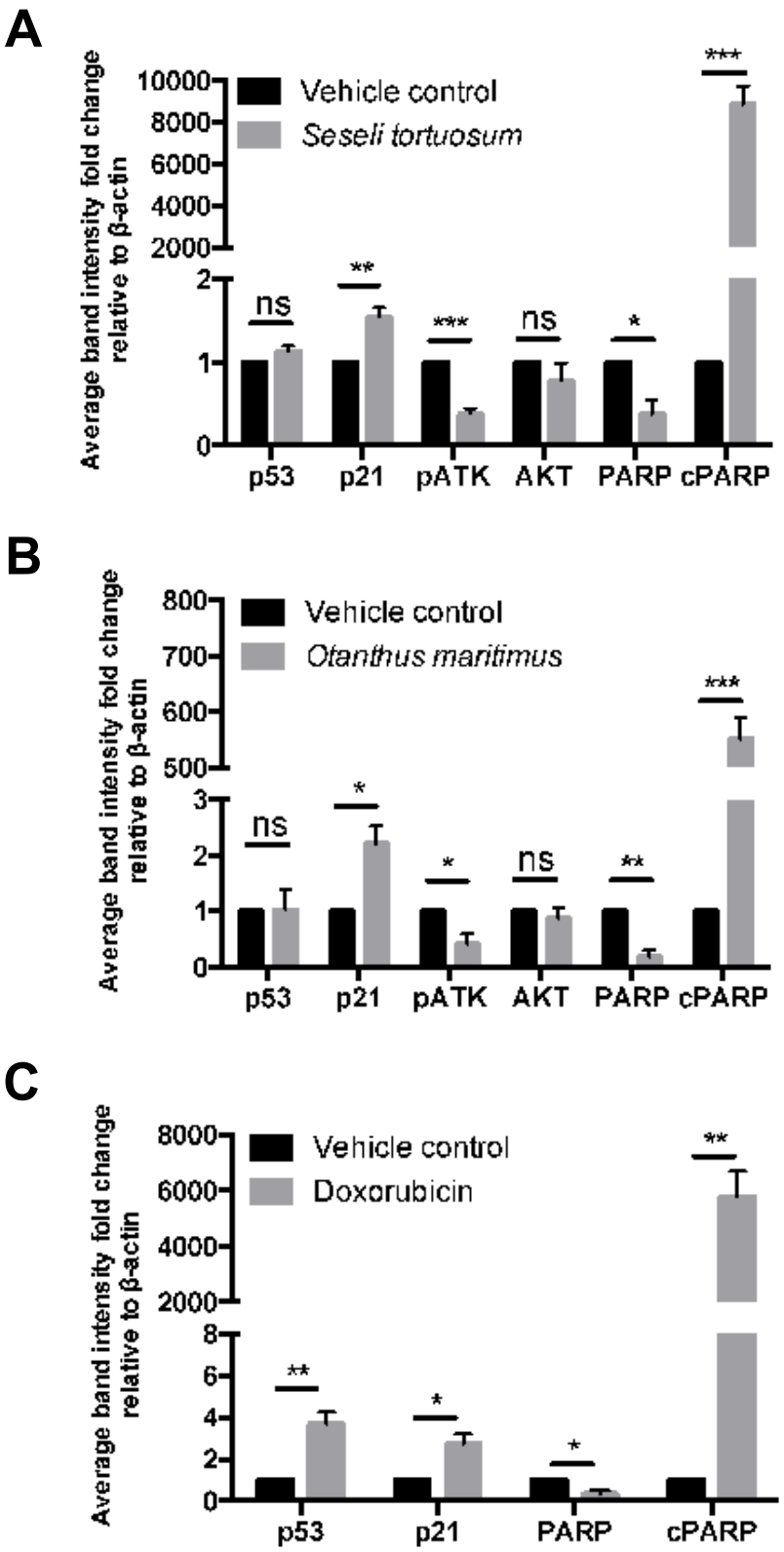
MCF7 cells were treated with either vehicle-only control (1 µL/ml DMSO), 1 µL/ml S. tortuosum EO extract, 1 µL/ml O. maritimum EO extract, or 2 µM Doxorubicin for 24 hours. Brightfield images were obtained using an inverted microscope (Axio Vert, Zeiss, UK). Scale bar represents 100 µm.



