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PROTECTIVE EFFECT OF EXTRA-VIRGIN OLIVE OIL ON CANCER: TOWARDS DEVELOPING A CERTIFICATION APPROACH

BY

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ΠΕΡΙΛΗΨΗ

Το βασικό συστατικό της Μεσογειακής Διατροφής είναι το Έξτρα-Παρθένο Ελαιόλαδο (Extra-Virgin Olive Oil, EVOO) το οποίο περιέχει φαινολικές ενώσεις που σχετίζονται με τις ευεργετικές ιδιότητες που αποκτώνται από την κατανάλωση του EVOO (Garcia-Martinez et al., 2018; Serra-Majem et al., 2019). Επιδημιολογικές μελέτες καταδεικνύουν πως EVOO πλούσια σε φαινολικά έχουν καρδιοπροστατευτικές ιδιότητες και αντικαρκινική δράση (Bartolí et al., 2000; Calza et al., 2001; Levi et al., 1999; Schwingshackl et al., 2018).

Ο καρκίνος είναι μια πολυπαραγοντική ασθένεια και η έναρξή της μπορεί να αποδοθεί τόσο σε γενετικούς όσο και σε περιβαλλοντικούς παράγοντες (Baena Ruiz & Salinas Hernández, 2014). Τα καρκινικά κύτταρα αποκτούν βελτιωμένες ικανότητες των φυσιολογικών κυττάρων, όπως είναι η επικράτηση σημάτων πολλαπλασιασμού, αποφυγή καταστολής ανάπτυξης, αντίσταση στον κυτταρικό θάνατο, επαγωγή αγγειογέννεσης κ.ά. (**Figure 6**) (Hanahan & Weinberg, 2011). Οι περιβαλλοντικοί παράγοντες είναι υπεύθυνοι για το 95% των περιπτώσεων καρκίνου (Irigaray et al., 2007). Η διατροφή διαδραματίζει έναν από τους ζωτικούς πυλώνες των περιβαλλοντικών παραγόντων και είναι υπεύθυνη για τις αποκλίσεις των περιπτώσεων καρκίνου ανάμεσα στις διαφορετικές γεωγραφικές περιοχές (Willett et al., 1995).

Στην παρούσα διπλωματική, θελήσαμε να διερευνήσουμε την βιολογική δραστικότητα δειγμάτων ελαιόλαδου με την ανάπτυξη *in vitro* δοκιμασιών οι οποίες προσομοιάζουν την *in vivo* δραστικότητα. Οι περισσότερες μελέτες έχουν πραγματοποιηθεί χρησιμοποιώντας συγκεκριμένες φαινολικές ουσίες απομονωμένες από το EVOO. Καθώς θέλαμε να εξάγουμε μια πιο άμεση συσχέτιση μεταξύ του EVOO και της επίδρασής του στην κυτταρική ανάπτυξη, επωάσαμε κύτταρα απευθείας με EVOO. Καταλήξαμε στο ότι θρεπτικό μέσο καλλιέργειας εμπλουτισμένο με EVOO αλλά και η απευθείας προσθήκη στα κύτταρα, αναστέλλει ειδικά την ανάπτυξη των καρκινικών κυττάρων χωρίς να αναστέλλει την ανάπτυξη των ενδοθηλιακών κυττάρων. Πρόσθετα, για πρώτη φορά αναλύθηκαν ελληνικά μονοποικιλιακά EVOO με γνωστή την περιέκτικότητά τους σε φαινολικές ενώσεις,

κάτι που μας δίνει την δυνατότητα να διερευνήσουμε εάν η δράση τους στην κυτταρική ανάπτυξη οφείλεται σε γνωστές φαινολικές ενώσεις ή όχι. Επίσης, με την παρούσα μελέτη δείξαμε ότι η μορφολογία και η μεταναστευτική ικανότητα των καρκινικών κυττάρων μετά από επώαση με EVOO μεταβάλλεται. Τέλος η επίδραση των ελαιολάδων ήταν ειδική στα καρκινικά κύτταρα καθώς βασικές αποκρίσεις ενδοθηλιακών κυττάρων, όπως ανάπτυξη και μετανάστευση, δεν επηρεάστηκαν μετά από επώαση με διάφορα ελαιόλαδα.

ABSTRACT

A main component of the Mediterranean Diet (MD) is Extra-Virgin Olive Oil (EVOO) which contains phenolic compounds that are associated with the beneficials effects of EVOO consumption (Garcia-Martinez et al., 2018; Serra-Majem et al., 2019). Epidemiological studies indicate that EVOO rich in phenols has a cardioprotective effect (Dimitrios Boskou, 2008; Nocella et al., 2017) and lower cancer incidence (Bartolí et al., 2000; Calza et al., 2001; Levi et al., 1999; Schwingshackl et al., 2018).

Cancer is a multifactorial disease and its onset can be attributed both to genetic and environmental factors (Baena Ruiz & Salinas Hernández, 2014). Cancer cells acquire deregulated functions of normal cells like enhanced proliferation, evading growth suppressors, resisting cell death, inducing angiogenesis etc. (**Figure 6**) (Hanahan & Weinberg, 2011). Environmental factors account for 95% of cancer incidences (Irigaray et al., 2007). Diet is one of the pivotal pillars of environmental factors and is responsible for the variation of cancer incidences among different geographical regions (Willett et al., 1995).

In this Thesis, we wanted estimate biological activity of olive oil samples by developing of *in vitro* assays that reconstitute the *in vivo* activity. Most of the studies have been carried out using specific phenolic compounds isolated from EVOO. As we wanted to exert a more direct correlation of EVOO and its effect on cell growth, we directly treated cells using EVOO. We concluded that EVOO-enriched culture medium and direct addition of EVOO on cell culture specifically inhibit cancer cell growth without inhibiting primary endothelial cell growth. In addition, for the first time EVOOs from Greece have been analyzed regarding their phenolic concentration, which gives as the opportunity to investigate whether any effect on cell growth is due to known phenolic compounds or not. Also, in this Thesis we showed that the morphology and migration ability of cancer cells is altered. Finally, the effect of EVOO was specific for cancer cells as basic responses of endothelial cells, like cell growth and migration, were not altered after treatment using different EVOOs.

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ABBREVATIONS

ACS	American Cancer Society
AKT/PKB	Protein Kinase B
BAX	Bcl-2-like protein 4
BCL2	B-cell lymphoma 2
BIM	Bcl-2-like protein 11
CDK	Cyclin-dependent kinase
CO2	Carbon Dioxide
COX	Cyclooxygenase
CTL OIL	Commercial oil-control oil
DLD-1	Duke's type C colorectal adenocarcinoma
DMEM	Dulbebo's modified Eeagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECGS	Endothelial cell growth supplement
EDHF	Endothelium-Derived Hyperpolarizing Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to mesenchymal transition
eNOS	Endothelial Nitric Oxide Synthase
ERK1/2	extracellular signal-regulated kinase 1/2
EVOO	Extra-Virgin Olive Oil
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GTP	Guanosine triphosphate
HCT-116	Human Colorectal carcinoma
HIF	Hypoxia-inducible factor
HT	Hydroxytyrosol
HUVEC	Human Umbilical Vein Endothelial Cells

IAP	Inhibitors of apoptosis
IGF	Insulin Growth Factor
MAPK	mitogen-activated protein kinase
MCF-7	Michigan Cancer Foundation-7, Breast cancer cell line
MD	Mediterranean Diet
MMP	Matrix metallopeptidase
MUFA	Monounsaturated Fatty Acids
NADPH	Nicotinamide adenine dinucleotide phosphate
NH4CL	Ammonium chloride
NO	Nitric oxide
OLC	Oleocanthal
OLE	Oleacein
PBS	Phosphate Buffer Saline
PDGFR	Platelet-derived Growth Factor Receptor
PFA	Paraformaldehyde
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinases
PKC	Protein kinase C
PLC	phospholipase C
PLGF	Placental Growth Factor
PUMA	p53 upregulated modulator of apoptosis
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat sarcoma
RB	Retinoblastoma
ROS	Reactive Oxygen Species
RTK	Receptor tyrosine kinase
SFA	Saturated Fatty Acids
SUP	Supernatant-EVOO-enriched media
TGA	Triacylglycerols
TP53	Tumor protein p53
TRAIL	TNF related apoptosis inducing ligand

TSP-1	Thrombospondin-1
TYR	Tyrosol
UNESCO	United Nations Educational, Scientific and Cultural Organization
UV	Ultra-Violet
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VOO	Virgin-Olive oil
XIAP	X-linked inhibitor of apoptosis

Chapter 1 INTRODUCTION

1.1 Mediterranean Diet

According to United Nations Educational, Scientific and Cultural Organization (UNESCO), in 2010 the Mediterranean Diet (MD) was recognized as "intangible cultural heritage of France, Italy, Greece, Spain and Morocco" (*Mediterranean Diet - Intangible Heritage - Culture Sector - UNESCO*). MD was considered in 1986 from Ancel Keys et al. as a diet poor in saturated lipids with positive effects in cardiovascular system due to low cholesterol levels in blood (Keys et al., 1986). Following on from this observation, MD was identified as a diet rich in nutrients that have a high protective role in several diseases (Dinu et al., 2018; Martinez-Gonzalez et al., 2009).

MD, besides being a health and nutritional dietary pattern, is actually a way of life. This term is based on dietary traditions in around 20 countries throughout the Mediterranean region. Differences found in culture, economy, ethnicity, and religion among these countries are addressed for their dietary patterns, but the food comprising their diet is only partially the same due to reasons like seasonality and local production, methods of conservation etc. (Serra-Majem et al., 2019)

1.1.1 Components of the Mediterranean Diet

Basic components of the MD are increased consumption of vegetables, legumes, fresh fruit, non-refined cereals, nuts, and olive oil (especially extra-virgin olive oil), moderate consumption of fish, dairy products and alcohol (red wine during main meals) , and low consumption of red meat (Trichopoulou & Critselis, 2004).

