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Anti-cancer activity of *Citrus bergamia* essential oil and its constituents in *in vitro* experimental models of paediatric neoplasms

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ABSTRACT

Cancer represents one of the leading causes of death worldwide. In paediatric subjects, leukaemia and brain tumours showed the highest incidence in 2020. The plant kingdom has always been a great source for bioactive compounds for treating several illnesses, including cancer, and *Citrus* fruits stand out among the others. For my PhD project, I focused on the anti-proliferative activity of the essential oil (BEO) of *Citrus bergamia* (bergamot) Risso & Poiteau and its constituents in *in vitro* experimental models of paediatric neoplasms.

The first step of the project was performing the quali-quantitative characterization of BEO and its furocoumarin-free fraction (BEO-FF). The chemical profile of these plant matrices showed that they are composed of a volatile part (< 95%) rich in monoterpenes, and a non-volatile one, containing coumarins, results in line with those present in literature.

The second step of my PhD project originated from a previous assumption by which bergamottin (BRG) and 5-geranyloxy-7-methoxycoumarin (5-G-7-MOC), two coumarins found in BEO, have been claimed to be relevant in the anti-proliferative effects exerted by BEO in the SH-SY5Y human neuroblastoma cells. Therefore, this step was designed to verify this and to assess the mechanisms underlying the anti-proliferative effect of both compounds. The results of this step demonstrate that BRG and 5-G-7-MOC are able to reduce the proliferation of SH-SY5Y cells, inducing apoptosis and increasing sub-G0/G1 phase cell population. Furthermore, the pro-oxidant activity of the two coumarins was demonstrated, which increased reactive oxygen species and consequently reduced

mitochondrial membrane potential. Moreover, BRG and 5-G-7-MOC were able to modulate apoptosis-related factors both at protein and gene level. As a final point, we evaluated the synergistic effect of their combination, finding that the highest synergy was observed at a concentration ratio similar to observed in the whole BEO, supporting our initial hypothesis. These results deepen the knowledge on the effect of BRG and 5-G-7-MOC in SH-SY5Y cells, stressing the relevance of their cooperation in achieving this effect.

Acknowledged the clinical relevance of multi-drug resistance (MDR) in cancer treatment, the last step of my PhD project was to evaluate the effectiveness of BEO and BEO-FF in acute leukemic lymphoblasts and in their doxorubicin-resistant counterpart (i.e., CCRF-CEM and CEM/ADR5000, respectively). Both fractions showed similar antiproliferative activity in both cell lines, as showed by cell viability assays. Flow cytometric analyses indicated that this effect was achieved by inducing apoptosis, although with different extent for BEO and BEO-FF, and confirmed by the increase of population in sub-G0/G1 phase, meaning hypodiploidy. To assess their role against MDR, likewise for coumarins present in BEO, both bergamot fractions were combined with doxorubicin at different ratios. If in CCRF-CEM cells neither BEO nor BEO-FF elicited any relevant effect, in CEM/ADR5000 cells, the whole BEO brought a strong synergistic effect, restoring the sensitivity of resistant cells to doxorubicin. Therefore, these results corroborate the hypothesis that a phytocomplex, like BEO, can be an appropriate weapon to fight MDR exploiting their collateral sensitivity, a phenomenon by which cells

resistant to a specific antineoplastic agent may be particularly sensitive to another compound or a multitude of them.

Keywords: cancer; citrus fruits; bergamot essential oil; coumarins; multidrug-resistance.

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1. INTRODUCTION

1.1. The incidence of cancer

The unceasing scientific progress has led to a constant enhancement of the quality of life to such an extent that, in highly industrialized areas, people have plenty of food, medications and technology at their disposal. Although this can be considered as an achievement, the other side of the coin is that abundance often brings misuse. Therefore, unhealthy dietary patterns, irregular pharmacological therapies and over-exposition to technological devices are acknowledged causes of the so-called “civilization diseases”, that are type-2 diabetes, obesity, auto-immune and neurodegenerative diseases, cardiopathies as well as cancer.

On this line, the International Agency for Cancer Research (IACR) has gathered in the 2020 the evidence on cancer incidence and relative mortality worldwide (Sung et al., 2021). From this report, it is apparent the correlation between population’s wealth and the diagnosis of cancer which is more than 1.6- and 1.5-fold higher for males and female, respectively, living in areas with high income respect with the average of the entire world population (Figure 1).

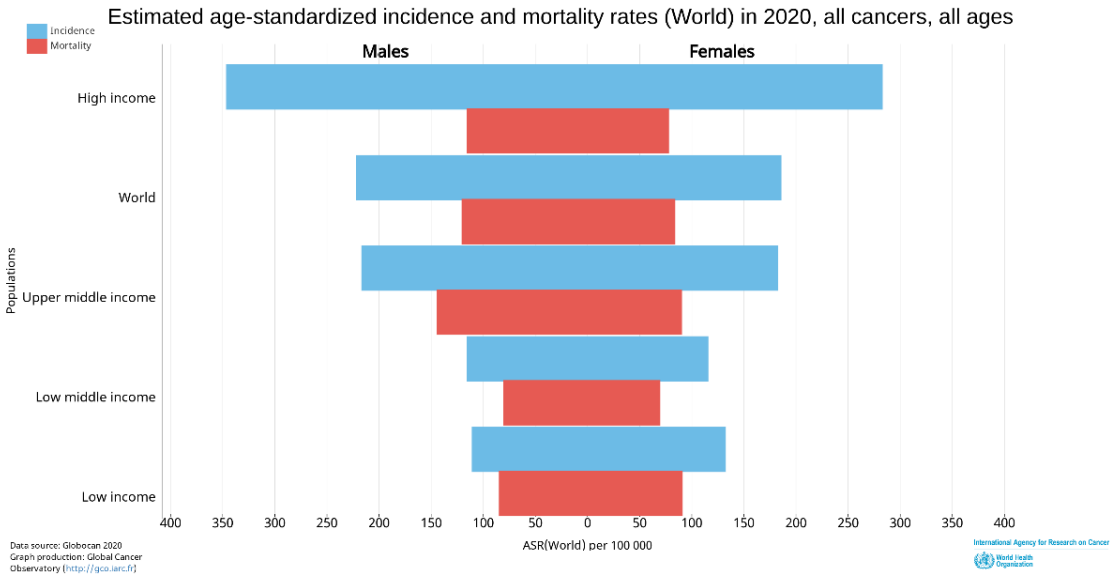

Figure 1. Incidence (blue bars) and mortality (red bars) of cancer based on income rate (from Globocan 2020).

Noteworthy, the mortality of cancer in wealthy areas is nearly comparable with that of the other world areas despite higher incidence, and this is due to the fact that the former ones possess a more efficient health system with cutting-edge equipment and top-trained medical personnel.

Nevertheless, the events elapsing from diagnosis to the resolution of the neoplasm are several, and these may include debilitating pharmacological therapies, difficult and risky surgical procedures in order to avoid nefarious outcomes that cancer unfortunately carries, along with a great impact on socio-economic costs. Therefore, scientific community is in the constant search of novel and more effective approaches for preventing or treating malignancies, to lower their incidence or improving the therapeutical path, respectively. This is crucial for each patient, yet primarily for younger ones which are, along with elderly, the most sensitive to first-line chemotherapy.

According to IACR, the cancer types with the highest incidence worldwide in 2020 for subjects being up to 9 years old are leukaemia and brain tumours, with 50,000 and 17,500 new cases, respectively (Figure 2).

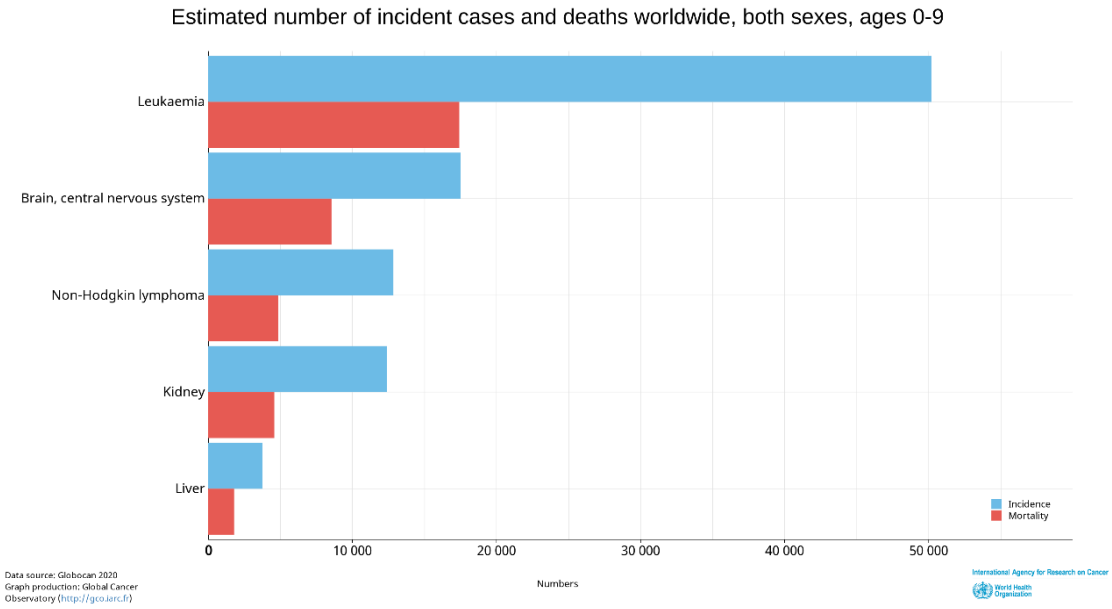

Figure 2. New cases (blue bars) and deaths (red bars) of paediatric cancers in 2020 (from Globocan 2020).

1.2. Acute lymphoblastic leukaemia (ALL)

The acute lymphoblastic leukaemia (ALL) is the most recurrent form of leukaemia in paediatric age (Miranda-Filho et al., 2018). Likewise acute myeloid leukaemia, ALL is caused by a series of acquired genetic aberrations. Malignant transformation usually occurs at level of the pluripotent stem cell, although it may occasionally involve and address stem cells with a more limited capacity for self-renewal. Abnormal proliferation, clonal expansion, aberrant differentiation, and reduced

apoptosis lead to the replacement of normal blood elements with malignant cells.

ALL is diagnosed after a complete blood count coupled with a peripheral blood smear, followed, if positive, by histochemical, cytogenetic and immunophenotyping studies. The most common symptoms of ALL are due to altered haematopoiesis resulting in anaemia, thrombocytopenia and granulocytopenia. Anaemia may manifest with fatigue, pallor, dyspnoea, tachycardia, and chest pain. Thrombocytopenia may cause general bleeding (i.e., gum, nasal, heavy menstrual), bruising and epistaxis, events particularly serious when affecting intracranial or intra-abdominal areas. Granulocytopenia may lead to high risk of a wide variety of infections (Onciu, 2009).

The first step of the treatment for paediatric ALL consists of an induction therapy with corticosteroids, anthracyclines, vincristine and asparaginase in order to completely eradicate leukemic cells from the bone marrow and restore the physiological white blood cell production. Usually, this phase allows a complete remission of a high percentage of patients, yet the occurring of leucopoenia may increase the onset of severe adverse reaction, underlining the importance of supporting therapy in this crucial stage of the treatment (Kato and Manabe, 2018). Induction therapy is followed by a multi-agent consolidation phase that includes methotrexate coupled with a re-induction therapy. After this, maintenance therapy is required for a couple of years to decrease the percentage of possible events during the remission. Furthermore, in the last decade, germline

variations have been recognized as similarly important contributors to understanding the aetiology and sensitivity of ALL to treatment.

Recent scientific advancements allowed a more individualized therapeutic approach based on genomic features (i.e., somatic and germline) and treatment response. In this regard, the introduction of novel agents specifically developed for precise molecular targeting as well as immunotherapy has vastly increased the life expectancy of many leukemic patients (Vrooman and Silverman, 2016). In this frame, tyrosine kinase inhibitors (i.e., imatinib, dasatinib, nilotinib, ponatinib and bosutinib) represent the principal treatment for ALL patients carrying the Philadelphia chromosome, hence with a new gene called BCR-ABL. Moreover, the monoclonal antibodies blinatumomab, which binds CD19 of B-cells and CD3 of T-cells and hence presenting tumour cells to immune ones, and inotuzumab ozogamicin, which binds CD22 of B-cells carrying inside them the cytotoxic agent to which it is bound (i.e., ozogamicin), are useful in treating relapsing ALL cases. Finally, CAR T-cell therapy involves the *ex vivo* genetical alteration of patient's T-cells in order to present specific receptors (i.e., chimeric antigen receptors, CARs) on their surface, able to recognize leukemic cells and destroy them.

1.3. Multi-drug resistance (MDR) in ALL

Unfortunately, the treatment of children ALL, as well as many other neoplasms, may be affected by existing or acquired resistance to chemotherapeutic agents employed. Among the numerous cellular mechanisms responsible for multi-drug resistance (MDR), the

transmembrane protein P-glycoprotein (P-gp; Figure 3) has been linked to inauspicious outcomes in paediatric ALL patients (Swerts et al., 2006).

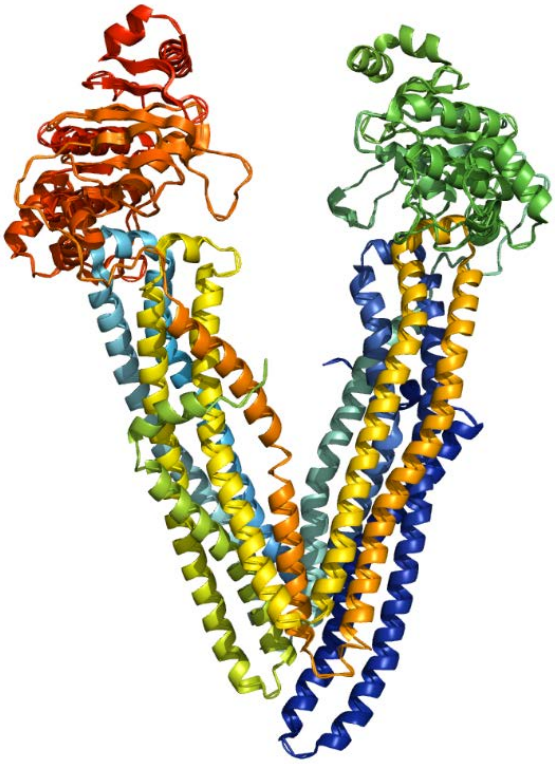

Figure 3. 3D structure of mammalian P-glycoprotein.

The clinical relevance of P-glycoprotein, that mediates the efflux of xenobiotics outside the cellular environment, along with other members of the ATP-binding cassette (ABC) transporter family, is that extruding several anti-neoplastic agents out of cancer cells, the appropriate concentration to achieve a therapeutic effect is not achieved, thus being an interesting target to be further studied in order to obtain more successful therapies (Efferth and Volm, 2017).

1.4. Neuroblastoma

Neuroblastoma (NB) is the most frequent solid neoplasm in infants. Indeed, approximately the 90% of cases occur in children of <5 years of age. Generally, neuroblastoma arises spontaneously, yet 1-2% appears to be inherited (Whittle et al., 2017). At diagnosis, performed by computer-assisted tomography or magnetic resonance imaging and confirmed by biopsy, almost 40-50% of children have localized or regional disease, while 50-60% have metastases. These can be into the bone marrow, bone, liver, lymph nodes, or, less commonly, to the skin or brain.

The clinical manifestations of NB depend on the site of the primary tumour and how the disease spreads. The most frequent symptoms are abdominal pain, discomfort, and a sense of fullness due to an abdominal mass. Metastases may induce bone pain, periorbital ecchymosis and proptosis, abdominal distension, and respiratory problems. When spinal canal is affected, children present focal neurological deficits or paralysis, whereas, in case of neck or upper chest metastases, Horner's syndrome (ptosis, miosis and anhidrosis), as well as paraneoplastic syndromes are likely to happen.