Species	IC_{50} (μL/mL)
	HEK293-T
Artemisia campestres subsp. *maritimum*	ND
Crithmum maritimum	ND
Eryngium maritimum	ND
Juniperus turbinata subsp. *turbinata*	ND
Othanthus maritimus	1.06
Seseli tortuosum	0.49

Supplementary Figure S4. Cytotoxic effect of EOs extracts from 6 dune plant species panel in a non-cancer cell line

HEK293-T cells were seeded at a density of 2x10 ^{4}cells per well in a 96-well plate. Triplicate wells were seeded per condition. Cells were treated with a range of EO concentrations (1:10 serial dilution 1 μL/ml – 10^{-5} μL/ml). Cells were exposed to treatment for 72 hours and an MTS assay was subsequently performed. Scatter plot represents cell viability expressed as percentage survival of control (n=3 independent experiments). IC50 value represents the average of n=3 independent experiments.



Supplementary Figure S5. Quantification of the effects of the impact of *O. maritimus* and *S. tortuosum* treatment on cell survival and cell death signaling.

MCF7 cells were treated with either vehicle-only control (1 µL/mL DMSO in media), 1 µL/mL *S. tortuosum* EO extracts *(A),* O. maritimum EO extracts (B) or vehicle control/2 µM Doxorubicin (C) for 24 and 48 hours. Graphs represent the semi-quantitative analysis of Western blots from Figure 4 (a.u. = arbitrary units of fold increase relative to β-actin). Significance values: * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Supplementary Table 1 – Details of antibodies used

Target	Manufacturer	Reference	Dilution	Origin Species
p53 (DO1)	Santa Cruz	sc-126	1:2000	Mouse mAb
p21	Cell signalling	2946	1:2000	Mouse mAb
AKT (pan)	Cell signalling	4691	1:1000	Rabbit mAb
Phospho AKT	Cell signalling	4060	1:2000	Rabbit mAb
PARP	Cell signalling	9542	1:1000	Rabbit pAb
ß-actin	Santa Cruz	sc-69879	1:10,000	Mouse mAb
Mouse HRP (2^o)	Dako	P0449	1:2000	Rabbit pAb
Rabbit HRP (2^o)	Dako	P0448	1:2000	Goat pAb

mAb, Monoclonal antibody*; pAb,* Polyclonal antibody

Supplementary Table 2-A. Essential oil composition.

Artemisia campestris **subsp.** *maritima* **(DC.) Arcang**.				
Peak	**Rt (min)**	**Identification**	**LRI** [a]	**Quantification** [b]
1	6.53	α-Pinene	922	2.99±0.07
2	7.90	Sabinene	961	2.09±0.01
3	8.16	β-Pinene isomer 1	968	64.34±0.95
4	9.86	*O*-Cymene isomer 1	1014	4.61±0.09
5	10.05	D-Limonene	1019	2.87±0.13
6	10.09	β-Phellandrene	1124	2.56±0.14
7	10.36	β-*cis*-Ocimene	1025	1.84±0.03
8	10.79	β-*trans*-Ocimene	1035	1.20±0.05
9	11.22	γ-Terpinen	1046	2.71±0.01
10	14.29	Neo-allo-ocimene	1116	2.02±0.03
11	16.61	Terpinen-4-ol	1167	1.45±0.07
12	17.29	α-Terpinoel	1182	1.23±0.06
13	35.53	γ-Muurolene	1611	10.10±0.51

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.

Supplementary Table 2-B. Essential oil composition.

Crithmum maritimum L.				
Peak	**Rt (min)**	**Identification**	**LRI [a]**	**Quantification [b]**
1	6.34	α-Thujene	916	0.47±0.01
2	6.53	α-Pinene	922	2.38±0.06
3	7.91	β-Phellandrene isomer 1	961	16.37±0.34
4	8.51	β-Pinene isomer 2	978	0.86±0.01
5	9.54	α-Terpinolene isomer 1	1008	0.82±0.03
6	9.89	*O*-Cymene isomer 1	1018	14.03±0.17
7	10.08	Carene	1023	0.64±0.02
8	10.37	*trans* β-*O*-cimene	1031	0.63±0.01
9	11.34	γ-Terpinene	1059	34.90±1.18
10	12.34	α-Terpinolene isomer 2	1088	0.47±0.01
11	14.30	Neo-allo-ocimene	1118	0.74±0.01
12	16.65	Terpinen-4-ol	1173	6.92±0.16
13	18.61	Thymol methyl ether isomer 1	1211	0.32±0.01
14	18.92	Thymol methyl ether isomer 2	1218	20.43±0.61

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.