Legumes are highly consumed due to their long time of preservation, easy preparation, and high nutritional value (Serra-Majem et al., 2019). Unrefined creals (wheat, rice etc) are the main source of carbohydrates and calories (Babio et al., 2009; Wirth et al., 2016). Fruits and vegetables are highly consumed as they are easily grown due to favorable climate conditions (Carlsson-Kanyama & González, 2009). Despite the

fact that fish are rich in omega 3 fatty acids, their contribution is compromised by contaminants. Production of dairy products was secondary as mainly sheep and goats were primarily raised for wool, milk and meat. Due to the price and other factors, consumption of red meat in the traditional Mediterranean Diet was low. The Mediterranean Diet is highly linked to a moderate consumption of red wine along with each meal in the European Mediterranean countries. The main component of Mediterranean Diet is extra virgin olive oil (EVOO) as it is the essential dietary fat. Its high consumption is tightly linked to high consumption of vegetables. From ancient times, longevity of the Mediterraneans is attributed to EVOO (Garcia-Martinez et al., 2018; Serra-Majem et al., 2019)

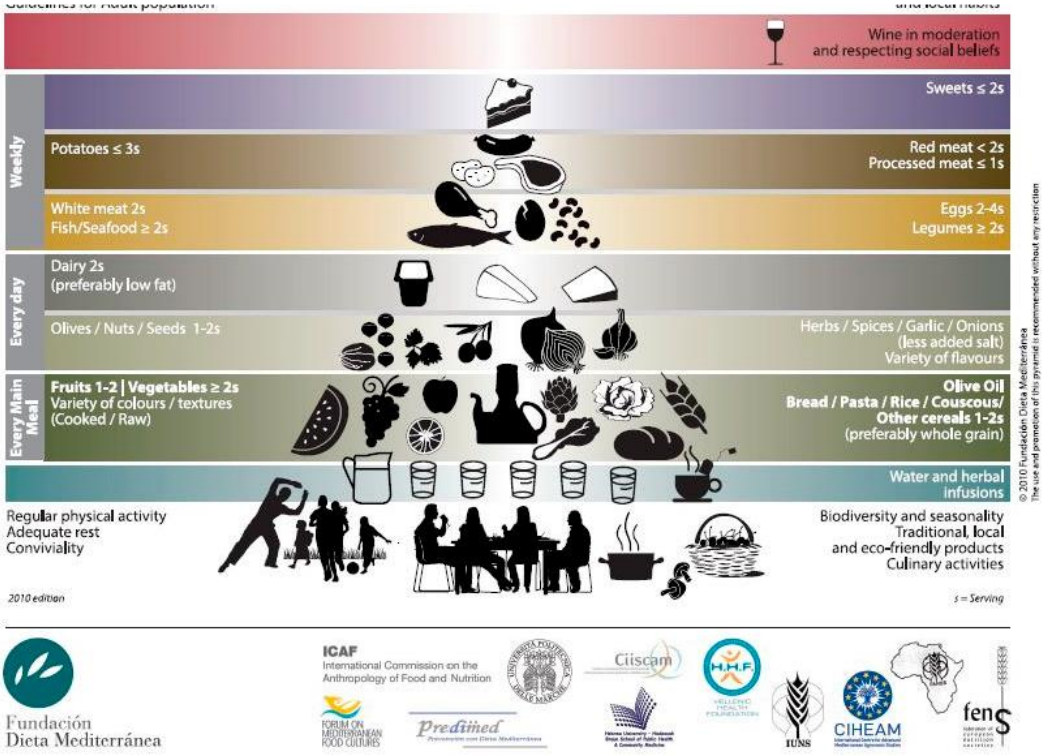


***Figure 1**: Mediterranean diet pyramid. Source: Fundacion Dieta Mediterrànea*

MD enclosures characteristics and values related with the preservation of tradition, habits, and environment (Dernini & Berry, 2015). Since 1960, there is an abundance of literature concerning the Mediterranean Diet and its positive, healthy, and nutritional benefits. It is important that the added value of MD is due the combination

of healthy foods and their nutritional benefits (Alsaffar, 2016; Bach-Faig et al., 2011; Bonaccio et al., 2012).

1.1.2 Benefits of the Mediterranean Diet

There is evidence that adherence to the Mediterranean Diet might have a protective effect against diseases concerning the cardiovascular system, cancer, diabetes, hypertension, Alzheimer and Parkinson's disease (Fung et al., 2009; Gao et al., 2007; Lopez-Garcia et al., 2014; Martínez-González et al., 2008; Scarmeas et al., 2009; Tektonidis et al., 2015). According to a number of studies, it is shown that the MD has been associated with reduced risk for developing various types of cancer, such as colorectal, liver, pancreas, and selected hormone-related cancers such as endometrial cancer (Farinetti et al., 2017; La Vecchia, 2009; Pelucchi et al., 2009).

1.2 Extra-Virgin Olive Oil (EVOO)

Olive oil is a very complex food that is influenced by thousands of years of history and culture in southern Europe, North Africa, and the Middle East, an area that produces over 95 % of the world's olive oil (Aparicio & Harwood, 2013).

Extra-Virgin Olive Oil (EVOO) is the main component of the MD (Piroddi et al., 2017). EVOO is mechanically extracted and can be consumed without any refining process (Angerosa et al., 2006). Thus, natural compounds, which are important for their nutritional value, are preserved and give rise to unique aroma and taste (Angerosa & Campestre, 2013).

There are 4 different types of olive oil according to their quality (Aparicio & Harwood, 2013):

1. EVOO is the finest Virgin Olive Oil (VOO), it is produced using mechanical extraction from the fruit and its free acidity, expressed as oleic acid, is not more than 0.8% (0.8g per 100g)
2. Virgin Olive Oil (VOO) is also produced using mechanical extraction, but its free acidity is not more than 2%
3. Olive Oil is a mixture of refined olive oil[1] and VOO which has a free acidity less than 1%
4. Olive-pomace oil[2] is a mixture of refined olive-pomace oil and VOO with free acidity less than 1%.

1.2.1 The Olive Plant and Olive Oil: A Brief History

Today the olive plant (*Olea europea*) is cultured all over the countries of the Mediterranean basin, particularly in Spain, Italy, Greece, Turkey, Tunisia, and Morocco. Consequently, a slow but continuous development of the civilizations

[1] Refined olive oil is the olive oil obtained from VOOs by refining methods which do not lead to alterations in the initial glyceridic structure

[2] Olive-pomace oil is the general term for the oil obtained by treating olive pomace with solvents, to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds. Olive pomace is the residual paste left over from the production of VOO and contains 5-10% of oil (Aparicio & Harwood, 2013)

growing various olive plant has been developed in the coastal territories of the Mediterranean and more inland areas of the Middle East. All ancient civilizations located around the Mediterranean basin and in the Middle East have left clear evidence that olive cultivation and oil production were activities that developed with the flourishing civilizations themselves (Vossen, 2007)

1.2.2 Cultivar Influence on Oil Composition and Properties

The differences in the composition and properties of different olive oils is an interaction between environment (climate, temperature, rainfall, and soil water status), agronomic factors (crop load, shading, leaf-to-fruit ratio, pruning, fertility, irrigation, and fruit maturity stage), and the specific cultivar (genetics) (Vossen, 2007). These variables influence chemical compounds that explain olive oil color (pigments), aroma (volatiles), and taste (phenols) and determine olive oil authenticity, traceability, and characterization (e.g., fatty acids, sterols, and hydrocarbons) through biochemical pathways and chemical reactions. Olive oil flavor can be altered, for example, from pest damage or mishandling of fruit during and after harvest that lead to fermentation but can also occur due to poor oil-processing and storage techniques. Lower-quality defective oils have a high free fatty acid percentage, peroxide value, or UV absorbance reading. (Aparicio & Harwood, 2013)

Cultivar characteristics may vary by tree vigor, productivity, precocity, alternate bearing, cold hardiness, flowering and maturity dates, susceptibility to certain diseases, fruit size, pit-to-pulp ratio, oil content, profiles of major and minor chemical compounds, and sensory characteristics (Kalua et al., 2005)

The choice of which cultivar to grow is one of the most important decisions in growing any horticultural crop, but it is more important for olives that live a long time and take several years to come into full bearing. There are over 1,200 documented olive cultivars in the world (Bartolini et al., 2005), but only approximately 30 represent the vast majority of the production. These cultivars dominate in specific growing areas due to their superior characteristics (Vossen, 2007).

1.2.3 Olive Processing

The mechanical extraction of oil from olives involves releasing it from the fruit tissues in a way that the droplets can merge into larger drops until they form a continuous liquid phase (Aparicio & Harwood, 2013). Olive oil is mainly located inside the vacuoles of the mesocarp cells but is also somewhat scattered inside the colloidal system of the cytoplasm and, in small amounts, in the epicarp and endosperm (Runcio et al., 2008).

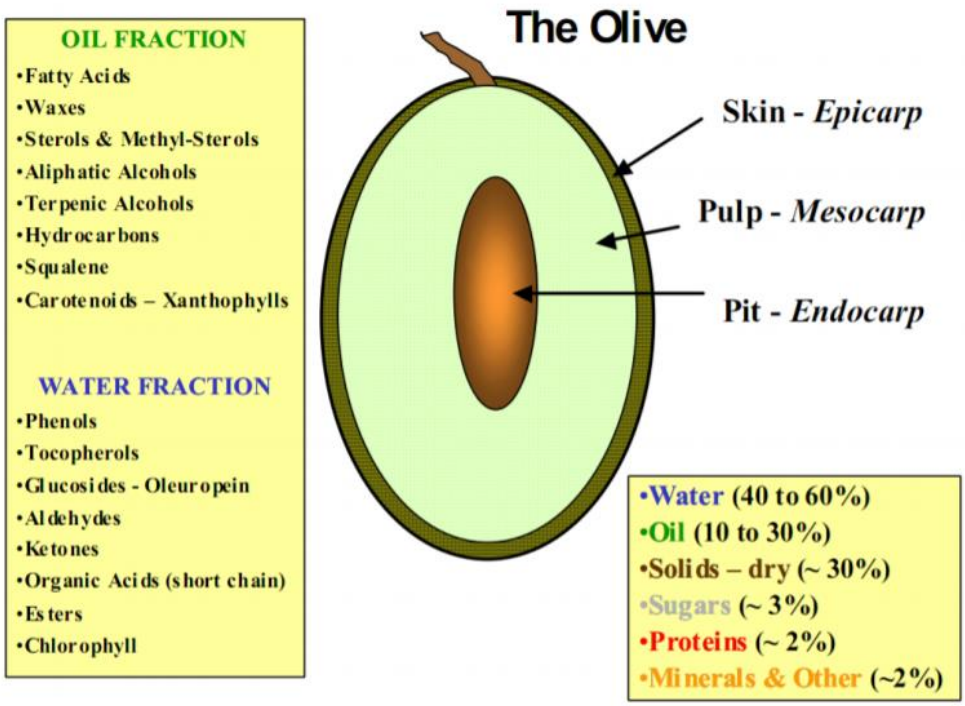


Figure 2:*Olive parts* (https://cordis.europa.eu/project/id/605357, Phenolive, 2016)

Oil enclosed inside the vacuoles can be released by mechanical means, although oil dispersed inside the cytoplasm is quite difficult to extract and is generally lost with the pomace or vegetation water.

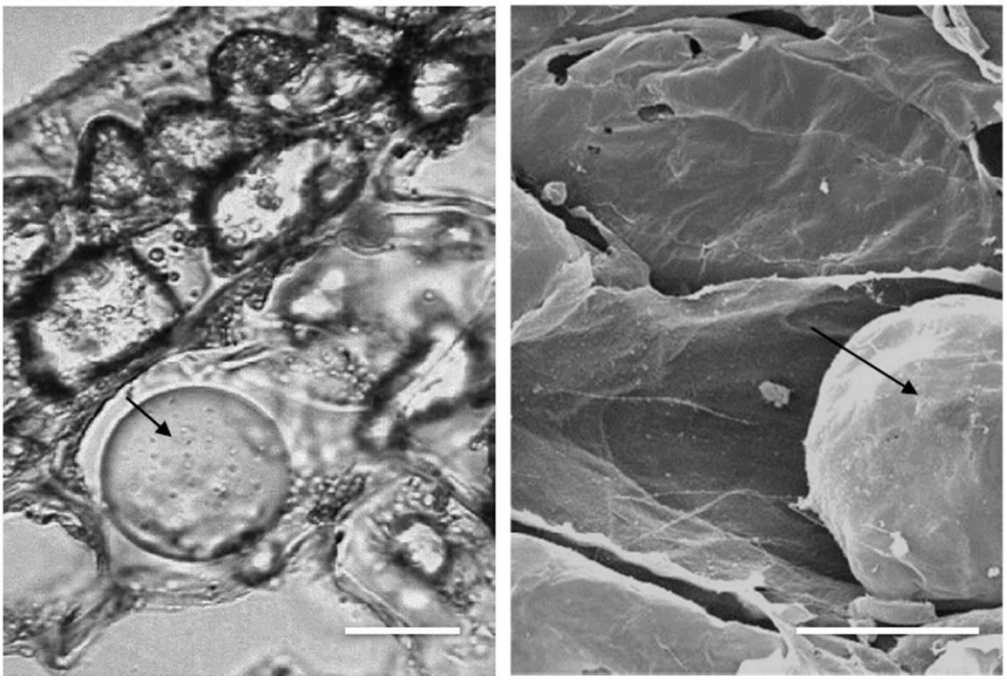


Figure 3: *Olive mesocarp cells with oil droplets (arrows) by (left) light microscopy and (right) scanning electron microscopy. Bars = 30 µm* (Lanza & Ninfali, 2020)

Moreover, when olive paste is prepared by crushing and malaxation operations, an emulsion may sometimes form, which hinders the subsequent separation of the oil. This is in part due to the droplets of emulsified oil being surrounded by lipoproteins, which prevent them from coalescing (Aparicio & Harwood, 2013; Runcio et al., 2008).