The treatment of NB is based on risk category. Therefore, surgical resection is important for low- and intermediate-risk disease. Nonetheless, it is often delayed until adjuvant chemotherapy is given to improve the chances of a successful resection. For children with medium-risk disease, chemotherapy is usually required, and cyclophosphamide, doxorubicin, cisplatin, carboplatin, ifosfamide and etoposide are typically employed. In case of high-risk NB, a more intense chemotherapy is followed, coupled

with stem cell transplantation and isotretinoin treatment. Radiotherapy and immunotherapy, with monoclonal antibodies specific for ganglioside G2 (i.e., dinutuximab and naxitamab), represent a valuable approach in incurable cases.

1.5. Pre-clinical models of paediatric neoplasms

Given the great demand for new and more effective treatments to treat such a fragile group as children, pre-clinical research is the first step on the long road to the assessment of the potential of many new drug candidates, both of natural or synthetic origin, in a relatively short time.

In this frame, several *in vitro* models are available for basic research on leukaemia (Valle-Reyes et al., 2021). Some of them also represent valuable models for studying naturally occurring resistance in paediatric ALL. Indeed, from CCRF-CEM cells, which belong to the T-cell subtype and were originally from a 3-year-old female subject, CEM/ADR500 cells are derived, which are acknowledged as a complete and rich model of leukemic MDR (Kadioglu et al., 2016).

Regarding NB, several cell lines are currently employed in 2D and 3D (i.e., spheroids) *in vitro* models, as well as in xenograft *in vivo* ones (Nolan et al., 2020). The parental SK-N-SH, originally from a 4-year-old female subject, and its third subcloned SH-SY5Y cell lines represent the most frequently employed NB cell lines. SH-SY5Y display a neuroblastic phenotype (N-type), which is characterized by a conserved genetic profile (i.e., proto-oncogene MYCN and chromosomes 1p/11q) and a high proliferation rate, with microtubule instability, typical of a poor prognosis

and chemotherapy resistant NB. In addition, the high expression of dopamine hydrolase by SH-SY5Y make this cell line ideal for the study of neurobiology and neuronal diseases (Campos Cogo et al., 2020).

1.6. Essential oils and their components as anti-cancer agents

It is acknowledged that the plant kingdom has always been a source of valuable compounds for the prevention and treatment of various diseases, both acute and chronic. Although some of these (i.e., *Vinca* alkaloids, podophyllotoxin derivatives, taxanes, etc.) are currently used in clinical practice to treat various forms of cancer, the wide majority of natural drugs has been proved as interesting anti-cancer compounds only in pre-clinical studies.

In this context, essential oils (EOs) have drawn the attention of researchers for their potential antitumor properties (Lesgards et al., 2014). In particular, EOs are known to act by multiple pathways and mechanisms related to the inhibition of apoptosis, arrest of cell cycle, as well as anti-metastatic, anti-angiogenic and pro-oxidant effects. These outcomes are achieved by targeting tumour suppressor proteins (i.e., p53 and Akt), transcription factors (i.e., NF-κB and AP-1), MAPK-pathway, and detoxification enzymes (i.e., superoxide dismutase, catalase, glutathione peroxidase and reductase) (Gautam et al., 2014). Moreover, EOs have

been also showed to exert anti-cancer properties in different MDR cancer cell lines (Queiroz et al., 2014; Saab et al., 2012; Wu et al., 2016).

EOs are phytocomplexes composed mainly by a volatile component, rich in terpenoids, and a non-volatile component, characterised by compounds originated from the phenylpropanoid biosynthetic pathway.

Monoterpenes are plant secondary metabolites derived from the condensation of two isoprene units, and contribute to the flavour and scent of plants. Pre-clinical studies, performed both *in vitro* and *in vivo*, have demonstrated that some monoterpenes possess antimicrobial (i.e., viruses, fungi, bacteria), as well as anti-carcinogenic properties (Wojtunik-Kulesza et al., 2019). In particular, monoterpenes may hamper cancer progression by targeting several cellular and molecular mechanisms, such as the arrest of cell cycle or the induction of apoptosis (Crowell, 1999; Sobral et al., 2014).

Other relevant compounds found in EOs, despite in much lesser quantities, are coumarins. These are secondary plant metabolites, biosynthetically arising from the phenylpropanoid pathway, which have been claimed to possess anticancer properties (Kupeli Akkol et al., 2020). Furocoumarins, compounds characterized by the fusion of a furan ring to the 1-benzopyran-2-one scaffold, are present also in EOs and are acknowledged for their phototoxicity when interacting with ultraviolet A (UVA) radiation, hence triggering cytotoxic and mutagenic effects. Remarkably, this characteristic is currently exploited in clinical practice as PUVA therapy,

where “P” stands for psoralen, leading compound of the whole class of furocoumarins (Melough et al., 2018).

1.7. Pharmacological relevance of *Citrus bergamia* Risso & Poiteau

Among EOs, those extracted from *Citrus* fruits stand out for their wide panel of biological effects (Dosoky and Setzer, 2018). This because, these fruits are acknowledged to be endowed by defensive activities against both oxidative stress and inflammation (Ferlazzo et al., 2016a; Maugeri et al., 2019a; Musumeci et al., 2020), cardiovascular (Testai and Calderone, 2017) and neurodegenerative diseases (Cirmi et al., 2016c; Cirmi et al., 2021), various microbial infections (Cirmi et al., 2016a), and several types of cancer (Cirmi et al., 2016b; Cirmi et al., 2017; Cirmi et al., 2018).

In these regards, *Citrus bergamia* Risso & Poiteau (bergamot) has been the object of several studies to prove the pharmacological relevance of its derivatives (i.e., juice and EO; Figure 4).

Figure 4. *Citrus bergamia* Risso & Poiteau.

Bergamot is a tree belonging to the Rutaceae family (genus *Citrus*), endemic to the southern part of the of Calabria (Italy). It is assumed to be a hybrid between *Citrus aurantium* L. (bitter orange) and either *Citrus limon* L. (lemon) or *Citrus aurantiifolia* (lime), or a mutation of this latter. The juice, obtained by squeezing bergamot fruits, is rich of flavonoids and has been studied for its for its antimicrobial (Filocamo et al., 2015) and anticancer activities both *in vitro* (Delle Monache et al., 2013; Ferlazzo et al., 2016b) and *in vivo* (Navarra et al., 2020; Navarra et al., 2014), proposing its main components as the potential responsible for this effect (Visalli et al., 2014). Therefore, the flavonoid-rich extract of bergamot juice

was further studied. It was suggested it possessed antioxidant (Ferlazzo et al., 2016c; Ferlazzo et al., 2015) and anti-inflammatory properties, both *in vitro* (Curro et al., 2016; Risitano et al., 2014) and *in vivo* (Gugliandolo et al., 2018; Impellizzeri et al., 2015; Impellizzeri et al., 2016), interacting as well with the AMPK/SIRT1 axis (Maugeri et al., 2019b) and indicating its potential role as a remedy against inflammation-based illnesses (Marino et al., 2015).

Bergamot EO (BEO), the most relevant product obtained by bergamot peels and highly valued in perfume industry, is a phytocomplex composed of about 95% by monoterpenes and by a non-volatile portion consisting of coumarins (Costa et al., 2010). Its application in aromatherapy was widely studied (Mannucci et al., 2017), likewise its antimicrobial (Cirmi et al., 2016a), anti-inflammatory (Lombardo et al., 2020), cardioprotective (Mollace et al., 2008), and anti-cancer properties (Celia et al., 2013; Navarra et al., 2015).

2. AIM OF THE RESERCH

Given the great potentiality of essential oils, the common thread of my PhD project was the evaluation of the anti-cancer activity of BEO and its constituents in *in vitro* experimental models of paediatric neoplasms. In particular, the research group led by Prof. Michele Navarra previously evaluated the mechanisms underlying the antiproliferative effects of various fractions from BEO in SH-SY5Y neuroblastoma cell line, suggesting that bergamottin (BRG) and 5-geranyloxy-7-methoxycoumarin (5-G-7-MOC), two coumarins present in it, may play a crucial role in this activity (Navarra et al., 2015). Following this, one of the targets of my PhD project was to deepen the knowledge on their activity and their role in the effect of the whole BEO.

In parallel, since EOs have been assessed for their anti-cancer properties in different MDR cancer cell lines (Queiroz et al., 2014; Saab et al., 2012; Wu et al., 2016), another goal was to investigate the potentiality of BEO in counteracting MDR in an *in vitro* model of resistant ALL, assessing its mode of action and hence proving that the multi-target nature of phytocomplexes can be exploited to counteract MDR.

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3. MATERIALS AND METHODS

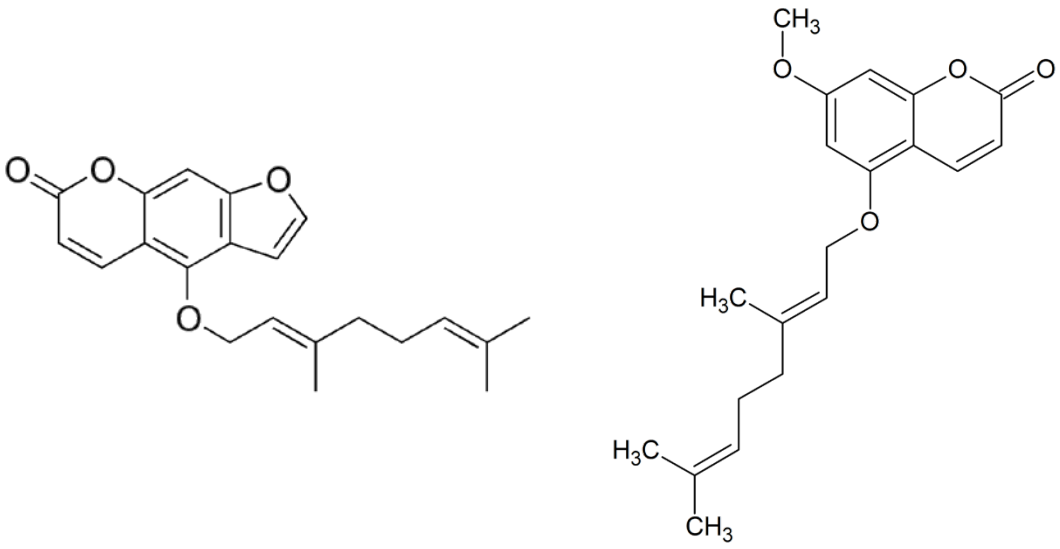
3.1. Quali-quantitative characterization of *Citrus bergamia* essential oil (BEO) and its furocoumarin-free fraction (BEO-FF)

BEO, obtained by cold pressing bergamot peels, and its extractive colourless fraction without furocoumarins (BEO-FF), obtained through distillation and fractionation of BEO, were kindly provided by “Baller s.r.l.” (Messina, Italy). The chemical characterization of both BEO and BEO-FF was performed by means of gas chromatography-mass spectroscopy (GC-MS) and high-performance liquid chromatography (HPLC), as previously described (Costa et al., 2010).

3.2. Cell cultures and treatments

Human neuroblastoma cell line SH-SY5Y was obtained originally from ATCC (Rockville, MD, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Acute leukemic lymphoblasts CCRF-CEM and its doxorubicin-resistant counterpart CEM-ADR5000 were kindly provided by Prof. Thomas Efferth of Johannes Gutenberg University Mainz (Germany). CCRF-CEM were cultured in the same manner as for SH-SY5Y cells. CEM-ADR5000 cells, overexpressing P-glycoprotein, were maintained by adding 5 µg/mL doxorubicin in the medium. Each cell culture reagent was from Sigma Aldrich (Milan, Italy) or Gibco (Life Technologies, Monza, Italy).

BRG and 5-G-7-MOC (Figure 5) were from Extrasynthese (Genay, France) and stock solutions (50 mM) were prepared in dimethylsulfoxide (DMSO), whereas BEO and BEO-FF were diluted 1:1 in a 1:9 H_2O/DMSO solution. These were further diluted in culture media to obtain the working concentrations.



Bergamottin (BRG)

5-geranayloxy-7-methoxycoumarin (5-G-7-MOC)

Figure 5. Molecular structures of bergamottin (BRG) and 5-geranayloxy-7-methoxycoumarin (5-G-7-MOC).

3.3. Cell viability and proliferation assays

In order to evaluate the anti-proliferative activity of BEO and BEO-FF as well as BRG and 5-G-7-MOC, we employed the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) test (Askari et al., 2019; Morisi et al., 2007) and resazurin assay (Kuete et al., 2017), as indexes of mitochondrial functionality, as well as the 5-bromo-2′-

deoxyuridine (BrdU) assay, as marker of DNA incorporation (Giofre et al., 2015). The IC50 of each treatment was calculated as non-linear regression using GraphPAD Prism 6 software.

3.3.1. MTT test

The MTT test was performed seeding the SH-SY5Y cells in 96-well plates at a density of $5x10^3$ cells/well. After 24 h, cells were treated with media containing the desired concentrations of BRG and 5-G-7-MOC (from 3.125 to 100 µM); in untreated cultures, only the medium was changed. Then, the plates were incubated for further 24, 48 and 72h, centrifuged and media changed with fresh one containing 0.5 mg/mL of MTT. Plates were left for 4 h to incubate until crystals of formazan formed. The supernatants were removed after centrifugation, and crystals were solubilized in 100 µl of 0.1 N HCl/isopropanol lysis solution. The absorbance was recorded by a microplate spectrophotometer at 570 nm with reference at 630 nm (iMark™ microplate reader, Bio-Rad Laboratories, Milan, Italy). Results of MTT assay were extrapolated as percentage of cell viability respect to untreated cells.

3.3.2. BrdU incorporation assay

For the BrdU assay, as marker of DNA incorporation, we employed a commercial kit (Merck Millipore, Darmstadt, Germany), following manufacturer's guidelines, as reported. Cells were exposed to BrdU for 2 h after treatments, then fixed and washed, prior adding the peroxidase-

conjugated anti-BrdU antibody. Lastly, we added the substrate and stopped the reaction to detect results with a microplate spectrophotometer at 450 nm (iMark™ microplate reader, Bio-Rad Laboratories, Milan, Italy). Similar to MTT, results were extrapolated as percentage of cell proliferation respect to untreated cells.

3.3.3. Resazurin assay

For the resazurin reduction assay, cells were seeded at a concentration of $1x10^4$ cells per well in 96-well plates, and then subjected to a panel of concentrations of both BEO and BEO-FF (from 0.5% to 0.001% v/v). After 72 h, 20 µl of resazurin (0.01%; Sigma-Aldrich) was added and plates incubated for 4 h. The fluorescence of resorufin, reduced form of resazurin, was measured by an Infinite M2000 Pro TM plate reader (ex. 544 nm, em. 590 nm; Tecan, Germany). Cell viability was expressed as percentage of cell proliferation respect to untreated cells.

3.4. Cytofluorimetric analyses

The fluorescence-activated cell sorting (FACS) was used to assess the role of the tested compounds in the induction apoptosis or interference with cell cycle progression (Celano et al., 2015; Cirmi et al., 2019).

3.4.1. Annexin V/propidium iodide staining

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining is an acknowledged procedure to measure whether apoptotic machinery is

involved, given the great affinity of annexin to phosphatidylserine exposed in early stages of apoptosis and that of PI, able to go through non-intact membranes, to the DNA. Briefly, $1x10^4$ SH-SY5Y cells were seeded in 6-well plates to adhere. The next day, cells were treated with 25 and 50 µM of BRG and 5-G-7-MOC for further 24 and 48 h. Subsequently, cells were collected by trypsinization, washed with PBS and resuspended in the binding buffer, following kit guidelines (eBioscience, Thermo Fisher Scientific). After, each sample was resuspended in 200 µl binding buffer solution made with 5 µL of Annexin-V-FITC and 10 µL of PI, gently vortexed and incubated at room temperature in darkness for 15 min. Samples were run on a Novocyte 2000 cytofluorimeter (ACEA Biosciences Inc., San Diego, California, USA). Doxorubicin 0.5 µM was employed as positive control.