Supplementary Table 2-C. Essential oil composition.

Juniperus phoenicea var. *turbinata* (Guss.) Parl				
Peak	**Rt (min)**	**Identification**	**LRI [a]**	**Quantification [b]**
1	6.79	α-Pinene	929	73.36±0.57
2	7.06	Camphene	937	0.63±0.01
3	8.04	β-Pinene isomer 1	965	1.37±0.04
4	8.56	β-Pinene isomer 2	980	3.23±0.10
5	9.09	α-Phellandrene isomer 2	996	0.75±0.02
6	9.82	*O*-Cymene isomer	1013	0.71±0.01
7	10.16	β-Phellandrene isomer 2	1021	11.75±0.65
8	12.31	α-Terpinolene isomer 2	1071	1.44±0.04
9	17.28	α-Terpineol	1187	0.77±0.04
10	24.00	α-Terpinyl acetate	1332	2.40±0.13
11	27.34	δ-Elemene isomer 2	1408	0.47±0.03
12	30.98	β-Cadinene	1495	1.06±0.03
13	32.22	Cyclohexanemethanol	1527	0.62±0.03
14	36.25	β-Guaiene	1630	1.42±0.00

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.

Supplementary Table 2-D. Essential oil composition.

			Eryngium maritimum L.	
Peak	**Rt (min)**	**Identification**	**LRI [a]**	**Quantification [b]**
1	5.08	Ethanone, 1-(2-methyl-2-cyclopenten-1-yl)-	869	0.422±0.002
2	6.51	α-Pinene isomer 1	921	3.87±0.07
3	8.00	β-Pinene	963	2.69±0.02
4	9.96	β-Terpineol, acetate	1016	0.317±0.001
5	20.11	(S)-(-)-Citronellic acid, methyl ester	1244	0.342±0.004
6	23.18	Elixene	1312	1.75±0.05
7	23.81	α-Copaene	1327	0.347±0.005
8	24.05	α-Guaiene	1332	0.26±0.01
9	25.05	α-Copaene	1355	3.62±0.08
10	25.31	β-Bourbonene	1361	0.614±0.002
11	25.57	β-Cubebene	1367	1.23±0.03
12	25.70	β-Elemen	1370	1.96±0.07
13	26.87	β-ylangene	1370	8.10±0.02
14	27.31	γ-Muurolene	1407	4.54±0.06
15	27.41	γ-Elemene	1410	4.78±0.06
16	28.32	Humulene	1431	1.225±0.004
17	29.53	D-Germacrene	1460	14.70±0.09
18	30.72	γ-Cadinene	1489	1.199±0.005
19	31.00	δ-Cadinene	1495	4.19±0.01
20	32.21	Elemol	1526	0.55±0.01
21	33.28	(-)-Spathulenol	1553	3.53±0.07
22	34.66	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-	1588	2.530±0.000
23	36.36	α-Cadinol	1632	2.04±0.04
24	36.74	Murolan-3,9(11)-diene-10-peroxy	1642	0.8054±0.0002
25	36.92	1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)-	1647	2.739±0.001
26	37.86	7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl-	1672	20.21±0.56
27	40.12	Cubedol	1733	3.782±0.002
28	41.29	2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester	1766	2.82±0.04
29	41.97	α-Glyceryl linolenate	1784	0.307±0.003
30	42.53	2-Butenal, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	1800	1.88±0.03
31	43.53	Aromadendrene oxide-(2)	1828	0.74±0.01
32	44.62	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	1858	0.959±0.001
33	44.83	Murolan-3,9(11)-diene-10-peroxy	1864	0.45±0.01
34	45.12	2-[4-methyl-6-(2,6,6-trimethylcyclohex-1-enyl)hexa-1,3,5-trienyl]cyclohex-1-en-1-carboxaldehyde	1872	0.49±0.01

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.