Until now, olive fruits for olive oil extraction were processed in mills by the following mechanical systems only: pressure, percolation, centrifugation.

1.2.4 Chemical Composition of Olive Oil

Olive oil mostly consists of triacylglycerols (TGAs) (98–99%), a diverse group of glycerol esters with different fatty acids. The predominant fatty acid present in olive oil TGAs is monounsaturated oleic acid (up to 83% *w*/*w*). There is also palmitic acid, linoleic acid, stearic acid, and palmitoleic acid making up the remainder of olive oil TGAs. There is a plethora of lipophilic or amphiphilic microconstituents present in VOO, among them, phytosterols, squalene, tocopherols, phenolic compounds, terpenic acid derivatives, etc.(Dimitrios Boskou, 2008; Luchetti, 2002; Ramírez-Tortosa et al., 2006). Phenolic compounds occur in the form of phenolic acids or alcohols, oleuropein

derivatives, lignans, and flavonoids. The content of polyphenols in EVOO may vary due to different mechanical processing, as depicted in **Figure 4** and it ranges from 50 to 1000 mg/kg. In more detail, it depends on the agronomic factors, the ripeness of olives, as well as extraction technology, along with storage or packaging processes (Baldioli et al., 1996; Bianco et al., 2002; Naczk & Shahidi, 2004; Tuck & Hayball, 2002).

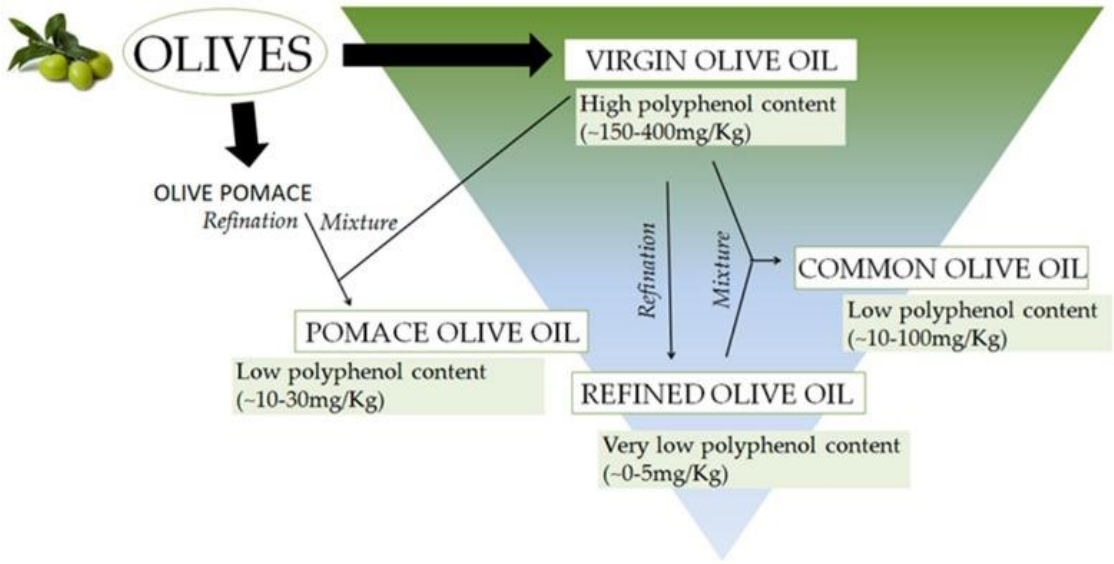


***Figure 4**: Polyphenol content in different kinds of olive oil according on different processes of oil extraction (Types of Olive Oil)*

The flesh of healthy olives contains about 2–3% of phenolic substances in the form of glucosides and esters. Virgin olive oil contains about 500 mg/L of polyphenols. The quantity and quality of polyphenols in olive oil is closely related to the process of olive milling and further processing. Therefore, virgin olive oils have substantially higher amounts of polyphenols than refined olive oils (Bianco et al., 2002; Naczk & Shahidi, 2004). The phenolic compounds in olive oil are mostly glycides (e.g., oleuropein), alcohols and phenols (tyrosol, hydroxytyrosol), and also flavonoids (D Boskou, 2015; Dimitrios Boskou, 2008; Ramírez-Tortosa et al., 2006). Phenolic compounds are mainly responsible for the characteristic gustatory property of virgin olive oil, namely the bitter taste. Some micro constituents of olive oil are soluble in water, and thus, the content of phenolic compounds that are present in olive oil depends to a large extent on the extraction process (D Boskou, 2015; Tuck & Hayball, 2002).

Among the minor components, the phenolic ones are relevant for the health effects attributed to EVOO (**Figure 5**) (Dimitrios Boskou, 2008). Epidemiological studies

indicate that dietary consumption of phenol enriched EVOO has a cardioprotective effect in Mediterranean populations (Romani et al., 2019) . The minor polar compounds include different subclasses among these: secoiridoids such as oleuropein (OLE) aglycone and oleacein, deacetoxyoleuropein, oleocanthal (OLC), phenolic alcohols with hydroxytyrosol (HT), and tyrosol (TYR), together with their secoiridoid precursors and traces of phenolic acids such as gallic acid, protocatechic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p- and o-coumaric acid, ferulic acid, and cinnamic acid. The flavonoids class is represented in small amounts; luteolin and apigenin are the flavones mostly found. The last class is composed of lignans, and the most representative compounds in EVOO are acetoxypinoresinol and pinoresinol (*CODEXALIMENTARIUS FAO-WHO*).

HT and OLE are valuable compounds for their high antioxidant capacity and for metal-chelating and free radical scavenging activities (Sroka & Cisowski, 2003). The above described lignans, pinoresinol and acetoxypinoresinol, also show antioxidant capacity (López-Biedma et al., 2016). HT is a molecule containing an ortho-diphenolic group that plays a significant role in EVOO.

EVOO Minor Polar Components	
Secoiridoids	(a) Oleuropein aglycone (b) Deacetoxy oleuropein (c) Oleocanthal and oleacin (d) Ligstroside aglycone
Phenolics	(a) Hydroxytyrosol (b) Tyrosol (c) Hydroxytyrosol glycole
Phenolic acids	(a) Gallic acid (b) Protocatechuic acid (c) *p*-Hydroxybenzoic acid (d) Vanillic acid (e) Caffeic acid (f) Syringic acid (g) *p*- and *o*-coumaric acid (h) Ferulic acid (i) Cinnamic acid
Flavonoids	(a) Luteolin (b) Apigenin
Lignans	(a) (+) Pinoresinol (b) (+) Acetoxypinoresinol

***Figure 5**: Minor compounds found in Extra-Virgin Olive Oil (Romani et al., 2019)*

One hundred grams of EVOO contains up to 25 mg of α-tocopherol and 1–2 mg of carotenoids, which are both potent antioxidants, as well as 20–500 mg of oleuropein and 98–185 mg of phytosterols (Visioli et al., 2020). Furthermore, it has been shown that 50 g of extra virgin olive oil contains up to 9 mg of oleocanthal, a phytochemical with ibuprofen-like COX-inhibitory activity (Beauchamp et al., 2005; Elnagar et al., 2011).

1.2.5 Olive oil certification

Certification is a multiplex issue that covers many aspects including characterization, adulteration, mislabeling, and misleading origin, among many others. Techniques like high-performance liquid chromatography (HPLC), gas liquid chromatography (GLC), and nuclear magnetic resonance (NMR) are being used to check and classify olive oil against international and specifications and legal regulations (Aparicio & Harwood, 2013).

Main parameters for the definition of quality of olive oils are color, taste, and aroma. These attributes are ascribable to compounds present in olive oil as the result of its mechanical extraction from the fruit and to the fact that it usually can be consumed without any refining process. Color is perhaps the most important sensory characteristic and it depends on the content of chlorophylls and carotenoids. Taste is probably attributed to secoiridoid compounds. Aroma seems to be related to chemical factors, such as the volatility and hydrophobic character, and to stereochemical structure of odorants rather than their concentration. Beside genetic factors, content in these compounds is affected by ripening degree and to the conditions adopted for oil extraction (Aparicio & Harwood, 2013).

According to worldwide standards, olive oils are graded upon their chemical composition (see the four types of olive oils in 1.2 Extra-Virgin Olive Oil (EVOO)) and their sensory components, as aroma, flavor, pungency and bitterness (*International Olive Council*; *Olive Oil Quality & Organoleptic Evaluation - Olive Oil Organoleptic Evaluation Laboratory Rethymno*). Sensory assessment is based on the positive and negative attributes. In positive attributes are included fruity, bitter and pungent, while negative attributes are fusty, musty, muddy and others. Positive attributes are determined by the quality of fruit produced on the tree while negative attributes are caused by human error (Mailer & Beckingham, 2006).

1.3 Cancer

Cancer is multifactorial disease and its onset can be attributed to genetic and environmental factors (Baena Ruiz & Salinas Hernández, 2014). It is the second leading cause of death globally and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer (World Health Organization). Exhaustive studies of these factors determined that genetic factors accounted for only 5% of cancer incidences, the other 95% is due to environmental factors (Irigaray et al., 2007). During cancer, normal cells are transformed into malignant cells following a multistep process. Malignant cells acquire enhanced features that are present in normal cells. These enhanced features can be illustrated in Figure 6. Tumors are complex tissues composed of multiple cell types that are organized in such a way to participate in heterotypic interactions with one another (Hanahan & Weinberg, 2011).

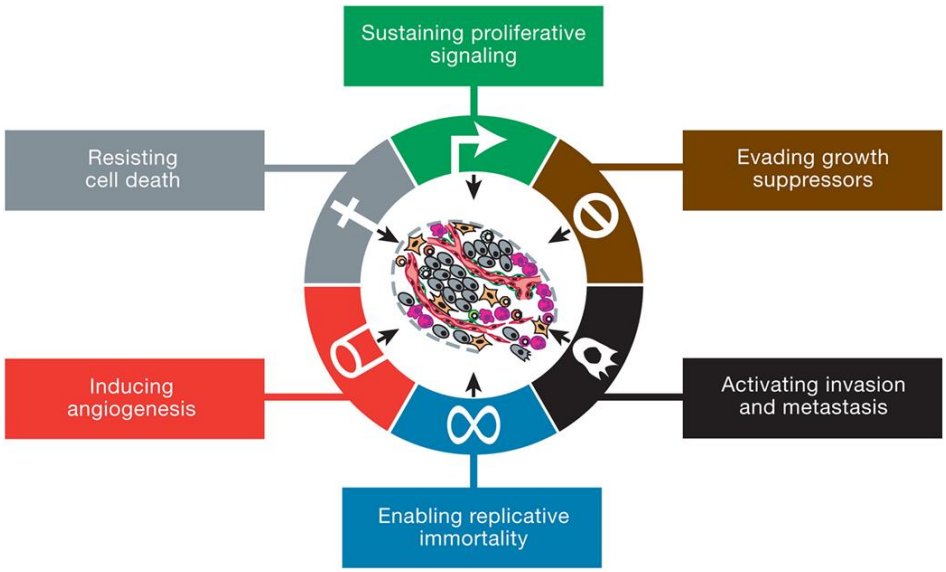


***Figure 6**: **The Hallmarks of cancer.** In this illustration are depicted the six hallmark capabilities of cancer* (Hanahan & Weinberg, 2011).