Similarly, CCRF-CEM and CEM/ADR5000 cells were seeded at $1x10^6$ cells in 6-well plates and treated for 24 h with both BEO and BEO-FF at various fractions of the IC50s obtained in resazurin assay (i.e., 1/8 or 0.125, 1/4 or 0.25, 1/2 or 0.5 and full IC50). The protocol for the Annexin V-FITC/PI staining was the same as for SH-SY5Y cells. Therefore, cells were washed with PBS, resuspended in binding buffer and stained with Annexin V and PI. Samples were read using a BD Accuri™ C6 (Becton-Dickinson).

3.4.2. PI staining

PI stoichiometrically binds to DNA allowing its precise quantification and the discrimination of cell phases. SH-SY5Y cells were seeded at a density

of $1x10^4$ cells in 6-well plates, treated accordingly for 48 h with 25 and 50 µM of both BRG and 5-G-7-MOC. Then, cells were harvested, washed with PBS and resuspended in ice-cold 70% EtOH, while gently vortexed. After 24 h at 4°C, cells were washed twice to remove any trace of EtOH and RNA was digested by RNase (10 mg/ml in PBS) at 37°C for 1h. In conclusion, 10 µl of PI (1 mg/ml; Sigma-Aldrich, Milan) were added to samples and immediately acquired by flow cytometry. Doxorubicin 0.5 µM was employed as positive control.

As for apoptosis evaluation, also for cell cycle analysis the protocol employed for CCRF-CEM and CEM/ADR5000 cells was the same as for SH-SY5Y line. Briefly, leukemic cells were seeded at $1x10^6$ cells in 6-well plates and treated for 24 h with several concentrations of both BEO and BEO-FF, like for apoptosis, washed with PBS, resuspended in ice-cold 70% EtOH, and kept at 4°C overnight. Cells were then washed, and RNA was digested enzymatically at 37°C for 1 h. Ten µl of PI (1 mg/ml) were added to samples, and immediately acquired by BD Accuri™ C6 (Becton-Dickinson).

3.5. Determination of redox balance

The reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi m$) were measured fluorometrically as markers of oxidative stress (Cirmi et al., 2021). SH-SY5Y cells were seeded in 96-well plates at a density of $5x10^4$ cells/well and, the next day, treated with increasing concentrations of BRG and 5-G-7-MOC (1-50 µM) for 24h. The quantification of ROS was gained employing the probe 2′,7′-

dichlorodihydrofluorescein diacetate (DCFH-DA; 25 µM; Sigma Aldrich), while the evaluation of ΔΨm by rhodamine 123 (R123; 10 µM; Sigma Aldrich) as reported (Ferlazzo et al., 2015). The fluorescence was acquired by a microplate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany; 485 nm Ex. and 535 nm Em. for DCFH-DA; 488 nm Ex. and 525 nm Em. for R123).

3.6. Gene expression studies

To evaluate gene expression, SH-SY5Y cells were seeded in 100 mm Petri dishes at a density of 1×10^6 cells/dish and, after 24 h, treated with BRG and 5-G-7-MOC (25 and 50 µM) for 24h. Doxorubicin 0.5 µM was used as positive control. The following day, total RNA was extracted using TRIzol reagent, according to the manufacturer's protocol. Then, equal amounts of extracted RNA (2 µg) were reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Thermo Fisher, Foster City, USA). The mRNA levels of B-cell lymphoma, B-cell lymphoma 2 (Bcl-2), (Bcl-2)-associated X protein (Bax), Bcl-Xl, p53 and caspase (CASP) 3 and 9 were assessed by real-time PCR (qPCR). The reactions were performed in a 96-well plate in 20 µL containing 1× SYBR® Premix Dimer Eraser™ (TaKaRa Bio Inc., Japan), 0.1 µM specific primers and 25 ng RNA converted into cDNA. The qPCR was carried out in a 7300 qPCR System (Applied Biosystems, Thermo Fisher) with the following program: one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. A standard dissociation stage was added to assess the primer specificity. β-Actin was used as the housekeeping control. The collected

data were evaluated using the $2^{-\Delta\Delta CT}$ relative quantification method (Maugeri et al., 2019b). The primer sequences are listed in the Table 1.

Table 1. Sequences of oligonucleotide primers used for real-time PCR.

Gene	NCBI Ref. Seq.	Primer sequence
p53	NM_000546.6	Forward: 5′-GTGTGGAGTATTTGGATGAC-3′ Reverse: 5′-ATGTAGTTGTAGTGGATGGT-3′
Bax	NM_138764.5	Forward: 5′-GGACGAACTGGACAGTAACATGG-3′ Reverse: 5′-GCAAAGTAGAAAAGGGCGACAAC-3’
Bcl-2	NM_000657.3	Forward: 5′-ATCGCCCTGTGGATGACTGAG-3′ Reverse: 5′-CAGCCAGGAGAAATCAAACAGAGG-3′
Bcl-XL	NM_138578.3	Forward: 5’-CGGTACCGGCGGGCATTCAG-3’ Reverse: 5’-CGGCTCTCGGCTGCTGCATT-3’
CASP3	NM_004346.4	Forward: 5’-AGCACCTGGTTATTATTCTTGG-3’ Reverse: 5’-GCTTGTCGGCATACTGTT-3’
CASP9	NM_001229.5	Forward: 5’-GCTCAGACCAGAGATTCG-3’ Reverse: 5’-ATCCTCCAGAACCAATGTC-3’
β-actin	NM_001101.5	Forward: 5′-TTGTTACAGGAAGTCCCTTGCC-3′ Reverse: 5′-ATGCTATCACCTCCCCTGTGTG-3′

3.7. Protein expression studies

The protein levels of apoptosis-related proteins were evaluated by Western blot. Briefly, SH-SY5Y cells were seeded at a density of 1×10^6 cells/dish in 100 mm Petri dishes, allowed to adhere overnight and then cells were treated with BRG and 5-G-7-MOC (25 and 50 µM) for 24h. Doxorubicin 0.5 µM was used as positive control. Total protein content was evaluated as previously described (Celano et al., 2015), using a Bio-

Rad Protein Assay (Bio-Rad Laboratories) and bovine serum albumin as standard. Proteins (30 μg/lane) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred on polyvinylidene difluoride membrane (PVDF; Merck Millipore). To block non-specific binding sites, 5% non-fat milk for 1h was employed. Next, membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-Bcl-2 (Santa Cruz Biotechnology, Segrate, Milan, Italy); rabbit anti-Bax (GeneTex, Irvine, USA) and rabbit anti-Bcl-XL, anti-p53, anti-CASP3 and anti-CASP9 (Cell Signaling Technology, Danvers, USA); rabbit anti-β-actin (Sigma-Aldrich). Membranes were washed thrice and incubated with horseradish peroxidise-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1 h. Chemiluminescence of protein bands was obtained employing Luminata Forte Western HRP Substrate (Merck Millipore) and quantified with C-Digit Blot Scanner (Li-COR Bioscience, USA). The protein expression was quantified using Image Studio software (Li-COR Bioscience, USA). The β-actin was used as housekeeping protein.

3.8. Drug combination treatments and analysis of synergistic effect

The effects of the combination between BRG and 5-G-7-MOC was assessed through the checkboard method by which SH-SY5Y cells, seeded in the same manner as for cell proliferation assays, were treated

with increasing concentrations (1-50 µM) of the abovementioned compounds combined at different ratios. Cell viability was assessed by MTT assay and results processed to determine their interactions.

Similarly, the effects of the combination of BEO or BEO-FF and doxorubicin in CCRF-CEM and CEM/ADR5000 cells were also evaluated. Therefore, cells were seeded in 96-well plates and treated with checkboard method of the EOs (20, 40, 60, 80 and 100% of IC50) and doxorubicin (0.1, 1, 10 and 100 µM) at different ratios. Cell viability was assessed by resazurin assay to proceed to determine their interactions.

Synergy scoring was determined using the SynergyFinder 2.0 software that exploits the ZIP calculation method, expressing the synergism as δ score. Positive δ values correspond to synergism, whereas negative ones to antagonism (Ianevski et al., 2020; Yadav et al., 2015).

3.9. Statistical analyses

One-way or two-way analysis of variance (ANOVA) were employed to analyse data, according to the assay, which are expressed as mean ± standard error of the mean (SEM). Multiple comparisons of the means of the groups were performed by the Tukey–Kramer test (GraphPAD Software). P values less than or equal to 0.05 were considered significant.

4. RESULTS

4.1. Chemical composition of BEO and BEO-FF

The quali-quantitative characterization of BEO showed that the volatile fraction, which represents the majority of the whole plant phytocomplex, is mainly composed by limonene, linalyl acetate, linalool, γ-terpinene and β-pinene (Table 2). Notably, the percentages of these components did not vary between BEO and BEO-FF, proving the effectiveness of the separation method. The non-volatile fraction of BEO, absent in BEO-FF, was mainly composed by the furocoumarins BRG and bergapten along with the coumarins citropten and 5-G-7-MOC (Table 2).

Table 2. Chemical composition of BEO and BEO-FF.

	BEO	**BEO-FF**
	Volatile fraction (%)	
Limonene	37,9	37,6
Linalyl acetate	36,0	34,4
Linalool	6,6	10,0
γ-Terpinene	5,3	5,6
β-Pinene	4,9	4,5
	Non-volatile fraction (ppm)	
BRG	20344	–
Bergapten	1815	–
Citropten	2119	–
5-G-7-MOC	1356	–

4.2. Cytotoxic activity of BRG and 5-G-7-MOC in SH-SY5Y neuroblastoma cell line

4.2.1. BRG and 5-G-7-MOC induce cytotoxic effects in SH-SY5Y cells

The effects on cell proliferation of BRG and 5-G-7-MOC were assessed by the MTT test (Figure 6A) and BrdU incorporation assay (Figure 6B).

As shown in Figure 6, both compounds were able to hamper viability of SH-SY5Y cell line significantly already at 25 µM, despite to different extent. In particular, BRG brough a significant reduction of cell proliferation at 72 h ($p<0.01$ and $p<0.001$ for MTT and BrdU, respectively), while 5-G-7-MOC already at 24 h ($p<0.01$ for both MTT and BrdU).

The IC50s extrapolated from the curves were 36.8 ± 3.8 µM for BRG and 46.9 ± 5.47 µM for 5-G-7-MOC at 72 h of treatment. In line of these results, 25 and 50 µM were chosen as concentrations for further studies. The percentage of DMSO (0.2%) present in the highest concentration (100 µM) served as vehicle control to confirm that no effect was caused by the solvent.

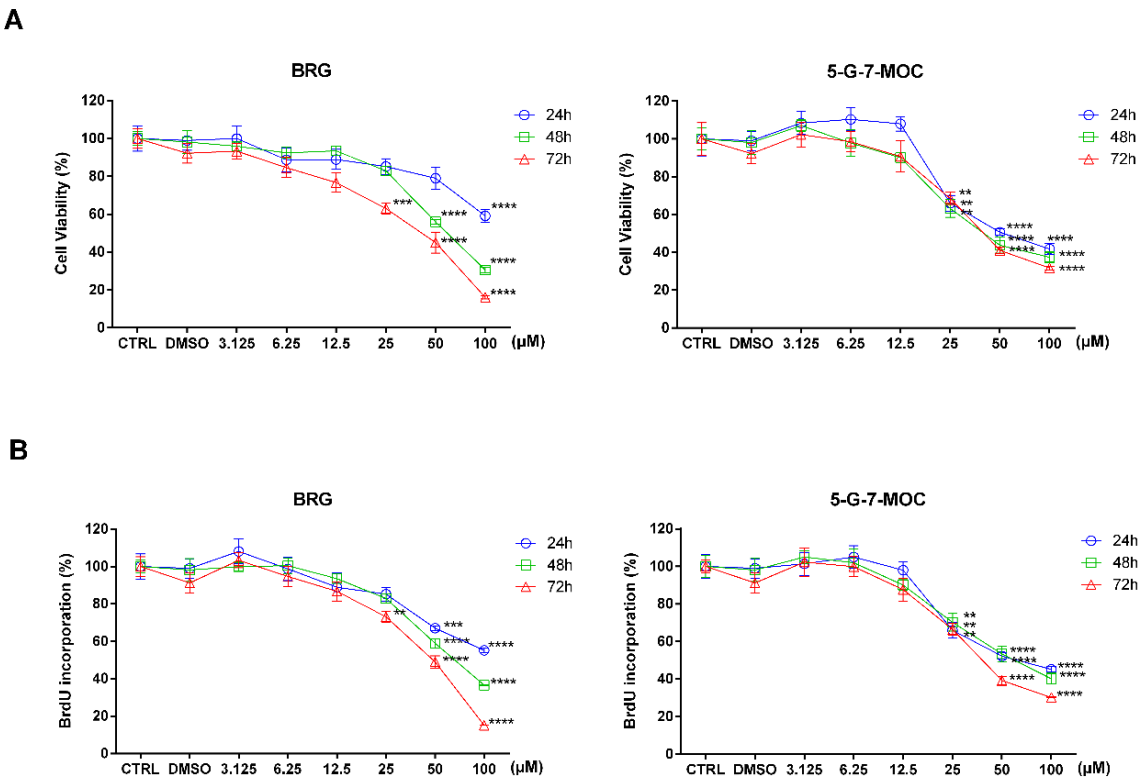


Figure 6. Effects of BRG and 5-G-7-MOC (3.125 to 100 µM) on SH-SY5Y neuroblastoma cell proliferation for 24-72 h. Viability rate was assessed by MTT test (A) and BrdU incorporation assay (B). Results are expressed as percentages ± SEM of the absorbance values detected in the control cells. Each concentration was tested eight-fold and three independent experiments were carried out (N=24). **p<0.01, ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

4.2.2. BRG and 5-G-7-MOC promote apoptosis in SH-SY5Y cells

In order to determine the type of cell death induced by BRG and 5-G-7-MOC in SH-SY5Y cells, Annexin V-FITC/PI cytofluorimetric assay was performed. The treatment with BRG at 25 and 50 µM increased the percentage of cells undergoing apoptosis, both early and late, up to 23.1%

and 29% (24 h) and up to 69.6% and 84.4% (48 h), respectively (Figure 7).