Supplementary Table 2-E. Essential oil composition.

Otanthus maritimus (L.) Hoffmanns. & Link				
Peak	**Rt (min)**	**Identification**	**LRI [a]**	**Quantification [b]**
1	6.51	α-Pinene	921	3.52±0.16
2	7.84	Sabinene	959	0.56±0.01
3	11.38	Safranal	1049	1.11±0.05
4	12.44	Verbenone isomer 1	1074	1.00±0.02
5	13.11	Chrysanthenone isomer 1	1090	21.55±0.29
6	13.35	3,5-Heptadien	1096	1.13±0.06
7	13.95	Chrysantenone isomer 2	1109	2.62±0.09
8	14.75	*trans* Pinocarveol	1126	1.00±0.03
9	15.04	10,13-Octadecadynoic acid	1133	0.75±0.04
10	15.69	Hyocholic acid	1147	0.92±0.01
11	15.88	*cis* Verbenol	1151	1.07±0.05
12	16.56	Terpinen-4-ol	1166	0.92±0.01
13	16.99	Chrysanthenone isomer 3	1176	0.87±0.01
14	17.77	Verbenone isomer 2	1193	0.86±0.01
15	18.43	trans Carveol	1207	2.08±0.12
16	18.95	Cinobufagin isomer 1	1219	0.93±0.02
17	19.25	Verbenone isomer 3	1226	1.84±0.06
18	19.44	(-)-Carvole	1230	0.71±0.01
19	20.11	Verbenyl acetate	1245	21.97±0.86
20	20.39	Cinobufagin isomer 2	1251	0.85±0.05
21	20.60	*p*-Mentha-1.8-dien-3-one	1255	4.92±0.17
22	21.51	5-Isopropenyl-2-methyl-1-cyclopentene-1-carbaldehyde	1276	0.83±0.04
23	23.40	*cis* Carvyl acetate	1318	0.94±0.04
24	23.54	Verbenone isomer 4	1321	1.55±0.05
25	25.47	Berkheyaradulene	1365	1.12±0.02
26	26.22	Chrysanthenone isomer 4	1382	11.92±0.58
27	26.41	Cinobufagin	1387	1.04±0.05
28	26.82	Docosahexaenoic acid, 1,2,3-propanetriyl esther	1396	0.87±0.02
29	27.18	Chysantenone isomer 5	1405	8.03±0.33
30	33.70	Docosahexanoic acid. 1.2.3-propanetryl ester	1564	0.87±0.01
31	36.18	2-Napthalenemethanol	1628	1.62±0.09

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.

Supplementary Table 2-F. Essential oil composition.

Seseli tortuosum L.				
Peak	**Rt (min)**	**Identification**	**LRI [a]**	**Quantification [b]**
1	6.34	α-Phellandrene isomer 1	916	1.01±0.01
2	6.58	α-Pinene	923	24.90±0.96
3	7.06	Camphene	937	1.17±0.01
4	7.88	Sabinen	960	4.92±0.02
5	8.09	β-Pinene isomer 1	966	24.52±0.01
6	8.51	β-Pinene isomer 2	978	1.79±0.05
7	9.52	α-Terpinolene isomer 1	1006	0.63±0.02
8	9.86	*O*-Cymene isomer 1	1014	4.98±0.03
9	10.03	Limonene	1018	7.98±0.36
10	10.38	*trans* δ-Ocimene	1026	4.83±0.03
11	10.79	*O*-Cymene isomer 2	1035	2.67±0.11
12	11.26	δ-Terpinene	1046	9.69±0.35
13	12.36	α-Terpinolene isomer 2	1072	0.90±0.02
14	14.32	Neo-allo-ocimene	1117	6.50±0.16
15	16.58	Terpene - 4-ol	1167	1.84±0.07
16	25.68	δ-Elemene isomer 1	1370	0.68±0.01
17	26.82	Caryophyllene	1396	2.07±0.09

[a] LRI, linear retention index determined on a DB-5 MS fused silica column relative to a series of n-alkanes (C8–C40). [b] relative % is given as mean ±SD, n=3.