Carcinogenesis involves the alterations of a variety of genes called cancer genes and can be grouped in four broad categories, activation of oncogenes, inactivation of tumor suppressors, evasion of apoptosis genes and defective DNA repair genes (Malarkey et al.). During carcinogenesis, each neoplasm arises from an individual cell that has accumulated at least 80 genetic alterations in cancer genes. In these alterations there are included some “driver” mutations that end up to cancer’s uncontrolled growth.

1.3.1 Sustaining proliferative signaling

It is without doubt that the most fundamental characteristic of cancer cells is their ability to sustain chronic proliferation. Cancer cells deregulate signals involved in production and release of growth factors, while in normal cells these signals are carefully controlled in order to ensure the homeostasis and maintenance of cell architecture and function (Hanahan & Weinberg, 2011). Growth factors bind to receptors in the surface of the cells to convey signals concerning the regulated progression through the cell cycle as well as cell growth. Coupled with cell growth is cell survival and energy metabolism.

Mitogenic signals are better understood in cancer cells (Hynes & MacDonald, 2009; Lemmon & Schlessinger, 2010; Perona, 2006; Witsch et al., 2010). Cancer cells built up alternate ways to sustain proliferative signaling: Through autocrine stimulation they can produce and secrete growth factor ligands while the respond is conveyed through receptors they express. Alternatively, stimulation of normal cells of the tumor-associated stroma from cancer cells can result in the support of cancer cells with growth factors so they can proliferate (Bhowmick et al., 2004; Cheng et al., 2008). Besides secretion of growth factors, cancer cells upregulate the expression of growth factor receptors to respond even in limiting amounts of growth factors, resulting in hyperresponsiveness. Another way to the extensive proliferation is the constitutive activation of signaling pathways downstream of growth factor receptors (Hanahan & Weinberg, 2011).

Normal cells use highly effective negative-feedback loops to ensure homeostatic regulation of the flux of signals coursing through the intracellular circuitry (Amit et al., 2007; Cabrita & Christofori, 2008; Mosesson et al., 2008; Wertz & Dixit, 2010). Proliferative signaling can be enhanced because cancer cells take advantage of them causing malfunctions, i.e. oncogenic mutation in *ras* gene (Ras oncoprotein) results in excessive proliferative signaling through MAPKs. Normal function of this protein is to operate as an intrinsic negative-feedback loop that ensures that active signal transmission is transitory.

Early research revealed that excessive expression of proliferative genes and the signals manifested by their products would result in increased cancer cell proliferation and

thus tumor growth. More research undermined this notion, revealing that overexpression of oncoproteins such as RAS, MYC and RAF can provoke counteracting responses from cells, specifically induction of cell senescence and/or apoptosis. Senescence is a viable but non-proliferative state. Normal cells oppose to maximal mitogenic stimulation entering in senescence and/or inducing apoptosis. These findings indicate that cancer cells adapt to excessive mitogenic signaling overcoming cell senescence and/or apoptosis (Collado & Serrano, 2010; Evan & d'Adda di Fagagna, 2009; Lowe et al., 2004).

1.3.2 Evading growth suppressors

Besides positively acting growth-stimulatory signals, cancer cells must overcome powerful circuits that negatively regulate cell proliferation; many of these circuits depend on the action of tumor suppressor genes (Hanahan & Weinberg, 2011). Normally, tumor suppressor genes inhibit cell proliferation. Genetic alterations in these genes combined with alterations in central proteins that participate in cell cycle checkpoints -like Cyclin-dependent kinases (Cdks), cyclins, p53 and RB (Retinoblastoma gene protein) proteins, Cdk inhibitors (p16 and p14)- have the ability to deregulate cell cycle of cancer cells giving them the ability to proliferate constitutively (Mirzayans et al., 2012). RB and p53 proteins play a pivotal role in two key complementary cellular regulatory circuits that govern the decisions of cells to proliferate or to activate senescence and/or apoptotic programs (Sherr & McCormick, 2002). Loss of RB protein results in non-controlled entrance of the cell to the S phase of the cell cycle (Burkhart & Sage, 2008). Likewise, p53 protein acts as a regulator for DNA damage. Its central role is to stop cell cycle progression in the presence of DNA damage (Deshpande et al., 2005). RB protein transduce growth-inhibitory signals that originate outside of the cell (mostly), while p53 receives inputs from stress and abnormality sensors within the cell's intracellular compartment (Hanahan & Weinberg, 2011).

1.3.3 Resisting cell death

Evidence support that apoptosis serves as a natural barrier to cancer progression (Adams & Cory, 2007; Lowe et al., 2004). Programmed cell death or apoptosis is a tightly regulated mechanism in which changes like reduction of nucleus size due to chromatin reduction and DNA damage can be observed morphologically (Nagata & Tanaka, 2017). During apoptosis biochemical alternations, like activation of caspases and loss of mitochondria function are also observed (Tomek et al., 2012).

Two major signaling pathways that convey apoptosis are the extrinsic pathway receiving and processing extracellular death inducing signals, like death receptor pathway, and the intrinsic pathway involving mitochondria sensing intracellular signals (Hanahan & Weinberg, 2011; Nagata & Tanaka, 2017). These two pathways have different triggers but share a common executing pathway, activation of caspase cascade resulting in DNA fragmentation, proteolysis of cytoskeleton and nuclear proteins, protein crosslinking, formation of apoptotic vesicles, expression of phagocytic ligands and, in the end, the recruitment of phagocytes (Elmore, 2007).

Cancer cells evolve a variety of strategies to eliminate apoptosis. Among most tumor cells, most common is the impairment of p53 tumor suppressor gene function, resulting in termination of the most critical sensor of DNA damage from the apoptosis signaling pathway. Alternatively, same results are observed from the excessive expression of antiapoptotic regulators (Bcl-2) or survival signals (Igf1/2), eliminating the expression of proapoptotic signals (Bim, Bax, Puma), or short-circuiting the extrinsic ligand-induced death pathway (Hanahan & Weinberg, 2011; Wong, 2011).

1.3.4 Inducing angiogenesis

Angiogenesis is the physiological procedure where new blood vessels are formed from already formed blood vessels. This procedure includes migration, differentiation and combination of endothelial progenitor cells to form progenitor blood vessels (Adair & Montani, 2010; Lapeyre-Prost et al., 2017). Both normal and cancer cells require sufficient nutrients and oxygen to survive. During embryogenesis, vasculogenesis results in the generation of endothelial cells and their assembly into tubes. Vasculogenesis is followed by angiogenesis (Hanahan & Weinberg, 2011). In adults, angiogenesis is strictly regulated and when activated, is only transient i.e. processes

of wound healing and female reproductive cycling. It is remarkable that cancer cells take advantage of this angiogenic switch resulting in continuous sprouting of new vessels and sustaining nutrients and oxygen delivery in tumor progression (Hanahan & Folkman, 1996).

Angiogenesis is governed by factors that either induce or oppose the event (Baeriswyl & Christofori, 2009; Bergers & Benjamin, 2003). Vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) are well-known inducer and opposer, respectively. VEGF orchestrates sprouting of new vessels during embryogenesis and in adults during wound healing. In malignancies~~,~~ VEGF is the central molecule for angiogenesis resulting tumor progression. Besides VEGF, other proangiogenic signals have the ability to sustain tumor angiogenesis, like fibroblast growth factor (FGF) (Baeriswyl & Christofori, 2009). Moreover, VEGF-mediated signaling in cancer cells results in tumorigenesis, combined with autorenewal and survival of cancer stem cells (Mercurio, 2019). On the contrary, TSP-1 is a opposer of angiogenesis evoking suppressive signals to counteract proangiogenic stimuli (Kazerounian et al., 2008).

1.3.5 Activating invasion and metastasis

Metastasis is a multistep process where each one step is tightly linked to the previous. Initiation of metastasis occurs when cancer cells locally invade normal tissue, then intravasate nearby blood and lymphatic vessels to transit to distant locations, extravasate into the parenchyma of distant tissues, form small nodules of cancer cells, and finally growth of small nodules to macroscopic tumors (Scully et al., 2012). Evidence showed that cancer cells use a developmental reversible regulatory program called Epithelial-Mesenchymal Transition (EMT) to invade, resist apoptosis, and to disseminate (Barrallo-Gimeno & Nieto, 2005; Klymkowsky & Savagner, 2009; Polyak & Weinberg, 2009; Thiery & Sleeman, 2006; Yilmaz & Christofori, 2009). This program enables cells to convert to a mesenchymal state where cells exhibit increased motility (Peinado et al., 2011).

1.4 Diet

Environmental factors can be categorized to pivotal pillars of lifestyle (i.e. tobacco, alcohol, physical activity), external stimuli (radiation, pollution, infections, etc.) and diet (Irigaray et al., 2007). They are responsible for the variation of cancer incidences among different geographical regions. These variations are certainly related to the human exposure to different modifiable factors which may either increase or decrease cancer risk (Willett, 1995).

In 1991, nutritional guidelines for cancer prevention were published by the American Cancer Society (ACS) (Weinhouse et al., 1991). They were last updated in 2012 and are based on scientific evidence on nutrition in relation to cancer prevention (L.H. et al., 2012). Decades ago, research started focusing in elucidating the relationship between diet and cancer. In the past decade there was a high number of well-designed studies that gave reliable and plausible data. However, approaches using isolated nutrients have been selected instead of dietary patterns (A.B. & J., 2010). Limiting factors that influence the ability to determine the association between dietary patterns and cancer are diverse pathophysiology, study durations, identification of etiologically relevant time window and measurement error in dietary assessment methods (Harmon et al., 2015).

As dietary patterns can be defined as the quantities, proportions or combinations of different types of food and the frequency they are consumed (United States Department of Agriculture, 2014). Dietary patterns based on regular consumption of fruit, vegetables and by consequence the intake of aliments rich in selenium, folic acid, vitamins (B-12 or D) and antioxidants (i.e. carotenoids) play a protective role in cancer onset (Donaldson, 2004).

1.5 Cancer and Diet

1.5.1 Extra-Virgin Olive Oil and Cancer

A number of cancers in humans are induced by carcinogenic factors present in our environment, including our food. Approximately one-third of all cancer deaths are related to dietary factors and reduced physical activity (Bail et al., 2016). The incidence of all kinds of cancer in Mediterranean countries is lower than in the rest of Europe and the USA (Casaburi et al., 2013). This is mostly accounted for by the lower incidence of large-bowel, breast, endometrial, and prostate cancers, which have been linked to dietary factors, particularly low consumption of vegetables and fruit and, to a certain extent, high consumption of meat (Elnagar et al., 2011). Moreover, epidemiological data suggest an inverse correlation between regular consumption of olive oil and cancer risk (Calza et al., 2001; Levi et al., 1999; Schwingshackl et al., 2018). That has been supported by animal studies that showed a protective effect of olive oil against UV-induced damage of the skin (Budiyanto et al., 2000) and its ability to prevent the colon crypt aberrant foci growth and colon carcinoma in rats (Bartolí et al., 2000)

The beneficial effect of olive oil against cancer has been attributed to its antioxidant properties due to the presence of oleic acid and minor components with biological activity, such as vitamin E, sterols, and polyphenols (Robert W. Owen et al., 2000). Carcinogens present in our diet can damage DNA directly by forming covalent adducts with DNA or indirectly after being activated from inactive procarcinogens or via their induction of ROS production. Other carcinogens are not genotoxic but stimulate cell proliferation, thereby increasing the probability of spontaneous occurrence of errors during DNA replication. Phenolic compounds can directly scavenge radical species by acting as chain-breaking antioxidants and suppress lipid peroxidation by recycling other antioxidants, such as α-tocopherol, by donating a hydrogen atom to the tocopherol molecule (Boonstra & Post, 2004; Kouka et al., 2017).