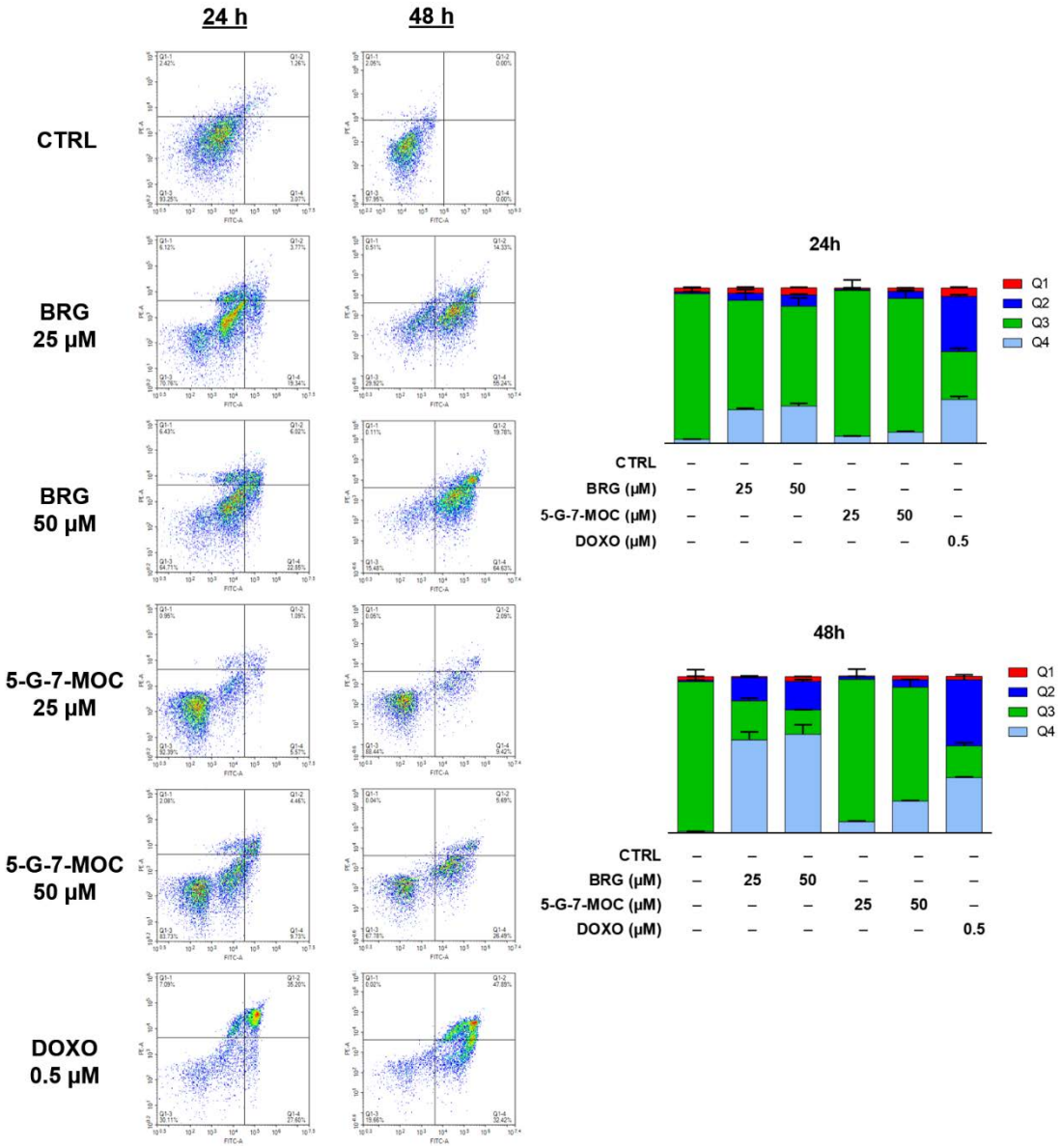


Figure 7. FACS analysis of apoptosis in SH-SY5Y cells exposed to BRG and 5-G-7-MOC. Representative Annexin V *vs* PI dot plots of the SH-SY5Y cells treated with 25 and 50 µM of both compounds and with 0.5 µM of doxorubicin (DOXO) for 24 and 48 h are displayed. Q3 contains the viable cells (Annexin V-/PI-), Q4 contains the cells in early apoptosis (Annexin V+/PI-), Q2 contains the cells in late apoptosis (Annexin V+/PI+), while Q1 contains the necrotic ones (Annexin V-/PI+). Histograms illustrate the percentages of cells present in the corresponding quadrants ± SEM of three experiments separately performed in triplicate (N=9).

The treatment of 5-G-7-MOC induced a slighter increase of apoptotic cells, compared to the other coumarin: 6.6% and 14.2% (24 h), while 11.5% and 32.2% (48 h) for 25 and 50 μM, respectively (Figure 7).

4.2.3. BRG and 5-G-7-MOC force SH-SY5Y cells out of the cell cycle

The PI assay was employed to assess the effect of BRG and 5-G-7-MOC on the cell cycle progression of SH-SY5Y cells.

Both compounds did not alter the ratio among G0/G1, S and G2/M phases, regardless of concentrations or testing times. Nevertheless, the treatment for 48h of SH-SY5Y cells with BRG and 5-G-7-MOC increased the percentage of cells in sub-G0/G1, phase where apoptotic cells gather and henceforward exiting the cell cycle, up to 2.7% and 8.3% for BRG 25 μM and 50 μM, respectively, and up to 1.8% and 4.2% for 5-G-7-MOC 25 μM and 50 μM, respectively (Figure 8).

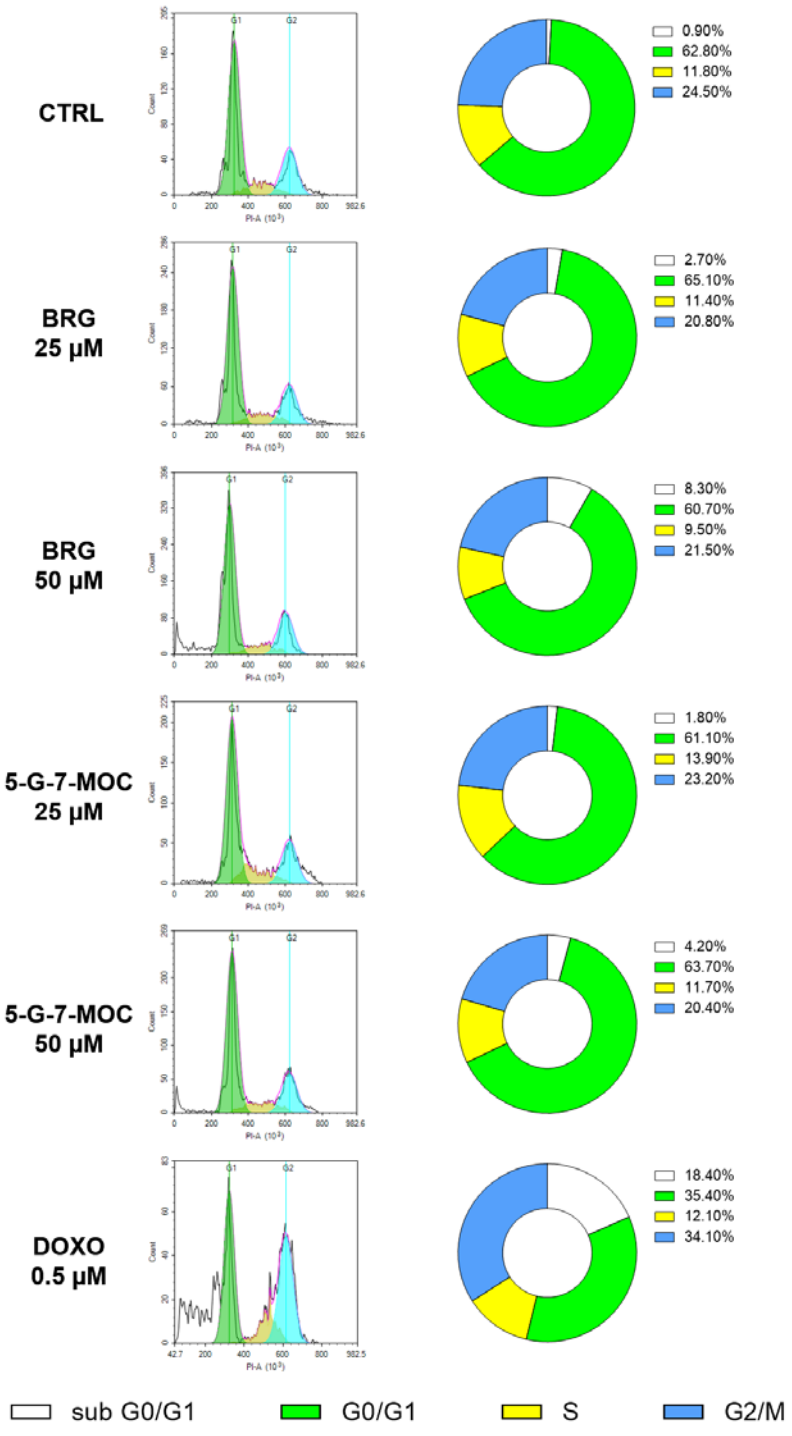


Figure 8. Influence of BRG and 5-G-7-MOC on cell cycle progression of SH-SY5Y cells. The effects of the treatment for 48h with BRG and 5-G-7-MOC at 25 and 50 µM and doxorubicin (DOXO) on cell cycle of SH-SY5Y cells were esteemed by the cytofluorimetric PI assay. The plots are representative of three different experimental sessions performed in triplicate (N=9). Percentages of cells present in each phase of the cell cycle are reported in the donut charts (sub G0/G1: white; G0/G1: green; S: yellow; G2/M: blue).

4.2.4. BRG and 5-G-7-MOC increase ROS production and impair ΔΨm

The production of ROS in SH-SY5Y cells exposed to BRG and 5-G-7-MOC at 25 and 50 µM for 24 h was evaluated employing the probe DCFH-DA, which is deacetylated in the cytosol and becomes fluorescent after interacting with intracellular radicals. As shown in Figure 9A, both compounds significantly increased ROS levels after 24 h of treatment in SH-SY5Y cells already at 5 and 10 µM for BRG and 5-G-7-MOC, respectively ($p<0.1$; Figure 9A).

The effect of the two coumarins on the ΔΨm was assessed by R123 staining. In compliance to the previous assay, BRG and 5-G-7-MOC significantly decreased the ΔΨm of treated SH-SY5Y from 5 and 10 µM for BRG and 5-G-7-MOC, respectively ($p<0.1$ and $p<0.01$, respectively; Figure 9B).

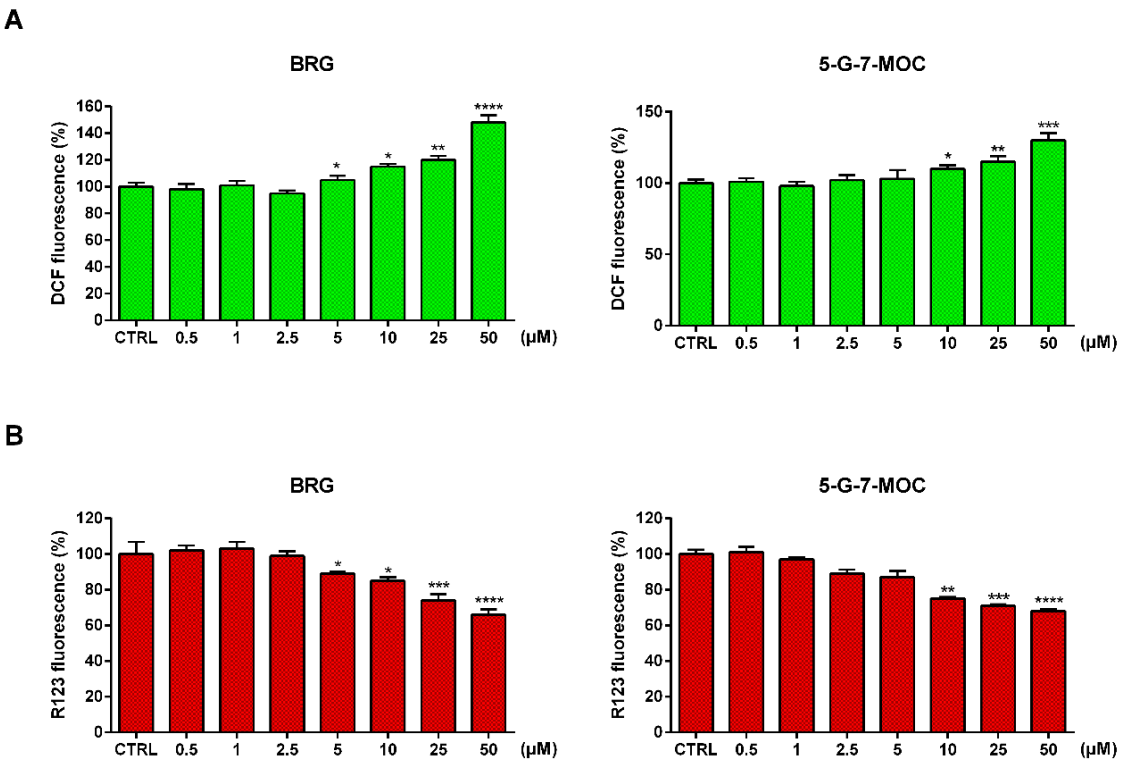


Figure 9. Levels of ROS and fall of ΔΨm in SH-SY5Y after treatment with BRG and 5-G-7-MOC. (A) ROS levels were assessed through the fluorescent probe DCFH-DA. (B) Variations of ΔΨm were evaluated through the cationic fluorochrome R123. Results of both analyses are expressed as percentages ± SEM of the fluorescence values detected in the control cells of three different experiments for eight replicates (N=24). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

4.2.5. BRG and 5-G-7-MOC modulate apoptosis-related factors in SH-SY5Y cells at both gene and protein level

In order to investigate the pathways involved in the anti-cancer effect of the two coumarins in SH-SY5Y cells, we evaluated both gene and protein levels of the most crucial factors linked to apoptosis. The treatment with BRG and 5-G-7-MOC at 25 and 50 μM for 24 h brought an increase of

gene expression of the pro-apoptotic BAX, p53 and both CASP9 and 3, as well as a decrease of Bcl-2 and Bcl-XL (Figure 10A). This result was significant for both BRG and 5-G-7-MOC 50 µM for p53, Bcl-2 and CASP9. The lowest concentration (25 µM), instead, reached a significant result only for BAX and CASP3 genes. These results reflected also at protein level, as assessed by Western blot (Figure 10B). The treatment of SH-SY5Y with both concentrations of BRG showed a significant increase of both BAX and p53 and a decrease of Bcl-2 and Bcl-XL protein levels. Moreover, the cleavage of pro-caspases 9 and 3 was significantly enhanced by BRG 50 µM, while only for CASP3, as regards to BRG 25 µM. Contextually, only the highest concentration of 5-G-7-MOC (50 µM) was able to provide a significant effect on the protein levels of all the factors here studied, except for Bcl-2 which was slightly decreased by 5-G-7-MOC treatment, yet not significantly.

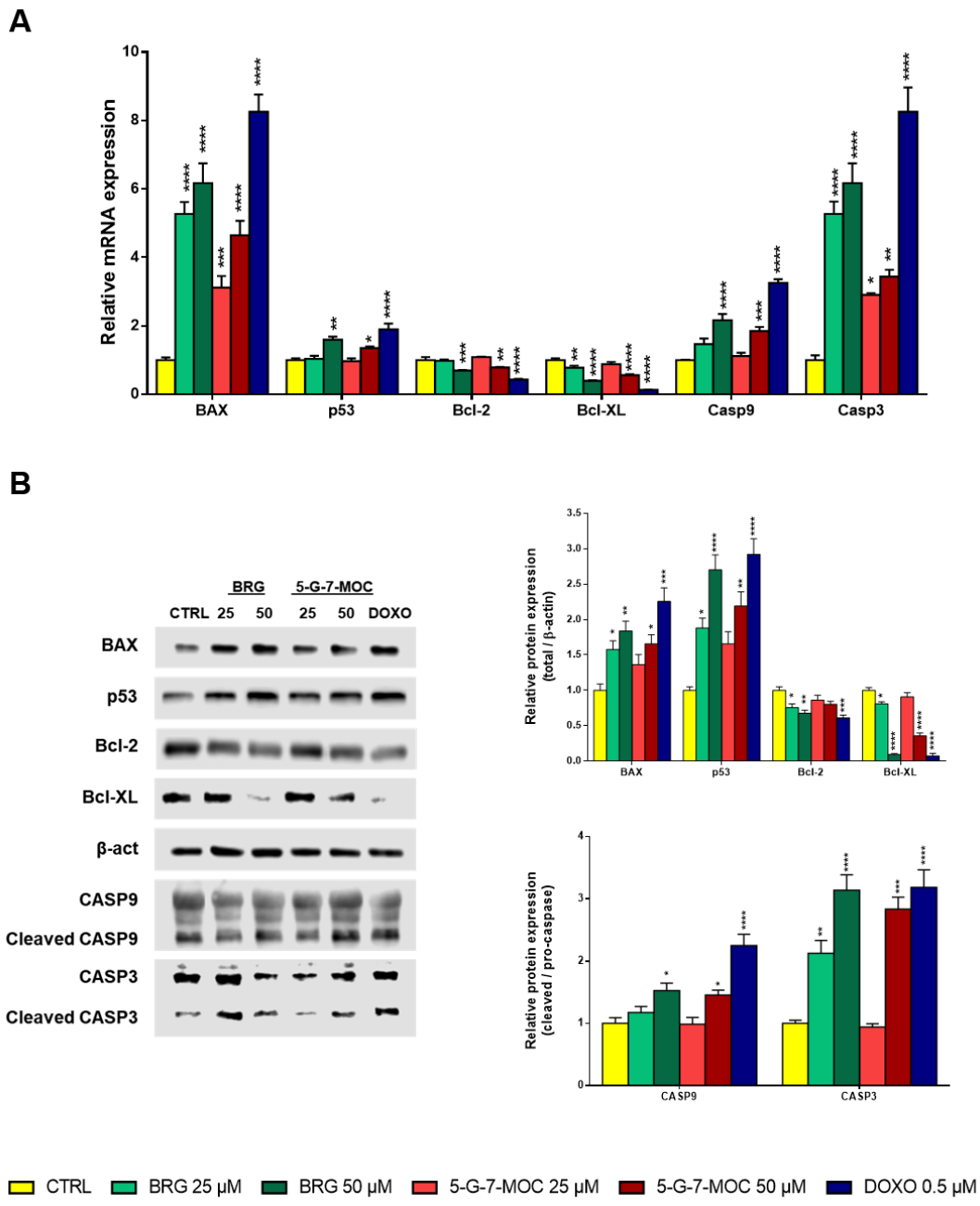


Figure 10. Apoptosis-related gene and protein levels modulation in SH-SY5Y cells treated with BRG and 5-G-7-MOC. Cells exposed to BRG and 5-G-7-MOC at 25 and 50 μM for 24h were processed for mRNA (A) and protein (B) expression studies. Relative quantities of mRNA were normalized to β-actin. Immunoblots of proteins are shown along with their densitometric analyses, on the right. The expression of BAX, p53, Bcl-2 and Bcl-XL was normalized against β-actin, while that of CASP3/9 against the relative zymogen. Results are expressed as fold change respect to untreated cells and expressed as mean ± SEM of three different sets of experiments performed in triplicate (N=9). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

4.2.6. BRG and 5-G-7-MOC synergistically cooperate to induce cytotoxic effects in SH-SY5Y cells

The synergism between the two coumarins was estimated testing their combination at different ratios and evaluating the inhibition of cell viability through MTT test, whose results have been processed by SynergyFinder 2.0 software (Figure 11).

The combination of BRG and 5-G-7-MOC proved to be overall synergistic with a Zero Interaction Potency (ZIP) score of 1.411, despite only for certain ratios of the two coumarins. Specifically, when the ratios of BRG/5-G-7-MOC were between 1:4 to 1:16, we observed the highest δ scores (δ = 4.52), and hence synergism (red area). Conversely, the treatment of SH-SY5Y cells with equimolar concentrations of BRG and 5-G-7-MOC or at a ratio of 2:1 led to a strong antagonistic effect (green area).

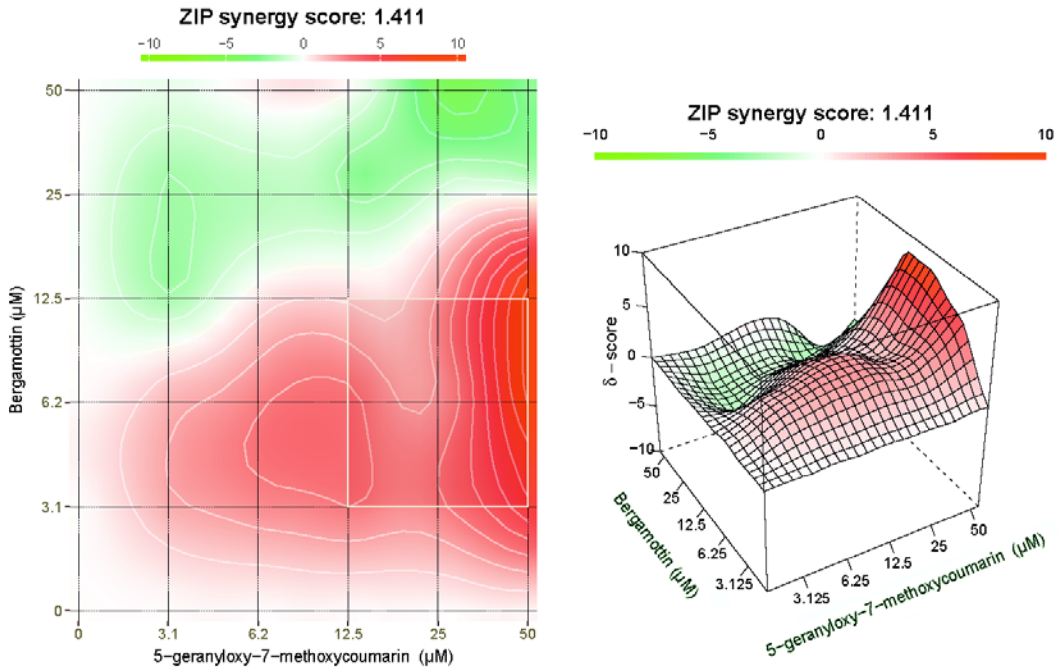


Figure 11. Synergistic effect of BRG and 5-G-7-MOC treatment in SH-SY5Y cells. Cell viability was assessed by MTT assay, and the effect of the drug combination was calculated and visualized using SynergyFinder 2.0 software through the ZIP reference model. The synergy score is expressed as the mean of all δ scores for the dose-response analysis, and the maps shown are representative of three different experimental sessions. The red, white, and green regions of the graph indicate the synergy, additivity and antagonism, respectively. The white square represents the highest synergistic area.

4.3. Anti-cancer effects of BEO and BEO-FF in CCRF-CEM and CEM/ADR5000 cell lines

4.3.1. BEO and BEO-FF reduce cell proliferation in CCRF-CEM and CEM/ADR5000 cells

The anti-proliferative activity of both BEO and BEO-FF was evaluated in CCRF-CEM leukemic cells and their doxorubicin-resistant counterpart CEM/ADR5000 by means of resazurin assay, (Figure 12).

As shown in Figure 12, both EOs hindered cell viability of both leukemic cell lines in a superimposable manner. Notably, CCRF-CEM cells were slightly more sensitive to the furocoumarin-free fraction of BEO, while the resistant ones to the whole BEO, despite both fractions significantly reduced cell proliferation already at 0.031% ($p<0.001$ and $p<0.0001$). The IC50s extrapolated from the curves were in CCRF-CEM cells 0.19 ± 0.05 % and 0.13 ± 0.03% for BEO and BEO-FF, respectively, whereas in CEM/ADR5000 cells the values were 0.11 ± 0.03 % and 0.18 ± 0.04% for BEO and BEO-FF, respectively, at 72 h of treatment. Further studies were performed based on these IC50 results.

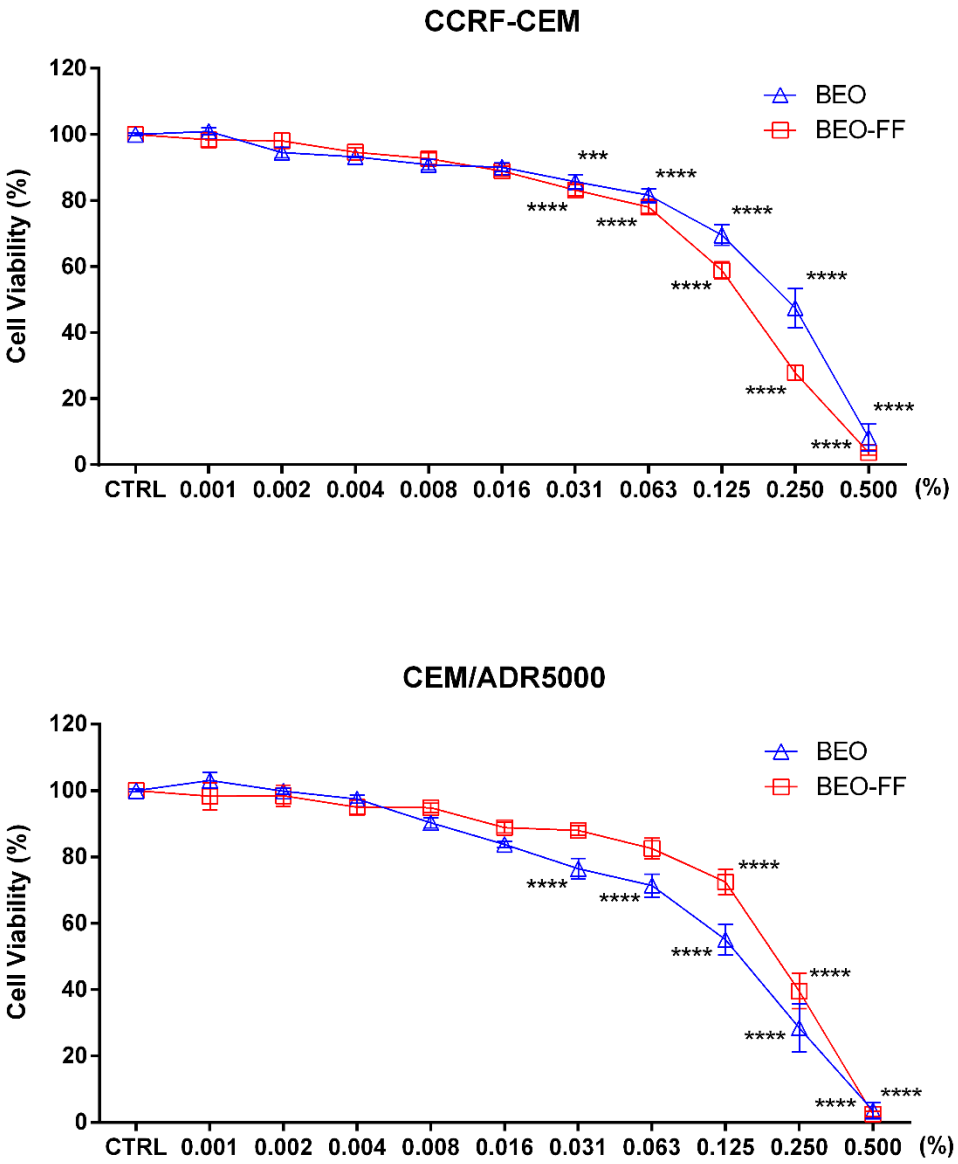


Figure 12. Effects of BEO and BEO-FF (0.001 to 0.5%) on cell proliferation of CCRF-CEM and CEM/ADR5000 cell lines for 72 h. Viability rate was assessed by resazurin assay. Results are expressed as percentages ± SEM of the fluorescence arbitrary units detected in the control cells. Each concentration was tested six-fold and three independent experiments were carried out (N=18). ***p<0.001 and ****p<0.0001 *vs* control (CTRL).

4.3.2. BEO and BEO-FF induce apoptosis in CCRF-CEM and CEM/ADR5000 cells

The treatment of CCRF cells with BEO and BEO-FF at concentrations spanning from 1/8 of the IC50 to IC50 for 48 h led to an increase of the percentage of apoptotic events, both early and late, only at the highest concentrations (Figure 13).

In detail, BEO treatment (IC50) augmented necrotic, late and early apoptotic cells up to 30.3%, 29.1% and 9.1%, respectively, whereas BEO-FF treatment (IC50) up to 5.1%, 4.1% and 6.8%, respectively (Figure 12). In parallel, the treatment of CEM/ADR5000 cells with BEO for 48 h did not induce any significant outcome at none of the concentration tested. Contrariwise, BEO-FF (IC50) increased necrotic, late and early apoptotic cells up to 14.9%, 18.3% and 14.8%, respectively, in this cell line (Figure 14).

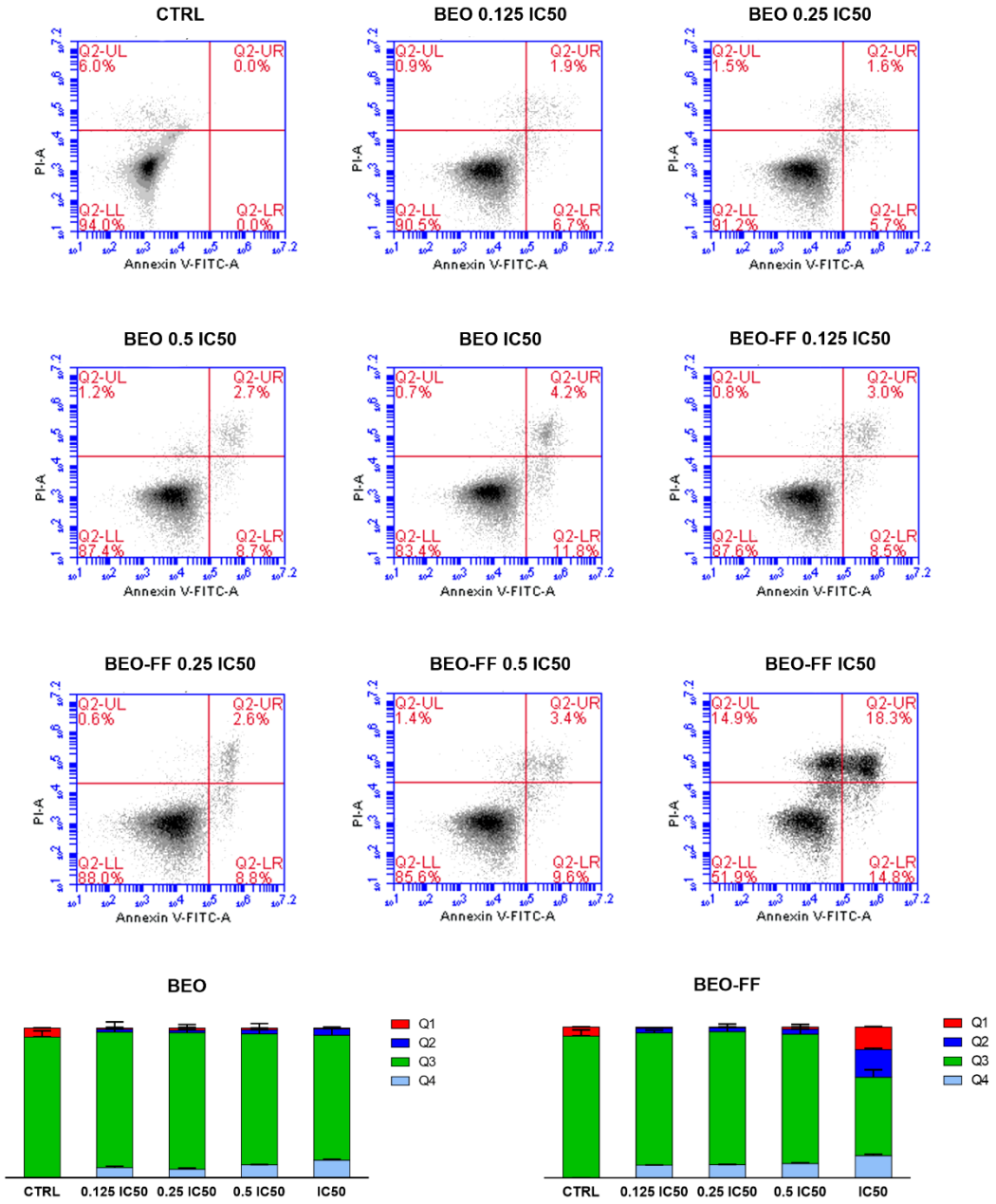


Figure 13. Detection of apoptosis in CCRF-CEM cells exposed to BEO and BEO-FF for 24 h. Apoptosis was assessed by means of the Annexin V-FITC/PI test. Representative Annexin V *vs* PI dot plots of the CCRF-CEM cells treated with the indicated concentrations of both essential oils for 48 h are displayed. Q3 contains the viable cells (Annexin V-/PI-), Q4 contains the cells in early apoptosis (Annexin V+/PI-), Q2 contains the cells in late apoptosis (Annexin V+/PI+), while Q1 contains the necrotic ones (Annexin V-/PI+). Histograms depict the percentages of cells present in the corresponding quadrants ± SEM of three experiments separately performed in triplicate (N=9).