1.5.2 Phenolics and Cancer

Accumulating evidence showed the favorable properties of minor though highly bioactive components of EVOOs, particularly the phenolic compounds, which have shown a broad spectrum of antioxidant, free radical scavenger and anti-inflammatory effects commonly associated with the origin of the main chronic diseases(Cárdeno et al., 2013). Additional studies have demonstrated the beneficial effects of polyphenols from olive oil and have been also related with their antiarrhythmic, platelet antiaggregant and vasodilatory effects (Cárdeno et al., 2013; Medina-Remon et al., 2013) Moreover, it has been described the vasoprotective effects of polyphenol consumption on blood pressure linked to their ability to increase endothelial synthesis of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)-mediated responses (Medina-Remon et al., 2013; Moreno-Luna et al., 2012). Among the endogenously produced genotoxic substances, the reactive oxygen species are the most important because they are continuously produced in all aerobic organisms (Boonstra & Post, 2004). For these reasons, oxidative stress has been strongly correlated to the onset of various degenerative diseases, particularly cancer (Halliwell, 2007). Chemo-preventive ability of olive oil could be attributable to the minor phenolic compounds that possess these antioxidant properties.

Polyphenols can interact with specific steps and/or proteins regulating the apoptotic process in different ways depending on their concentration, the cell system, the type or stage of the pathological process. Because of their ability to modulate cell death, olive polyphenols have been proposed as chemo-preventive and therapeutic agents (Giovannini & Masella, 2012; Visioli & Bernardini, 2011). In addition to the ability acting as a chemo-preventive agent with high antioxidant activity, the antitumor effects of olive oil phenols has been studied due to their capacity to inhibit proliferation and promote apoptosis in several tumor cell lines, by diverse mechanisms (Casaburi et al., 2013)

It is well established that the pathophysiology of common disease like cancer, cardiovascular disease, arthritis, and neurodegenerative disease are associated with chronic inflammation (McGeer et al., 2009; Solinas et al., 2009). The connection between inflammation and cancer can be thought of as consisting of two pathways: i)

an extrinsic mechanism, where a constant inflammatory state contributes to increased risk of developing cancer at certain anatomical sites (for example, the colon, prostate and pancreas) and ii) an intrinsic mechanism, where acquired genetic alterations triggers tumor development (for example an activation of various types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumor suppressor genes) (Mantovani et al., 2008). Members of the RAS family are the most frequently mutated dominant oncogenes in human cancer and activated oncogenic components of the RAS–RAF signaling pathway, in turn, induce the production of tumor promoting inflammatory chemokines and cytokines. Another oncogene, MYC, encodes a transcription factor that is over expressed in many human tumors; deregulated expression of this gene initiates and maintains key aspects of the tumor phenotype. In addition, MYC instructs the remodeling of the extracellular microenvironment, with inflammatory cells and mediators having important roles in this process (Campbell et al., 2002; Sumimoto et al., 2006).

The understanding of the relationship between inflammation and cancer is growing and the resulting improved knowledge base will undoubtedly allow for development of approaches to targeting inflammation in cancer that are worthy of clinical evaluation (Cárdeno et al., 2013).

In this sense, it is well-known that a deregulated cell proliferation and suppressed cell death provide the underlying basis for tumor progression (Evan & Vousden, 2001). Several studies have reported that the consumption of olive oil has a potential protective effect against several malignancies (Di Fronzo et al., 2007; R. W. Owen et al., 2004).

1.6 Phenolics and Endothelium function

1.6.1 Angiogenesis

Development of the vascular system is one of the earliest events of organogenesis. In mammals, first blood vessels of the fetus and the yolk sac develop de-novo by differentiation of endothelial stem cells (angioblasts) in situ resulting in the formation of an immature plexus, a process known as vasculogenesis. Then, the early vascular network gradually expands through processes such as germination, maturation and remodeling of the vessels leading to the development of a strictly organized and repetitive network, which consists of larger vessels branching into capillaries (**Figure 7**). Smooth muscle cells overlapping provides stability and regulate vascular tightness. The process of creating new vessels from pre-existing vessels is called angiogenesis (Carmeliet, 2005).

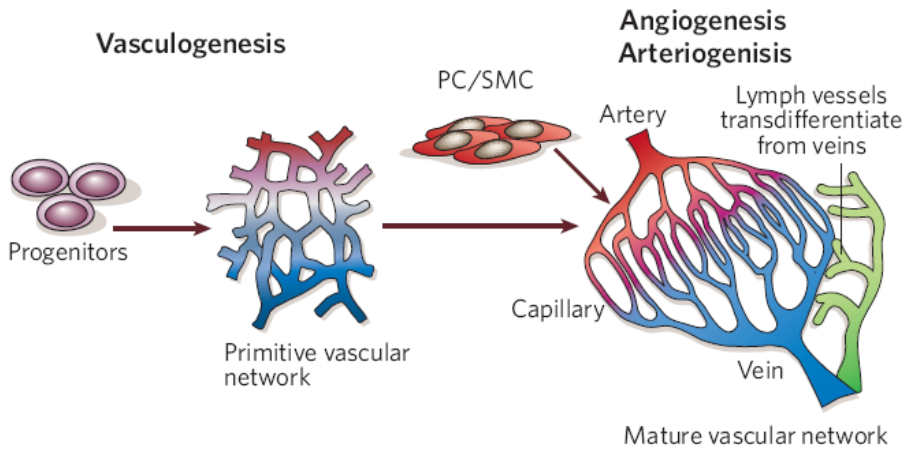


Figure 7: *Development of the vascular systems* (Carmeliet, 2005)

Members of the vascular endothelial growth factor (VEGF) family and their receptors (Vascular Endothelial Growth Factor Receptor-1/2/3, VEGFR-1/2/3) are the most important factors in vascular differentiation and angiogenesis, as they are necessary in the formation of early vessels during vascular differentiation and in the angiogenesis of the fetus and in the adult (Carmeliet et al., 1996).

VEGF signal transduction represents a critical step in controlling the rate of normal angiogenesis, while its overexpression plays an important role in the development of abnormal angiogenesis by deregulating the balance of its activators and inhibitors.

VEGF is an endothelial cell survival factor both in *in vitro* and *in vivo* models (Alon et al., 1995; Gerber et al., 1998). *In vitro*, VEGF inhibits serum-starved apoptosis through PI3K-dependent activation of the anti-apoptotic kinase Akt/PKB (Gerber et al., 1998). VEGF also induces the expression of the anti-apoptotic proteins Bcl-2 and A1, which inhibit activation of caspases and in turn increase the expression of two members of the IAP (Inhibitors of Apoptosis) family, survivin and XIAP (Tran et al., 1999) . *In vivo*, inhibition of VEGF results in extensive apoptotic changes in the vascular organs of the neonatal, but not adult, mouse (Gerber et al., 1999). In addition, it is observed that endothelial cells of newly formed vessels are dependent on VEGF. In contrast, endothelial cells of mature vessels, which are overlapped from pericytes, are independent from VEGF (Benjamin et al., 1999).

Vascular endothelium is essential to maintain a proper vasodilatation and regulates the metabolism of different molecules. Among these, molecules that are involved in coagulation system, platelets or circulating inflammatory mediators can lead to a dysfunction of the vascular endothelium (Borissoff et al., 2011). The binding of platelets each other and between platelets and circulating leukocytes produces co-aggregates which trigger leukocyte recruitment, and is important for plaque progression (Gleissner et al., 2008; Lievens et al., 2009).

1.6.1.1 VEGF family and its receptors

In mammals, the VEGF family consists of 5 members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PLGF (Placental Growth Factor) (Olsson et al., 2006a). Alternative splicing of several of the VEGF family members gives rise to isoforms with different biological activities (Bellou et al., 2009)

VEGF receptors, VEGF Receptor 1-3, share common regulatory mechanisms with other well-studied tyrosine kinase receptors (RTKs), such as PDGFR and EGFR (Epidermal Growth Factor Receptor). These mechanisms include dimerization and activation of tyrosine kinase, as well as the creation of a docking site for signal molecules. In addition, VEGFRs induce cellular responses like migration, survival, and proliferation of endothelial cells (Bellou et al., 2009; Olsson et al., 2006b; Donald R. Senger et al., 1983). Depending on the properties of the different ligands, VEGFRs form homodimers or heterodimers (Olsson et al., 2006a). Receptor dimerization is followed by kinase

receptor activity leading to receptor autophosphorylation. Phosphorylated receptors recruit interfering proteins and induce activation of signaling pathways comprising a set of messenger molecules.

1.6.1.2 VEGFR-2 signaling

VEGFR-2 is the major mediator of mitogenic and angiogenic effects, as well as the enhancement of vascular permeability induced by VEGF (Ferrara et al., 2003). Unlike VEGFR-1, the autophosphorylation of VEGFR-2 by VEGF is immediately detectable.

Only few molecules have been shown to interact directly with VEGFR-2. PLC-γ binds to the phosphorylated residue Tyr1175 and triggers the activation of a cascade of mitogen-activated protein kinases (MAPKs) / extracellular-regulated kinases 1/2, (ERK1/2) and endothelial cell proliferation (Takahashi et al., 2001). PLC-γ in turn activates PKC through the production of diacylglycerol and increased concentrations of intracellular calcium (**Figure 8**)

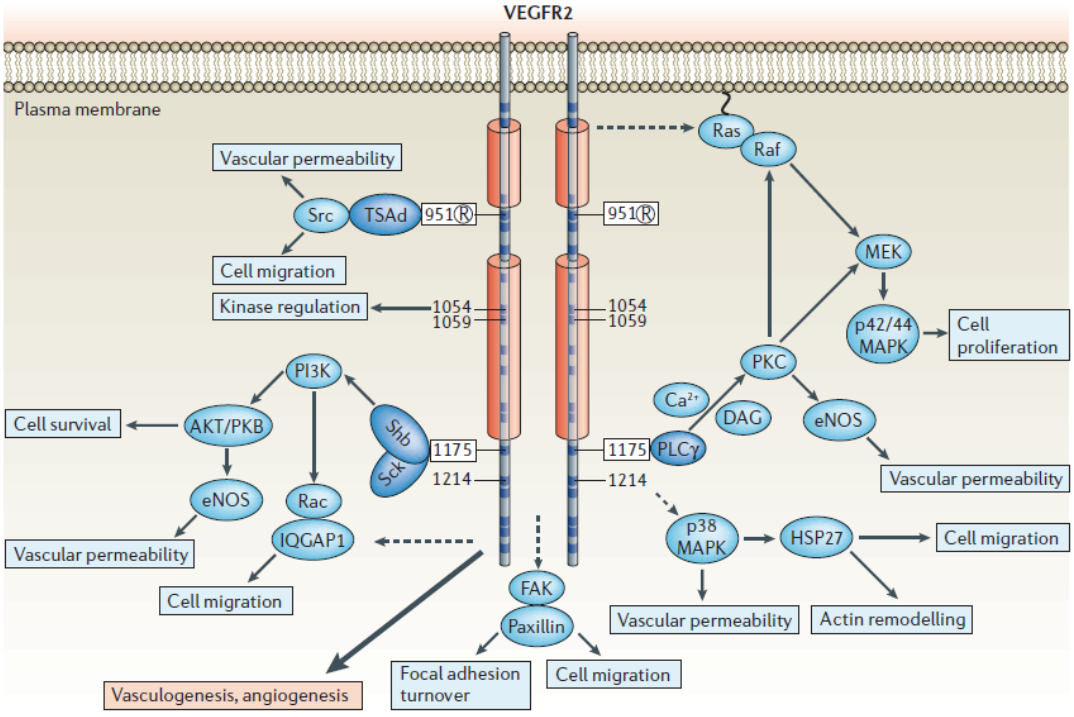


Figure 8:*VEGFR-2 signaling and its phosphorylation sites* (Olsson et al., 2006a)

Downstream activation of AKT / PKB (Serine / threonine kinase) by PI3K determines endothelial cell survival (Bagli et al., 2004; Fujio & Walsh, 1999). AKT / PKB kinase also regulates nitric oxide (NO) production through direct phosphorylation and activation of endothelial nitric oxide synthetase (eNOS).