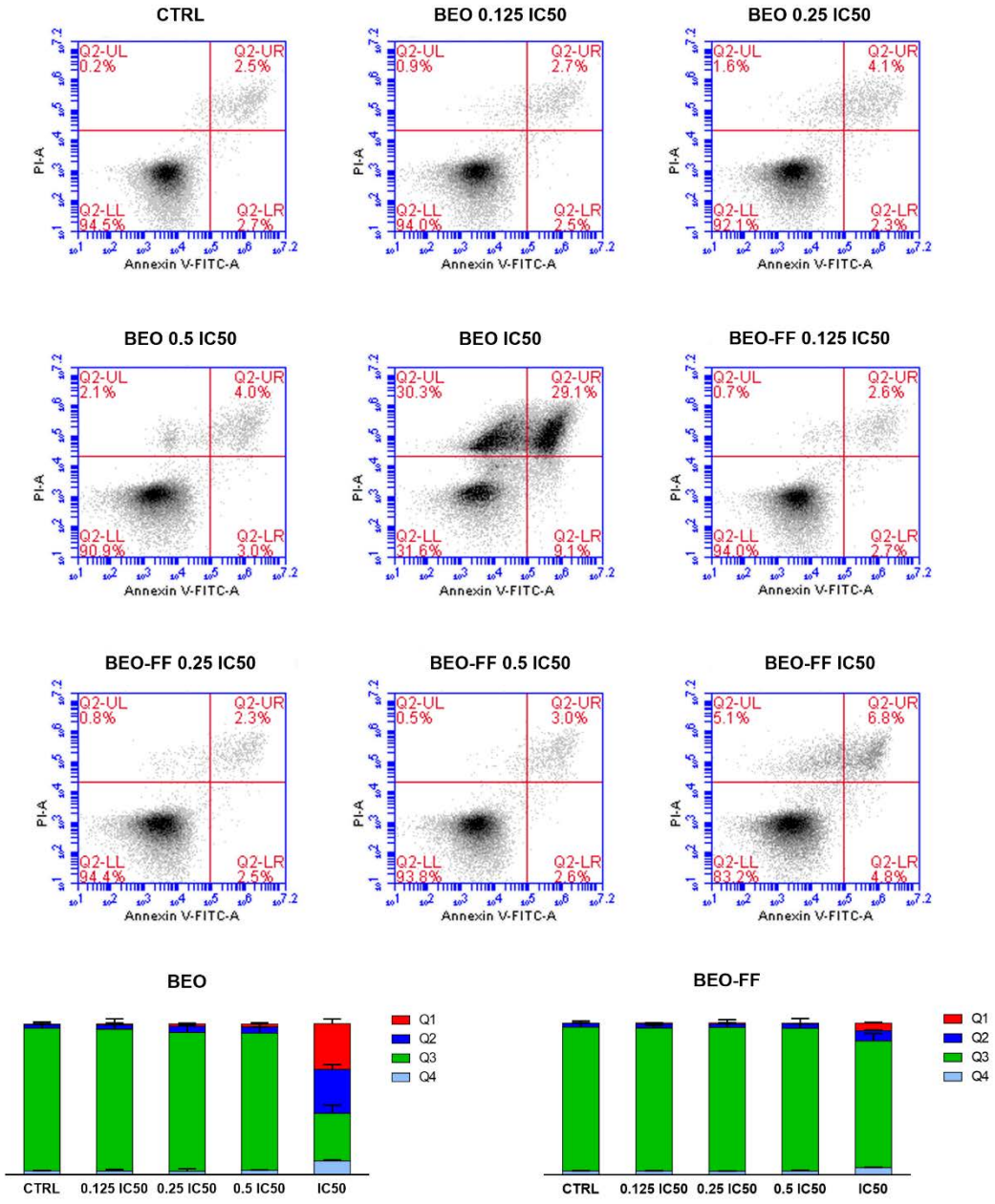


Figure 14 Detection of apoptosis in CEM/ADR5000 cells exposed to BEO and BEO-FF for 24 h. Apoptosis was assessed by means of the Annexin V-FITC/PI test. Representative Annexin V *vs* PI dot plots of the CCRF-CEM cells treated with the indicated concentrations of both essential oils for 48 h are displayed. Q3 contains the viable cells (Annexin V-/PI-), Q4 contains the cells in early apoptosis (Annexin V+/PI-), Q2 contains the cells in late apoptosis (Annexin V+/PI+), while Q1 contains the necrotic ones (Annexin V-/PI+). Histograms depict the percentages of cells present in the corresponding quadrants ± SEM of three experiments separately performed in triplicate (N=9).

4.3.2. BEO and BEO-FF alter cell cycle progression in CCRF-CEM and CEM/ADR5000 cells

The PI staining allowed to appreciate the effect of BEO and BEO-FF on cell cycle progression of both CCRF-CEM and CEM/ADR5000 cells. As shown in Figures 15 and 16, neither BEO nor BEO-FF altered the ratio among G0/G1, S and G2/M phases. However, the treatment for 24h of CCRF-CEM and CEM/ADR5000 cells with the two EOs increased the percentage of cells in sub-G0/G1, phase acknowledged to be populated by cells undergoing apoptosis, and hence exiting the cell cycle.

Specifically, only the highest concentration of BEO and BEO-FF was able to push out of the cell cycle up to 24.1% and 16.6% of CCRF-CEM cells, respectively (Figure 15).

In CEM/ADR5000 cells, the two bergamot fractions were able to increase cell population in the sub-G0/G1 phase already at half of the IC50 (17.9% and 10.9% for BEO and BEO-FF, respectively), and to a stronger extent for the highest concentration tested (32.3% and 12.3% for BEO and BEO-FF, respectively; Figure 16).

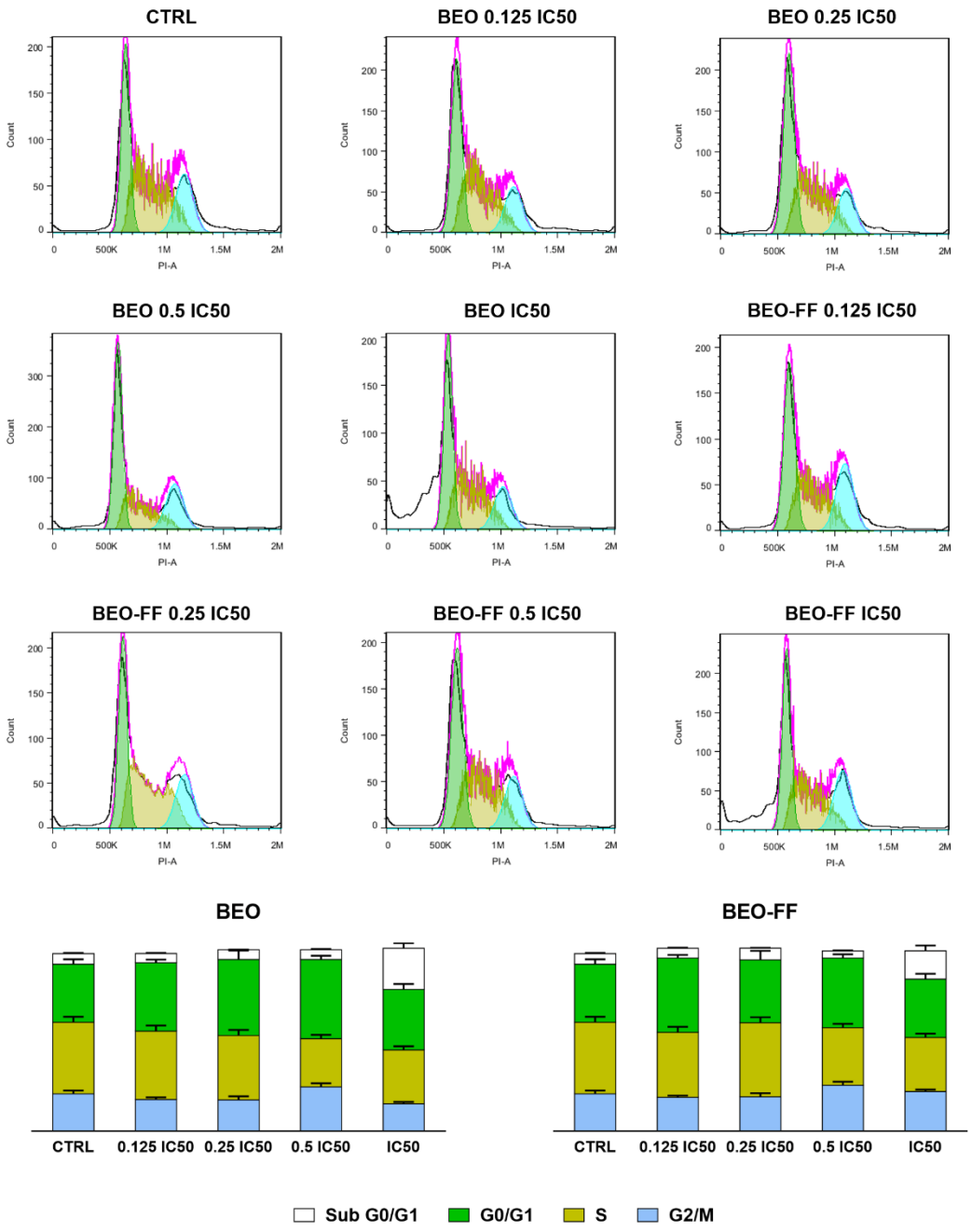


Figure 15. Influence of BEO and BEO-FF in cell cycle progression of CCRF-CEM cells exposed after 24 h. By the cytofluorimetric assay of propidium iodide staining, the effect of the exposure of CCRF-CEM cells to BEO and BEO-FF for 24 h was assessed. The plots are representative of three different experiments performed in triplicate (N=9). Percentages of cells present in each phase of the cell cycle are reported ± SEM in the histograms (sub G0/G1: white; G0/G1: green; S: yellow; G2/M: blue).

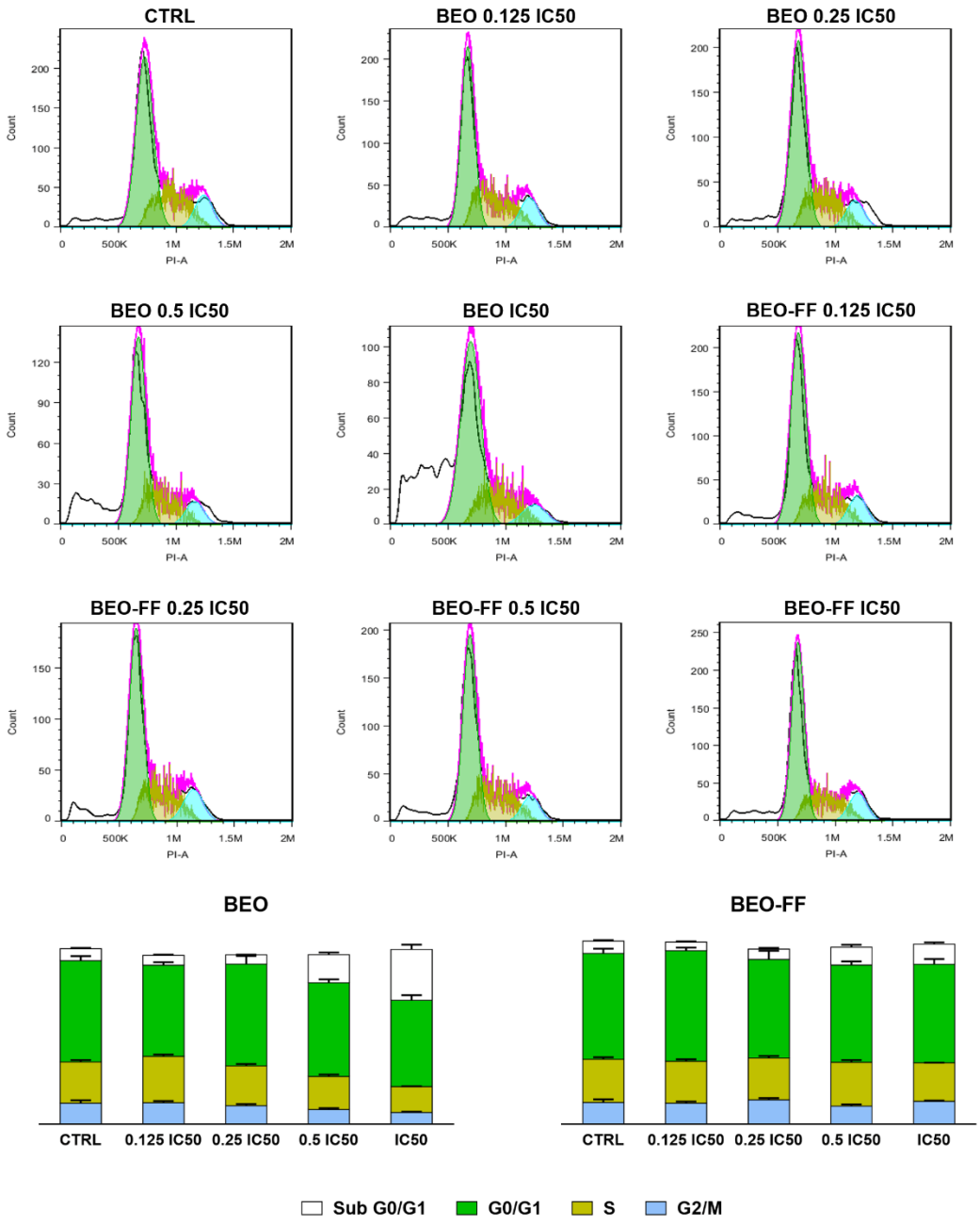


Figure 16. Influence of BEO and BEO-FF in cell cycle progression of CEM/ADR5000 cells exposed after 24 h. By the cytofluorimetric assay of propidium iodide staining, the effect of the exposure of ADR cells to BEO and BEO-FF for 24 h was assessed. The plots are representative of three different experiments performed in triplicate (N=9). Percentages of cells present in each phase of the cell cycle are reported ± SEM in the histograms (sub G0/G1: white; G0/G1: green; S: yellow; G2/M: blue).

4.3.3. BEO and BEO-FF modulate the response to doxorubicin in CCRF-CEM and CEM/ADR5000 cells

To investigate the role of BEO and BEO-FF in modulating the response of CCRF-CEM and CEM/ADR5000 cells towards doxorubicin, their combination at different ratios was evaluated in terms of inhibition of cell viability through resazurin assay. The obtained results have been processed by SynergyFinder 2.0 software.

In CCRF-CEM cells, the combination of BEO and doxorubicin proved to be overall synergistic with a ZIP score of 7.11, despite only for certain ratios (Figure 17). Conversely, when doxorubicin was combined with BEO-FF, a slight antagonism was found (δ = -0.59), effect strongly enhanced when both drugs were combined at the highest concentration (δ = -8.82; Figure 18).

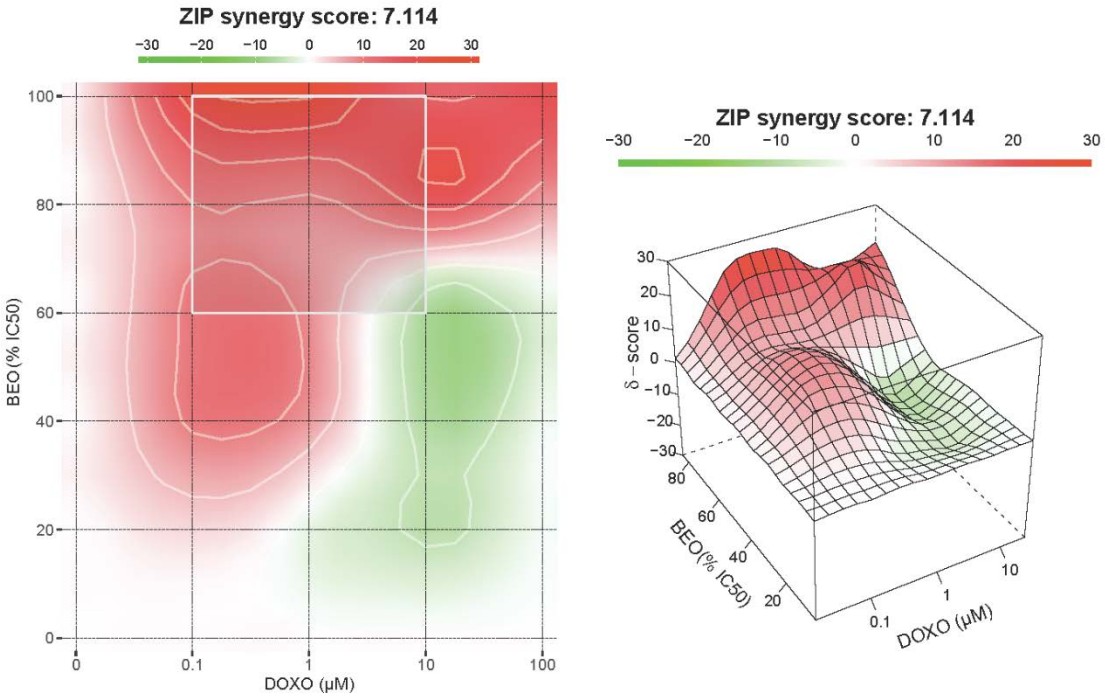


Figure 17. Synergistic effect of BEO and doxorubicin treatment in CCRF-CEM cells. Cell viability was assessed by resazurin assay and the effect of the combination between BEO and doxorubicin (DOXO) was calculated and visualized using SynergyFinder 2.0 software through the ZIP reference model. The synergy score is expressed as the mean of all δ scores for the dose-response analysis, and the maps shown are representative of three different experimental sessions. The red, white and green regions of the graph indicate the synergy, additivity and antagonism, respectively. The white square represents the highest synergistic area.

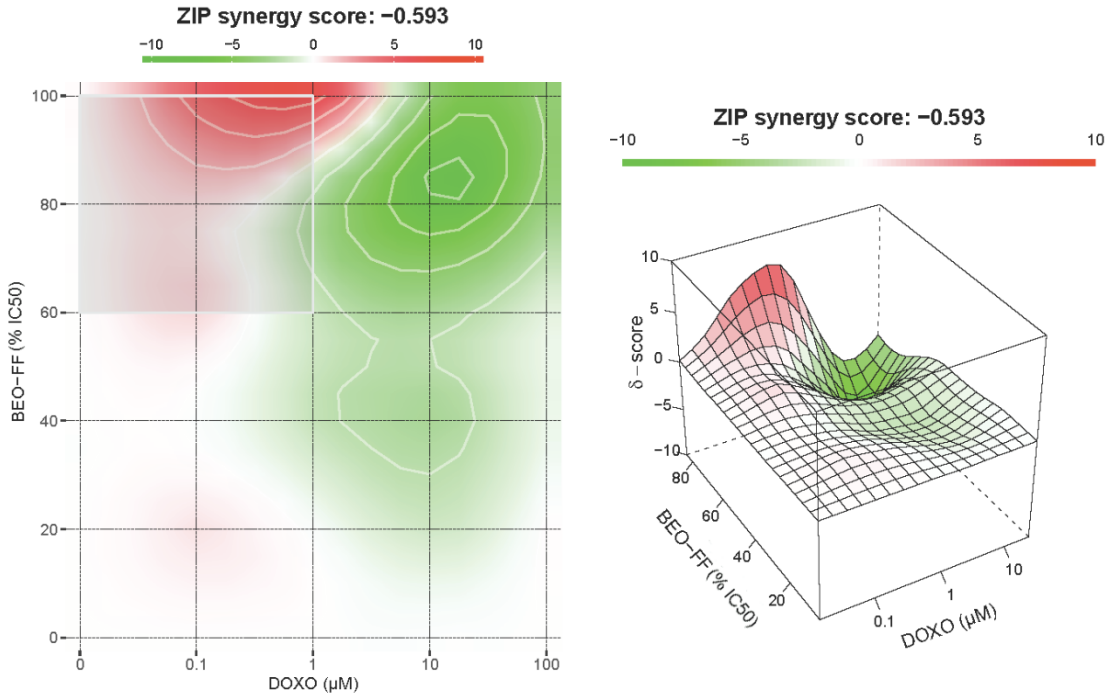


Figure 18. Synergistic effect of BEO-FF and doxorubicin treatment in CCRF-CEM cells. Cell viability was assessed by resazurin assay and the effect of the combination between BEO-FF and doxorubicin (DOXO) was calculated and visualized using SynergyFinder 2.0 software through the ZIP reference model. The synergy score is expressed as the mean of all δ scores for the dose-response analysis, and the maps shown are representative of three different experimental sessions. The red, white and green regions of the graph indicate the synergy, additivity and antagonism, respectively. The white square represents the highest synergistic area.

In CEM/ADR5000 cells, the results of the combination between BEO and doxorubicin showed interesting results, with an overall ZIP score of 11.49. Specifically, the strongest synergy was achieved when doxorubicin was tested at 0.1-1 μM together with BEO at 20-80% of IC50, with the highest peak of synergy (δ = 14.49) at 20% of IC50 (Figure 19). The combination of BEO-FF and doxorubicin, instead, proved to be far less synergistic than the whole counterpart, with a ZIP score of 2.71. Nevertheless, when BEO-

FF was combined at half of the IC50, it was clear a stronger synergistic effect for all concentration of doxorubicin tested, with a peak of ZIP score 8.15 (Figure 20).

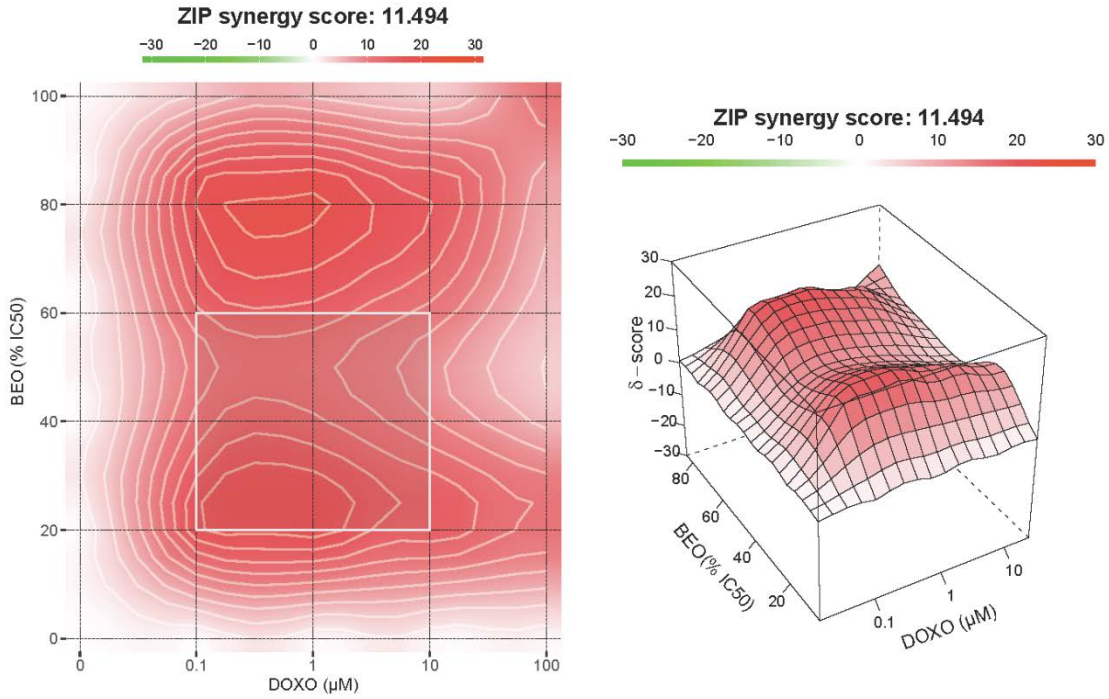


Figure 19. Synergistic effect of BEO and doxorubicin treatment in CEM/ADR5000 cells. Cell viability was assessed by resazurin assay and the effect of the combination between BEO and doxorubicin (DOXO) was calculated and visualized using SynergyFinder 2.0 software through the ZIP reference model. The synergy score is expressed as the mean of all δ scores for the dose-response analysis, and the maps shown are representative of three different experimental sessions. The red, white and green regions of the graph indicate the synergy, additivity and antagonism, respectively. The white square represents the highest synergistic area.

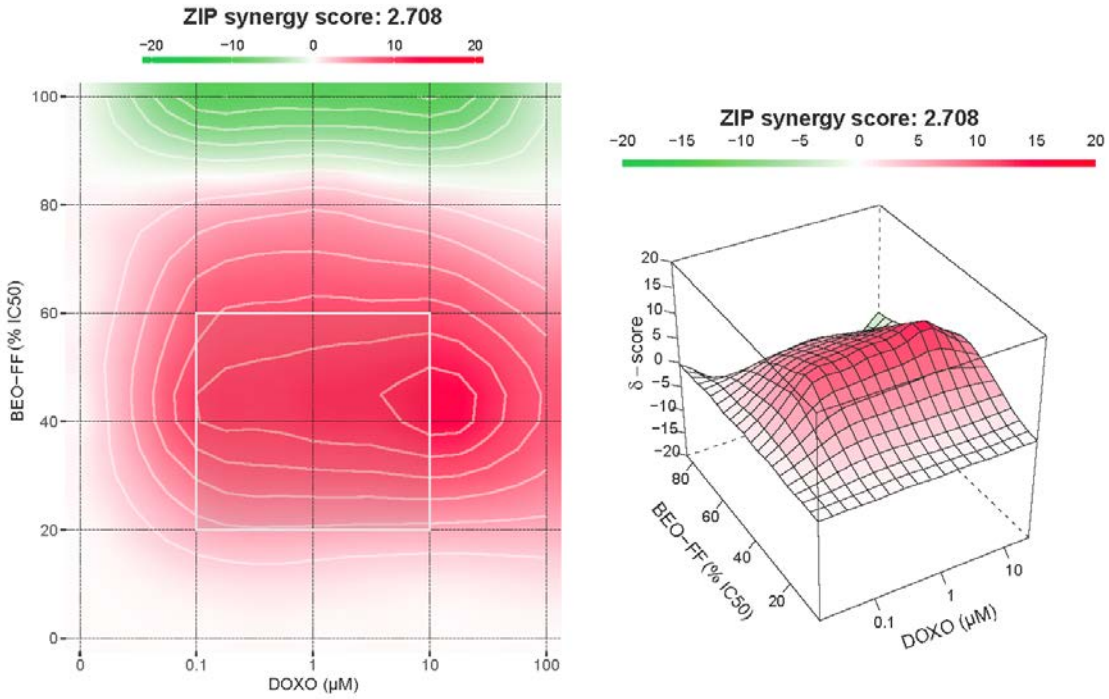


Figure 20. Synergistic effect of BEO-FF and doxorubicin treatment in CEM/ADR5000 cells. Cell viability was assessed by resazurin assay and the effect of the drug combination between BEO-FF and doxorubicin (DOXO) was calculated and visualized using SynergyFinder 2.0 software through the ZIP reference model. The synergy score is expressed as the mean of all δ scores for the dose-response analysis, and the maps shown are representative of three different experimental sessions. The red, white and green regions of the graph indicate the synergy, additivity and antagonism, respectively. The white square represents the highest synergistic area.

5. DISCUSSION

Citrus fruits have been exploited to improve human health over the centuries, thanks to the extraordinary variety of compounds they offer and which make these fruits such precious allies to fight or prevent different illnesses. From the parts these fruits are composed of, different active principles are obtained. Specifically, in the flavedo (i.e., peel), where the essential oil is obtained from, monoterpenes and coumarins are present; in the *albedo* (i.e. the intermediate spongy white portion), polyphenols are in abundance as well as in the endocarp (i.e., pulp), where the juice comes from, which is plenty of other micronutrients (Lv et al., 2015).

Among the great variety of *Citrus* fruits present worldwide, *Citrus bergamia* Risso & Poiteau has drawn the attention of the scientific community for the pharmacological features of its juice and EO (Cirmi et al., 2016a; Cirmi et al., 2016c; Cirmi et al., 2017; Ferlazzo et al., 2016a; Maugeri et al., 2019a; Musumeci et al., 2020). For its acknowledged pharmacological relevance, BEO was the main focus of my PhD project.

The first step was performed at Baller s.r.l. (Italy), where we chemically characterized both BEO and BEO-FF, which showed a chemical profile very similar to what was previously reported (Costa et al., 2010). These data demonstrated the reliability of the process employed to obtain a coumarin-less fraction because the monoterpene fingerprint remained untouched, while levels of coumarins were undetectable.

The second part of my PhD project was performed at the University of Messina (Italy) and was based on the assumption that BEO was able to induce anti-proliferative effect in human neuroblastoma SH-SY5Y cells as

whole, along with fractions of BEO deprived of both furocoumarins or terpenes which proved to be almost as effective as the whole BEO (Navarra et al., 2015). This part was carried out at the University of Messina (Italy). Focusing at the quali-quantitative composition of those fractions, we saw that linalool and linalyl acetate, among monoterpenes, as well as BRG and 5-G-7-MOC, among coumarins, were in common among the three fractions. Although it was previously claimed that those monoterpenes were inactive in SH-SY5Y cell line regardless the high concentrations employed (Russo et al., 2013), many other studies reported the effects of linalool and linalyl acetate in different experimental models (Ashrafizadeh et al., 2019; Itani et al., 2008; Jia et al., 2013; Koziol et al., 2014; Navaei Shoorvarzi et al., 2020; Prashar et al., 2004; Wojtunik-Kulesza et al., 2019).

In this line, these monoterpenes were assessed at the concentration present in the BEO at the highest concentration tested previously (Navarra et al., 2015). In a preliminary set of experiments, it was proved that just after 24h they showed a strong anti-proliferative activity alone and in combination, without noticing any noteworthy difference (Figure 21). From this, it was deduced that these monoterpenes were definitely the major players in the anti-proliferative activity of BEO.

In parallel, when BRG and 5-G-7-MOC were tested at the concentration present in BEO (0.03%) for 24h, no significant anti-proliferative effect either alone or in combination together was seen. Interestingly, when linalool, limonene or linalyl acetate were put together with the mixture of

coumarins, the cytotoxic effect increased respect to the combination of monoterpenes alone (Figure 21).

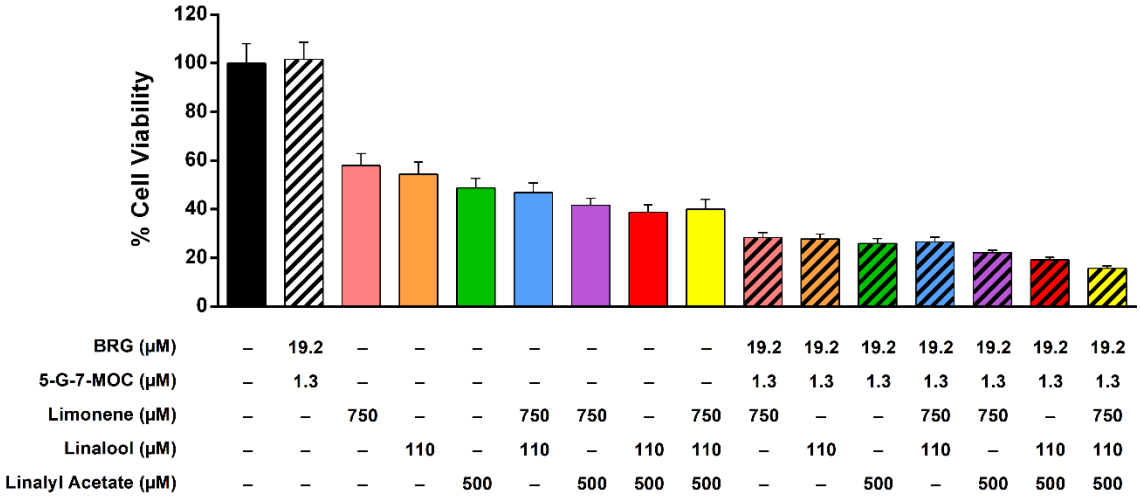


Figure 21. Effects on SH-SY5Y cell proliferation after the exposure for 24 h to limonene, linalool and linalyl acetate alone or in combination with BRG and 5-G-7-MOC. Viability was assessed by MTT test and results are expressed as percentages ± SEM of the absorbance values detected in the control cells (black bar). Each concentration was tested eight-fold and three independent experiments were carried out.