VEGF does not appear to induce strong mitogenic signals in endothelial cells and the importance of Ras-Raf-MEK-MAPK pathway downstream of VEGFR-2 is unclear. However, VEGF induces Ras activation in human umbilical vein endothelial cells (HUVECs) and Ras has been implicated in the angiogenetic phenotype of endothelial cells (Meadows et al., 2001).

1.6.1.3 Macroscopic overview of VEGF expression in cancer

To sustain self-sufficiency or autonomy, tumor cells express VEGF and VEGFRs acting in an autocrine manner mostly (Cao et al., 2012; Hamerlik et al., 2012; Mak et al., 2010; D R Senger & Van De Water, 2000). VEGF is also secreted by stromal cells, including macrophages, endothelial cells, and fibroblasts. Autocrine VEGF signaling is generally characteristic of more aggressive types of cancer, including poorly differentiated carcinomas (Cao et al., 2012; Hamerlik et al., 2012; Mak et al., 2010). These carcinomas as characterized by embryonic gene expression pattern and activation of key developmental pathways, like angiogenesis an epithelial-to-mesenchymal transition (EMT) (Ben-Porath et al., 2008; Goel & Mercurio, 2013). T regulatory cells are attracted by VEGF in order to inhibit an immune response against tumor cells. It is likely that autocrine VEGF signaling in tumor cells is driven by hypoxia-inducing factor (HIF) (Mimeault & Batra, 2013).

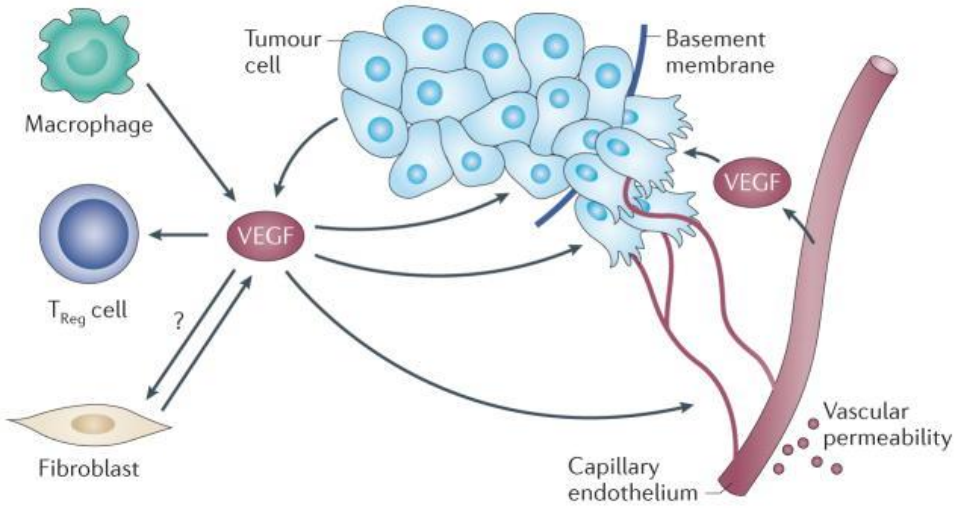


Figure 9: *VEGF functions in tumors* (Goel & Mercurio, 2013)

1.6.2 Role of phenolic compounds on the endothelium

Some environmental factors have been associated with impairments in vascular endothelium function, which produce an imbalance of the redox system, due to the increase of oxidative stress, and a decrease of nitric oxide availability at the vascular site (Ambrose & Barua, 2004; Brunner et al., 2005). Meals rich in EVOO have been described to have a favorable effect on the postprandial vasomotor function of the endothelium, enhancing the vasodilator capacity during this phase, in comparison to meals rich in saturated fatty acids (SFA). Thus, a 4-weeks period of consumption of Mediterranean diet rich in EVOO was associated with an improvement in endothelial function versus a SFA-rich diet in hypercholesterolemic patients (Fuentes et al., 2001). The same improvement was observed in elderly subjects who adhered to a Mediterranean diet, where the main source of monounsaturated fatty acids (MUFA) was provided by EVOO, and supplemented or not with the antioxidant coenzyme Q10 (Yubero-Serrano et al., 2012), mediated by a lower inflammation and oxidative stress, with a higher bioavailability of nitric oxide (Yubero-Serrano et al., 2012, 2013). An in vitro study analyzed the effect of the phenolic fraction from EVOO on the vascular endothelial growth factor (VEGF)-induced angiogenic responses of human endothelial cells. Human cultured endothelial cells with the EVOO phenolic fraction significantly reduced the VEGF-induced cell migration, inhibited the expression of MMP-2 and MMP-9, and reduced VEGF-induced intracellular ROS by modulating NADPH oxidase activity, compared with fasting control serum (Calabriso et al., 2016). Specifically, hydroxytyrosol (HT) was reported to increase nitric oxide production in endothelial cell culture (Storniolo et al., 2014). In a randomized controlled trial it was shown that healthy individuals who consumed oleuropein extracts showed a decrease in systolic and diastolic blood pressure (Lockyer et al., 2017).

According to literature, as described above, it is known that EVOO has beneficial effects on reducing the risk of diseases like these concerning the cardiovascular system and cancer (Casaburi et al., 2013; Fuentes et al., 2001). Most of the studies have been carried out using specific phenolic compounds isolated from EVOO (Elnagar et al., 2011; Fogli et al., 2016b). In Greece, very few studies have assessed the chemical profile of monovarietal origin EVOOs (Karkoula et al., 2012, 2014) while another study

assessed the chemical profile of olive-leaf extracts and their anti-proliferative effect on cancer and endothelial cells (Goulas et al., 2009). However, different EVOOs from different places have not been examined in order to investigate the effect of place of origin.

In our study, we examined the effect of 11 different Greek EVOOs of monovarietal origin on proliferation, migration, and survival of different cell line. In addition, for the first time EVOOs from Greece have been analyzed regarding their concentration in phenolic compounds, which gives us the opportunity to investigate whether any effect on non- or cancer cells is due to known phenolic compounds that are characterized and analyzed.

THESIS AIMS

Plant-derived dietary components contain high concentrations of **phytochemicals,** compounds devoid of any nutritional or energy-generating value that are responsible for the protective effects of plant-based diets. An abundance of different phytochemical classes, composed of many metabolites, have been identified in plant-based diets and shown to have biological activity *in vitro* and in experimental animal models. Olive oil as one of the components of the MD diet does contain several classes of phytoestrogens that have been shown to exert certain biological activities (see above). Several assays have been developed to monitor the quality of the olive oil and related products, which are based on chemical analysis by measuring the level of the main metabolites of certain classes of phytochemicals. Then, depending on the concentration levels of certain metabolites an indirect estimation of the potential health benefit of the said olive oil is deduced. Whereas this methodological approach at present represents probably the best solution to tackle the issue of the potential health benefits of olive oil, it still has several drawbacks.

The chemical analysis does not include measurement of all the phytochemical classes and certainly not all the metabolites. Therefore, any estimation regarding the health benefits of the olive oil under testing will have to rely only on the value of 1-2 metabolites (probably the most abundant) of selected phytochemical classes. Any conclusions based on such partial values will not provide an accurate picture of the health benefits of olive oil samples. For instance, some combinations of phytochemicals might be synergistic on certain enzymes (or other targets) and inhibitory on others. Considering that most probably there are many metabolites that remain to be identified and that metabolites of lower concentration might be more active than the abundant metabolites, then predicting biological activity by measuring the level of few phytochemical metabolites of ingested with olive oil, is not an accurate process.

The best approach, at present, towards estimating biological activity of olive oil samples is the development of *in vitro* assays that reconstitute the *in vivo* activity.

These assays should be in principle easy, reliable and scientifically sound. If possible, these assays should be amenable to automation to increase the sample throughput and reduce the time. Finally, the availability of a relevant mouse model for validation would be advantageous.

Phytochemicals protect against cancer, degenerative (Alzheimer's) and heart disease, lower blood pressure, reduce risk of diabetes, and exhibit antioxidant as well as anti-inflammatory activity. For most of these diseases or states *in vitro* assays can be developed.

Aim of this Thesis is to investigate the protective effect of Greek EVOO against cancer focusing on developing a certification approach that is based

i. On several *in vitro* assays that reconstitute the hallmarks of cancer, and
ii. On sampling that does not discriminate between the different phytochemical classes and their metabolites

Chapter 2 MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 DLD-1 cells

DLD-1 cells were cultured in 10cm dishes at 37°C, 5% CO_2 using McCoy's 5A (HyClone) medium supplemented with 10% FBS (Gibco), 100U/mL Penicillin and 100mg/mL Streptomycin (Gibco). Cells were passaged every 2 days in a ratio of 1 to 4. During passaging, cells were washed with 10mL PBS (PAN-biotech) and incubated with Trypsin (Gibco) for 2-3 minutes at 37°C. Thereafter, cells were dissociated, resuspended in 10mL fresh medium, and transferred to new dishes.

2.1.2. HCT-116 cells

HCT-116 cells were cultured in 10cm dishes at 37°C, 5% CO_2 using DMEM high glucose (PAN-biotech) medium supplemented with 10% FBS, 100U/mL Penicillin and 100mg/mL Streptomycin. Cells were passaged every 2 days in a ratio of 1 to 4. During passaging, cells were washed with 10mL PBS and incubated with Trypsin for 1-2 minutes at 37°C. Thereafter, cells were dissociated, resuspended in 10mL fresh medium, and transferred to new dishes.

2.1.3 Differences between DLD-1 & HCT-116 cells

Both cancer cell lines are colorectal, DLD-1 (Duke's type C colorectal adenocarcinoma) cells harbour the mutation E545K in PI3KCA and the mutation S421F in TP53, while HCT-116 (colorectal carcinoma) cells harbour the mutation H1047R in PI3KCA and they have wild type TP53 (Ahmed et al., 2013; Samuels et al., 2005). Both cell lines are diploid and heterozygous for each mutation.

2.1.4 MCF-7 cells

MCF-7 cells were cultured in 60mm dishes at 37°C, 5% CO_2 using DMEM high glucose medium supplemented with 10% FBS, 100U/mL Penicillin and 100mg/mL Streptomycin. Cells were passaged every 2 days in a ratio of 1 to 3. During passaging, cells were washed with 5mL of PBS and incubated with Trypsin for 2-3 minutes. Thereafter, cells were dissociated, resuspended in 5mL fresh medium, and transferred to new dishes.