This confirmed the initial hypothesis on their relevant role in the anti-proliferative effect of BEO (Navarra et al., 2015) and led further investigations on their activity in neuroblastoma cell line. The anti-cancer activity, as well as the underlying mechanisms, of BRG was previously evaluated in different *in vitro* and *in vivo* models (Ko et al., 2018), whereas that of 5-G-7-MOC was reported only in colon cancer cells (Patil et al., 2013). In this first report of the anti-proliferative activity of both compounds in SH-SY5Y cells, they hindered cell viability with different efficacy.

Afterward, to assess which type of cell death these coumarins induced in neuroblastoma cells, firstly, it was investigated whether these compounds interfere with the progression of SH-SY5Y cells during cell cycle through cytofluorimetric analyses. Cell cycle is characterized by checkpoints strictly complied by normal cells; in case genomic aberrations or defects occur, cells may undergo cell cycle arrest (Stewart et al., 2003). In this study, neither BRG nor 5-G-7-MOC altered the ratio among the G0/G1, S and G2/M phases respect to controls. On the other hand, we witnessed an increase of the cell population in the sub-G0/G1 phase, typical of hypodiploid cells, after the treatment with BRG and, though to a slighter extent, 5-G-7-MOC, thus suggesting that these coumarins might force cells to exit cell cycle and go in apoptosis. For this reason, we verified this hypothesis through Annexin-V/PI staining, a specific assay aimed at evaluating whether the apoptotic process started and at which stage (i.e., early or late) cells are. Apoptosis is a process finely regulated in healthy cells, and, hence, its dysregulation represents an acknowledged tumoral marker (Hanahan and Weinberg, 2011). In this experimental model, BRG induced apoptosis in SH-SY5Y cells at both 25 and 50 μM already at 24h of treatment, whereas 5-G-7-MOC only at 48h.

Oxidative balance in normal cells is precisely balanced, as well in tumours. Nevertheless, in cancer cells, ROS levels are higher as a mechanism of keeping the process of DNA impairment continuing. However, pushing ROS levels even further in those cells is known to start a nefarious vicious cycle. This because, overcoming anti-oxidant defence will lead to an extensive DNA damage, deposition of Bax protein on mitochondrial

membrane, a decrease of its potential and, hence, additional ROS production (Nguyen and Pandey, 2019). Here, BRG and 5-G-7-MOC induced ROS overproduction in our *in vitro* model, as well as the impairment of $\Delta\Psi m$.

Apoptosis can be unleashed also by an irreparable DNA damage that afterward activates downstream proteins aimed at regulating this event. Among these factors, p53 represents one of the most relevant promotors of the apoptotic machinery which triggers downstream proteins like BAX and Bad, well-known pro-apoptotic factors, and hinders the anti-apoptotic ones of the Bcl family (i.e., Bcl-2 and Bcl-XL) after genomic impairment. These proteins cooperate to activate or inhibit the caspase cascade, which sequentially leads to apoptosis (Reed, 2001). In our study, we witnessed a decrease of both Bcl-2 and Bcl-XL, whereas BAX and p53 increased, as a distinct sign of cells undergoing apoptosis. This was coupled to the cleavage, and hence activation, of both caspases 9 and 3, suggesting that BRG and 5-G-7-MOC follow the intrinsic apoptotic pathway. The effect observed at protein level reflected those obtained at gene level.

After proving the anti-proliferative effect of both BRG and 5-G-7-MOC alone in neuroblastoma cell line, it was wondered how these two compounds might affect the activity of the whole BEO, as highlighted above, being at concentrations in the essential oil far below from the IC50s extrapolated in this study. Therefore, it was evaluated whether their combination could be synergistic or antagonistic through the employment of a computational technique. Several approaches have been established so far to assess pharmacological interdependence among drugs; the

Loewe's additivity and Bliss independence drug interaction models are the most relevant and accepted, whereas the Zero Interaction Potency (ZIP) is an innovative protocol derived from the previous ones (Yadav et al., 2015). The synergy scores obtained shed light on the fact that the most synergistic combinations between BRG and 5-G-7-MOC are when their ratios range from 1:4 to 1:16. Surprisingly, in the BEO, as well as in the fractions studied before (Navarra et al., 2015), the ratio between BRG and 5-G-7-MOC is about 1:14, value that falls perfectly within the range of the most synergistic area of the maps obtained by drug combination software. Therefore, this may explain why, despite the low concentrations of the two coumarins in BEO, they play together a relevant role in its activity, supporting the monoterpene counterpart.

These results assume a relevant implication since it is well-known that synergy among compounds present in natural products is crucial given their complex nature (Caesar and Cech, 2019), and hence their simultaneous multitarget capacity may lead to greater effect than that attainable by single molecules (Cirmi et al., 2017; Ferlazzo et al., 2016a; Maugeri et al., 2019b). Therefore, when using phytocomplexes, the synergism in natural products is an exceptional tool from a potential therapeutic approach, due to the fact that stronger effects can be obtained by employing lower concentrations (Atanasov et al., 2021; Efferth and Koch, 2011). This is particularly important in children who are sensitive to even small variations in the dosage of anti-neoplastic agents.

The third part of my PhD project integrates in this frame, whose results has been sent for publication. Therefore, I focused on the evaluation of

the whole BEO along with BEO-FF as anti-proliferative agents in CCRF-CEM cells and their doxorubicin-resistant counterpart at the Johannes Gutenberg University Mainz (Germany). This because resistance to chemotherapy and molecularly targeted therapies are major problems in current cancer research and therapy (Holohan et al., 2013). In particular, cancer survival among paediatric subjects may be highly hampered due to the occurrence of intrinsic and/or acquired MDR (Kuttesch, 1996). The mechanisms of drug resistance are numerous and complex. One of them is mediated by the overexpression of ATP-binding cassette (ABC) transporters able to efflux drugs out of the tumours cell. ABC proteins exert numerous physiological and protective activity, being involved in the regulation of local permeability at level of the blood brain barrier (BBB), blood cerebrospinal fluid barrier (BCFB), blood testes barrier (BTS) and placenta. Moreover, in the liver, gastrointestinal tract and kidney, ABC proteins extrude metabolites and toxins, as well as are implied in cellular lipid transport and homeostasis (Gillet et al., 2007). Finally, avoiding accumulation of carcinogens inside the cells, they prevent carcinogenesis in healthy tissues. Pathologically, overexpression of multidrug resistance (MDR), a subfamily of ABC proteins, in some cancer cells lead to the failure of cancer chemotherapy, because it causes the efflux of several anticancer drugs from the cells. P-glycoprotein (P-gp), also known as MDR1, is member of ABCB1 subfamily, the first discovered and the most studied of all ABC transporters. This because it is highly expressed in the whole human body. In the intestinal epithelium, it pumps xenobiotics back into the intestinal lumen; in the kidney proximal tubule cells, it ejects them

into urine-conducting ducts; in liver cells, it extrudes metabolites into bile ducts; in the capillary endothelial cells composing BBB, BCFB, BTS and placenta, where limits passage across those barriers. In addition, some cancer cells also express large amounts of P-gp that, pumping out various anticancer drugs, making these cells multidrug resistant. Hence, inhibition of P-gp may improve chemotherapy, increasing intracellular drug concentrations and their therapeutic effects. Indeed, during the last decades, many efforts have been made to discover new therapeutic agents acting as P-gp inhibitors (Kathawala et al., 2015). However, although numerous compounds have been successfully studied both in in vitro and in vivo models, their highly toxicity and adverse side effects did not permit their clinical application. Clinical trials with third-generation drugs (Fracasso et al., 2004; Kruijtzer et al., 2002) are without encouraging results. Hence, the scientific interest for P-gp inhibitors in oncology shifted towards natural compounds, which generally exert lower side effects and have better tolerability than synthetic drugs. At the beginning of 2000, the research group led by Prof. Efferth started a research program on molecular pharmacology and pharmacogenomics of natural products derived from Chinese herbs. In this frame, in order to identify novel P-gp inhibitors from natural origin, they tested numerous phytochemicals for their activity towards CCRF-CEM human leukaemia cells and sublines overexpressing P-gp, selected for resistance to certain established anti-cancer drugs (Adams et al., 2007a; Adams et al., 2007b; Efferth et al., 2002; Mahringer et al., 2010). Results of these studies suggested that some natural compounds derived from medicinal plants

used in traditional Chinese medicine are inhibitors of P-gp and cytotoxic towards CCRF-CEM and drug-sensitive subline cells, both alone and in combination with well-established chemotherapeutic agents. This suggests that P-gp inhibitors from natural origin may represent a novel candidate for improving the efficacy of cancer combination therapy regimens to treat leukaemia (Abdallah et al., 2015; Eichhorn and Efferth, 2012).

Within the compounds found mainly in BEO, monoterpenes such as limonene, linalool, linalyl acetate and β-pinene have been already tested on multi-drug resistant cell lines. In particular, limonene did not actually affect the efflux of [3H]-digoxin in both LLC-PK1 (porcine kidney-derived cell line) and L-MDR1 cell lines (LLC-PK1 stably transfected with the human MDR1 gene), as elucidated by Zhang and Lim (Zhang and Lim, 2008). Although in different subline, the LLC-GA5-COL150 (LLC-PK1 cell line established by transfection of human MDR1 cDNA encoding P-gp), Yoshida and collaborators (Yoshida et al., 2005), obtained similar results. Linalool, if used alone, was shown to exert weak antiproliferative effects against MCF-7 WT breast cancer cells and MCF-7 AdrR resistant cells, whereas, in combination with doxorubicin, increases the cytotoxicity of doxorubicin in a dose-dependent fashion (Ravizza et al., 2008). This indicates that linalool in both MCF-7 parental cell line and MCF-7 AdrR cells, otherwise resistant to doxorubicin alone, exerts significant synergistic effect along with the chemotherapeutic agent (Ravizza et al., 2008). In LLC-GA5-COL150 (porcine kidney-derived cell line transfected with human MDR1 cDNA encoding P-gp), linalool instead was shown not

to vary [3H]-digoxin efflux (Yoshida et al., 2005). In CCRF-CEM leukemic cells, it was shown that linalool exerts a relevant anti-leukemic activity (Chiang et al., 2003). Hassan and co-workers (Hassan et al., 2008) characterized the anti-tumour activity and mechanism of action of linalyl acetate in 14 cancer cell lines, highlighting that small cell lung carcinoma and colorectal cancer cell lines were the most sensitive to the drug. Moreover, a greater tumour selectivity was observed against chronic lymphocytic leukemic cells compared to normal mononuclear cells. Only limited effect of some of the classical mechanisms of multi-drug resistance on the activity of linalyl acetate was noted which makes it potentially interesting for drug-resistant patients (Hassan et al., 2008). Finally, β-pinene, as well as other monoterpenes, was found to largely increase the intracellular accumulation of [3H]-digoxin in the resistant porcine kidney-derived cell line LLC-GA5-COL150 (Yoshida et al., 2006).

Bergamottin and bergapten, the two furocoumarins present in BEO in higher amounts, were shown to significantly increase the cell/medium ratio of [3H]-vinblastine uptake in colon cancer cell line Caco-2 (Ohnishi et al., 2000). Moreover, it was demonstrated that bergamottin showed relevant inhibition of cell growth in both CCRF-CEM cell line and in CEM/ADR5000 drug-resistant subline, respectively (Adams et al., 2007b).

In this field, several preclinical studies suggested that natural compounds may play a potential role against MDR in cancer (Kumar and Jaitak, 2019). Noteworthy, EOs have been assessed for their anti-cancer properties in different MDR cancer cell lines (Queiroz et al., 2014; Saab et al., 2015; Wu et al., 2016), suggesting their potentiality.

In this study, BEO and BEO-FF have shown anti-proliferative activity in both CCRF-CEM and CEM/ADR5000 cells without any appreciable difference. Indeed, the IC50s extrapolated from cell viability are quite similar, although the resistant cells were slightly more sensitive to the whole BEO, while CCRF-CEM to the furocoumarin-free counterpart.

This outcome also reflected data obtained by the Annexin V/PI staining to evaluate the eventual involvement apoptosis. As a matter of fact, only the exposure of CCRF-CEM cells for 48h with BEO-FF brought a robust increase of cells undergoing both late apoptosis and necrosis. Conversely, CEM/ADR5000 cells richly populated Q1 and Q2, namely for necrosis and late apoptosis, when exposed to the whole BEO.

Similar to what was observed for the coumarins of BEO in SH-SY5Y, cell cycle phases of both leukemic cell lines were not altered in their ratio when treated with both bergamot fractions. Nevertheless, sub-G0/G1 phase population increased, meaning higher hypodiploid cells, when both CCRF-CEM and CEM/ADR5000 cells were exposed to the IC50s of BEO, whereas to a lesser extent to BEO-FF.

After proving that BEO and BEO-FF showed anti-proliferative activity in both CCRF-CEM and CEM/ADR5000 cells via activating the apoptotic machinery, the next step was to evaluate whether these two fractions were able also to increase the sensitivity to doxorubicin of these two cell lines. Following the same protocol used for the coumarins present in BEO, the combination of BEO and doxorubicin at different ratios showed overall a low synergistic effect in CCRF-CEM, effect almost flattened when the anti-neoplastic agent was combined to BEO at concentration lower than 80%

of the IC50. Conversely, the furocoumarin-free fraction showed a slight antagonistic effect in the same cell line, proving that neither BEO nor BEO-FF elicited any relevant effect in the sensitive leukemic cell line.

Interestingly, in CEM/ADR5000 cells, in which doxorubicin IC50 is almost a ten thousand times higher than in the sensitive counterpart (Lu et al., 2020), both fractions showed synergistic effects. Specifically, BEO-FF proved to be synergistic only at IC50 lower than the 80%, whereas BEO demonstrated a strong synergistic effect reaching a peak when BEO was at 20% of IC50 and doxorubicin from 0.1 to 1 µM, hence far below its IC50 if CEM/ADR5000 cells were treated alone. Given the furocoumarin content of the whole BEO, such high synergy can be expected due to well-known P-gp inhibition properties of these compounds (Raad et al., 2006).

Overall, these data corroborate the hypothesis that the multi-target nature of phytocomplexes can be exploited to counteract MDR. On this line, the research group led by Prof. Navarra (Cirmi et al., 2016b) and other researchers (Amin et al., 2009; Efferth and Koch, 2011; Liu, 2004; Surh, 2003) suggested that a single biologically active molecule, even if used at high concentrations, could not be sufficient in preventing or treating cancer because several different pathways are involved in malignant progression. Therefore, employment of complex mixtures of biologically active substances, such as those present in whole fruits and vegetables, juices or their extracts, increases the chances of success against cancer (Cirmi et al., 2017). The multitarget specificity of natural products might represent an advantage in cancer therapy, since tumour cells can more easily develop resistance to mono-specific single drugs rather than to multi-

specific combinations of bioactive phytochemicals in plant extracts (Saab et al., 2015). Moreover, MDR cells are not generally resistant to all drugs. Some drugs surprisingly reveal hypersensitivity, i.e., they kill multidrug-resistant cells with even better efficacy than their drug-sensitive parental cell lines. This phenomenon has been defined as collateral sensitivity (Efferth et al., 2020; Saab et al., 2015). Interestingly, Saab and collaborators claimed that not only isolated phytochemicals, but also plant extracts could provoke collateral sensitivity, and that multiple compounds may jointly interact to kill MDR cells more efficiently than parental sensitive cells. Therefore, it is conceivable that the effectiveness against MDR cells of a collaterally sensitizing phytocomplex, like BEO where many different compounds coexist, might surmount novel resistance mechanisms, because it is much more unlikely that cancer cells acquire resistance against the multitude of bioactive molecules present in it and hence, in the view of a multi-target therapy, this may represent an appealing approach to overcome this clinically relevant problem.

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