2.1.5 Isolation of Human endothelial cells from neonatal umbilical cord vein

HUVECs (Human Umbilical Vein Endothelial Cells) are isolated from the umbilical cord vein of neonates according to the protocol described by Jaffe and colleagues (Jaffe et al. 1973). Until processed, the umbilical cord is maintained in phosphate buffer (PBS) on ice. Initially, the cords are washed externally with PBS. The vein of the cord is then palpated and opened on both sides. Three-way stop cocks are attached, which are tied with surgical thread. Subsequently, the vein in the cord is washed with PBS until the blood is removed and the solution is clear. The vein is filled using a syringe on one side with collagenase solution in PBS (0.1%, Collagenase type IA, Sigma), then incubated at 37 °C for 12 minutes in a jar with preheated PBS. 12 minutes later, this solution is collected along with detached cells, after applying 2 syringes at both ends of the cord. One of them contains 5% FBS M199 (Gibco), which inactivates the collagenase while the other is empty. By applying pressure, the medium is transported along the strand, from side to side, pulling the detached cells. The procedure is repeated twice, and the cell suspension is collected in a centrifuge tube. Then, the suspension is centrifuged for 5 minutes at 1400 rpm at room temperature. The supernatant is removed, and the cell pellet is resuspended in M199 supplemented with FBS, ECGS, Pen/Strep, and Heparin (Sigma). Cells are then plated on 10 cm plates which are pre-coated with 4ml rat type I collagen (BD Biosciences Collagen Type I RatTail).

2.1.6 HUVECs

Cells were cultured in 10cm dishes using M199 medium supplemented with 20% FBS, 100U/mL Penicillin, 100mg/mL Streptomycin, 0.05mg/ml Endothelial Cell Growth Extract (ECGS) and 0.05U/ml heparin. Dishes were preincubated with 4mL of collagen rat type I for 20 minutes in 37^{o}C. Thereafter, these dishes were washed 2 times with 10mL of PBS. Passaging of the cells was conducted every 3 days in a ratio of 1 to 3. During passaging, cells were washed with 10mL PBS, incubated with Trypsin for less than 1 minute. Subsequently, they were dissociated, resuspended in 10mL fresh medium, and transferred to new dishes. All nutrients were filtered for retention of insoluble particles. Cells were used up to passage 5.

2.1.7 Freezing cells

All cell lines were frozen the same way. Cells were passaged as described above and cultured at a confluency of 60-70%. They were dissociated and resuspended in 900uL heat-inactivated FBS with the addition of 100uL DMSO (Sigma). The cryovials were quickly transferred in -80^{o}C freezer for 24hrs before transferred to liquid nitrogen.

2.1.8 Thawing cells

All cell lines were thawed the same way. 20mL of fresh medium was added in the 10cm dish (collagen pre-coated dishes for HUVE cells). Cells were incubated in a 37^{o}C waterbath for 30sec and platted in 10cm dish. Medium was changed the day after thawing and cells were cultured as described above.

2.2 Biochemical Methods

2.2.1 Staining using Phalloidin

All cells were treated the same way except for HUVECs. Coverslips for HUVECs were coated with collagen rat type I so the cells can attach on the glass surface.

After cell treatment, cells were washed 2 times using PBS, fixed in 3.7% PFA (Merck) for 15 minutes and quenched with 50mM NH_4Cl (in PBS) for 15 minutes. Subsequently,

cells were permeabilized with 0.1% Triton X-100 (Sigma) (in PBS) for 5 minutes. Phalloidin (stock 2mM) was added in a ratio 1:100 (in PBS) for 20 minutes. The samples were mounted in Mowiol (EMD Millipore) mounting medium. Imaging was carried out using a Leica SP5 Confocal Microscope.

Phalloidin (Sigma) is a highly selective bicyclic peptide that is used for staining actin filaments (also known as F-actin). It binds to all variants of actin filaments in many different species of animals and plants. Typically, it is used conjugated to a fluorescent dye, such as Rhodamine. The tetramethyl rhodamine dye can be easily detected with a fluorescent microscope at Ex/Em = 546/575 nm.

2.3 Live imaging using IncuCyte® system

The IncuCyte® Live Imaging System was used. IncuCyte® is a live imaging system which can be used for a variety of techniques as it can monitor cells while growing in an incubator. Parameters and analysis can be controlled through its dedicated software.

2.3.1 Cell Growth

For the growth capability of the cells, the IncuCyte® live imaging system (Sartorius) was used. 10,000 cells were seeded in a 96-well plate in duplicates or triplicates for each condition. For HUVECs, 96-well plates were coated with collagen rat type I, incubated in 37^{o}C, 5% CO_2 for 20 minutes and washed 2 times with PBS. After an overnight incubation of the cells, each treatment was performed, and the plate was placed in the IncuCyte®. Pictures were obtained every 2-4 hrs for 48 hrs using 10x lens.

2.3.2 Wound healing assay for migration

The test migration, cells were seeded in a 24-well plate and incubated in a 37^{o}C, 5% CO_2 incubator (collagen rat type I coated plates for 20 minutes for HUVECs with 2 washes using PBS). Once cells were confluent, a scratch was made in each well using a 200p tip in a cross-shape manner. After 1 PBS wash, cells were treated with each

condition and the plate was placed in IncuCyte® for 16-24hrs using 4x lens and an interval of 2hrs.

Analysis of the images was performed using the first and the last picture (0h and 16h or 24h after) using Fiji-ImageJ.

2.3.3 Apoptosis-Necrosis assay using Propidium Iodide (PI)

Cells were seeded in a 96-well plate. After an overnight incubation in 37ºC, 5% CO_2, medium was replaced with serum free medium and cells were treated with EVOO (Extra-Virgin Olive Oil)-enriched medium or directly with EVOO. The plate was placed in IncuCyte for 72h taking images every 4h using 10x lens and red filter. After 24h, 48h and 72h, Propidium Iodide (PI, *Propidium Iodide*, *ThermoFischer Scientific*) was added in each well at a final concentration of 2ug/mL. As positive control H_2O_2 was used which causes necrosis at a concentration of 10mM.

2.3.4 Apoptosis assay

Cells were seeded in a 96-well plate. After an overnight incubation in 37ºC, 5% CO_2, medium was replaced with serum free medium and the addition of IncuCyte® Green Caspase Reagent (Sartorius) in a ratio of 1 to 1000 for a final concentration of 5uM. Each EVOO was added in a ratio 1:50. As positive control TRAIL (ImmunoTools) was used (ligand capable of initiating apoptosis in cancer and transformed cells (Wang & El-Deiry, 2003) at a concentration of 35ng/mL dissolved in 0.1%HSA plus IncuCyte® Green Caspase Reagent. As negative controls we used 1) HSA 0.1% plus IncuCyte® Green Caspase Reagent, 2) IncuCyte® Green Caspase Reagent alone in plain culture medium, and 3) cells in full medium without IncuCyte® Green Caspase Reagent since seeding. The plate was placed in the IncuCyte® for 48hrs with an interval of 2hrs using 10x lens and green filter.

The Caspase-3/7 Green Reagent is specially formulated for use in the Incucyte® Live-Cell Analysis System and can be added directly to tissue culture wells using a no wash, mix and read protocol to acquire live cell images of cells undergoing caspase-3/7 mediated apoptosis (**Figure 10**). In addition, the Caspase-3/7 Green Reagent is non-

perturbing to cell growth and morphology. When added to tissue culture medium, this inert, non- fluorescent substrate crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent staining of nuclear DNA (**Figure 11**). Kinetic activation of caspase-3/7 can be monitored morphologically using live cell imaging, and quantified using the Incucyte® Basic Analyzer (*Incucyte® Caspase-3/7 Green Reagent for Apoptosis*).

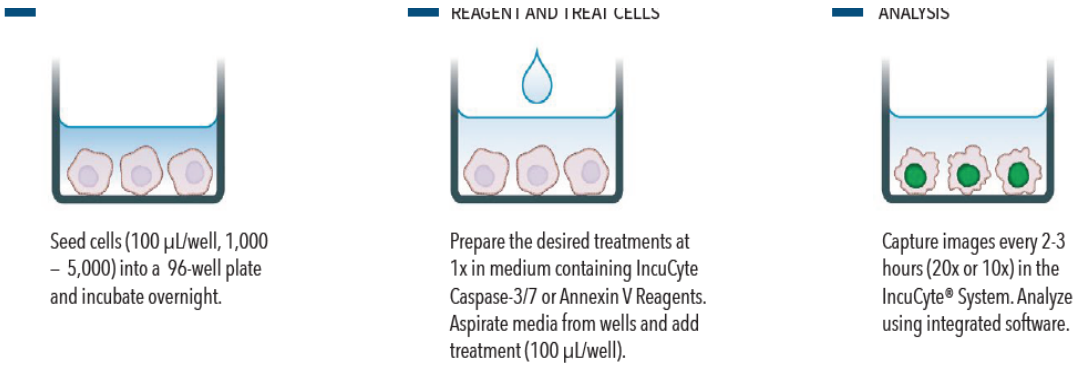


***Figure 10**: IncuCyte caspase-3/7 Green Reagent for apoptosis protocol for non-adherent cell lines*

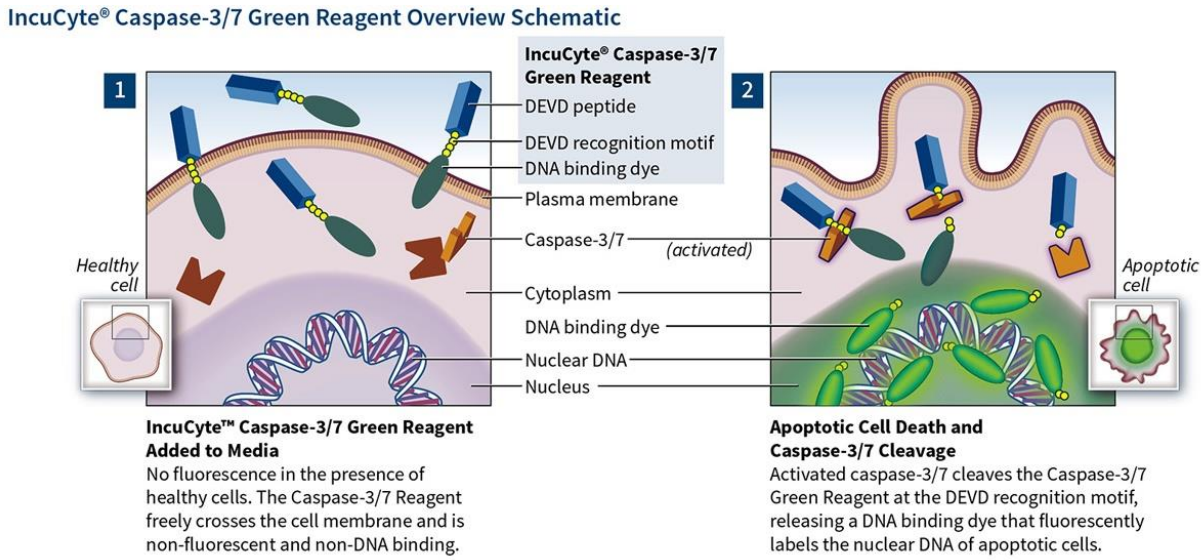


***Figure 11**: IncuCyte Caspase-3/7 Green Reagent for apoptosis schematic overview of DEVD recognition motif coupled to NucView 488, a DNA intercalating dye.*

2.4 Treatment using Extra-Virgin Olive Oil (EVOO)

2.4.1 Extra Virgin Olive Oil (EVOO) enriched medium

EVOO was added in serum free medium (Plain medium) in a ratio EVOO:medium 1:25. The mixture was vortexed for 20sec and an incubation at room temperature for 5 minutes. Cells were treated with each supernatant (Sup) carefully avoiding the oily phase on top of the mixture. 1:50 ratio was prepared from 1:25 by diluting 1:2. 1:100 ratio was prepared from 1:50 by diluting 1:2.

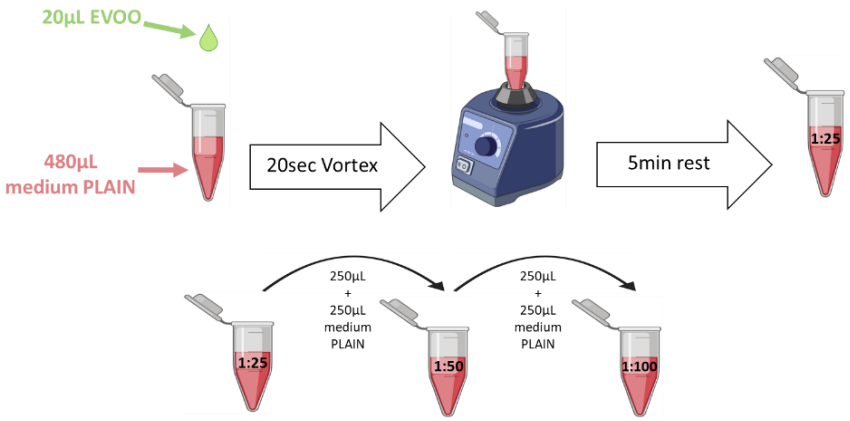


***Figure 12**: Schematic overview of production of EVOO (or Corn oil)-enriched culture medium and every dilution used in experiments.*

EVOO enriched medium contains the more hydrophilic components of EVOO, including the phenolic compounds.

2.4.2 Direct use of EVOO

EVOO was added directly in culture medium in a ratio of 1:25, 1:50 or 1:100 depending on the final volume of each plate, i.e. in 96-well plate, in a volume of 100ul 4ul of EVOO were added.

2.5 Extra-Virgin Olive Oil chemical analysis

Chemical analysis of each Extra-Virgin Olive Oil (EVOO) used in this thesis was performed by Prokopis Magiatis in NKUA, Department of Pharmacognosy & Natural

Products Chemistry according to Karkoula et al., 2012, 2014. Each EVOO is monovarietal. The chemical composition is shown in the table below.

Number of EVOO	1	2	3	4	5	6	7
EVOO	Elegia 2018	Eleagnos 2018	Eleagros 2018	Eleagnos 2019	Biotrichonis 2019	Biotrichonis 2019	Elegia 2019
Producer	Nikoloulia	Marinopoulos	Zoris	Marinopoulos	Trapeziotis	Koumasis	Nikoloulia
Variety	Koroneiki	Koroneiki	Koutsourelia	Lianolia-Koutsourelia	Kalamon	Koutsourelia	Koroneiki
Oleocanthal	165	176	72	250	152	148	165
Oleacein	124	129	38	214	47	66	117
Oleocanthal + Oleacein (index D1)	288	305	111	465	198	214	282
Listroside aglycon (monoaldehyde form)	23	28	<5	163	21	21	62
Oleuropein aglycon (monoaldehyde form)	48	46	<5	221	13	26	87
Listroside aglycon (dialdehyde form)	181	197	<5	395	35	116	190
Oleuropein aglycon (dialdehyde form)	92	107	<5	253	0	90	143
Total tyrosol derivatives	369	401	72	808	207	285	418
Total hydroxytyrosol derivatives	264	282	38	688	59	182	348
Total polyphenols analyzed	**633**	**683**	**111**	**1,497**	**266**	**467**	**765**

Number of EVOO	8	9	10	11	12	13
EVOO	HOLYoil 2018	HOLYoil 2020	Golden Tree 2020	Olive leaves enriched EVOO	Corn Oil - No phenolics	Control Oil - No phenolics
Producer	Martinou	Martinou	Zotou	Therianos		
Variety	Koroneiki	Koroneiki-Askoutha-Kolovi		-	-	-
Oleocanthal	185	285	461	-	-	-
Oleacein	60	211	247	-	-	-
Oleocanthal + Oleacein (index D1)	245	496	62	-	-	-
Listroside aglycon (monoaldehyde form)	39	114	65	-	-	-
Oleuropein aglycon (monoaldehyde form)	44	204	109	-	-	-
Listroside aglycon (dialdehyde form)	102	342	78	-	-	-
Oleuropein aglycon (dialdehyde form)	36	294	633	-	-	-
Total tyrosol derivatives	330	740	390	-	-	-
Total hydroxytyrosol derivatives	135	709	708	-	-	-
Total polyphenols analyzed	**466**	**1450**	**1,023**	**-**	**-**	**-**

***Table 1**: Chemical analysis of each EVOO to identify phenolic compounds and their concentration*

Chapter 3 RESULTS

3.1 Cell growth during EVOO treatment

Initially we investigated the effect of various EVOOs on cell growth either using the EVOO enriched medium or after direct treatment of cells with EVOO. The ratio between EVOO:medium was 1:25, following the literature (Goren et al., 2019)

3.1.1 DLD-1 cells treated using EVOO-enriched media and direct addition of EVOO

When DLD-1 cells were treated for 48 hours using the EVOO enriched media in a ratio of 1 to 25, cell growth was significantly inhibited, as shown in **Figure 13**. Cells were cultured in serum free medium, since FBS contain factors that stimulate cell growth and we considered that we could not be able to observe the effect of EVOO on cell growth under fully stimulated conditions.

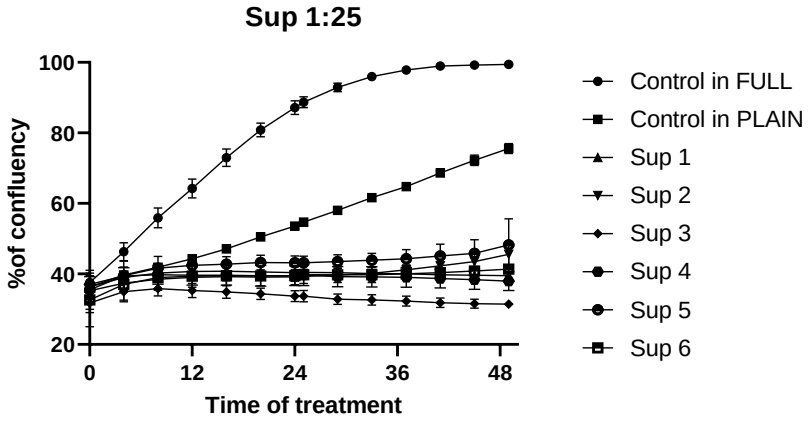


***Figure 13**: **Effect of EVOO-enriched medium on DLD-1 cell growth.** DLD-1 cells were treated with 6 different EVOO-enriched plain culture medium in a ratio EVOO:medium 1:25. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*

We also added the EVOOs directly to DLD-1 cells in full medium (10% FBS). The results are shown in **Figure 14**.

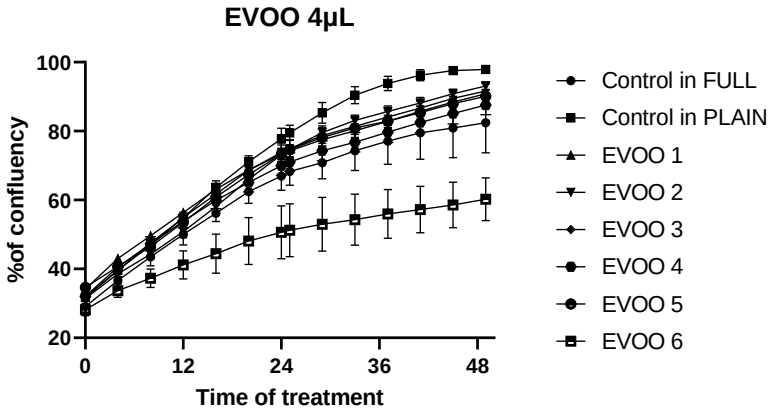


Figure 14: ***Effect of EVOOs on DLD-1 cell growth after direct addition in culture medium.*** *DLD-1 cells were treated directly with 6 different EVOOs, Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software.*

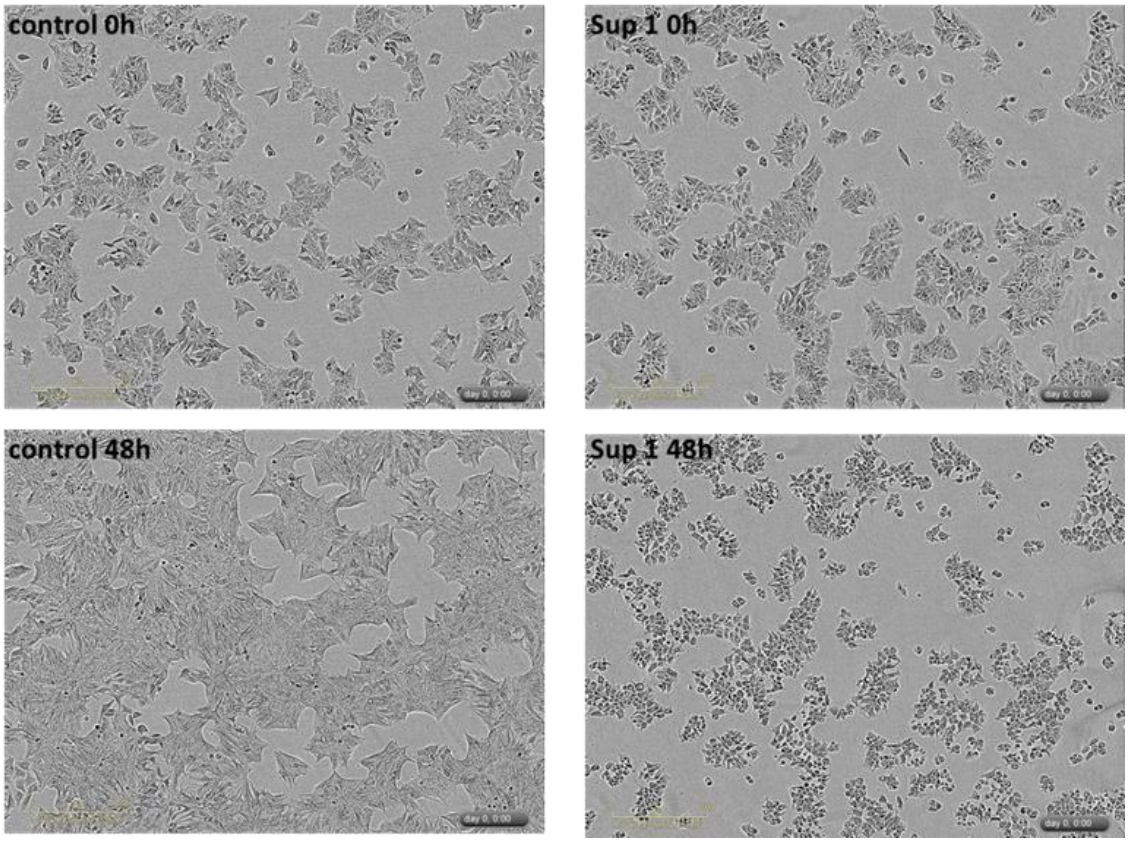


Figure 15: ***Effect of EVOO1-enriched medium on DLD-1 cell growth.*** *DLD-1 cells were treated or not with EVOO1-enriched medium at a ratio EVOO1:Medium 1:25., Cell growth was monitored using IncuCyte for 48h, taking image every 4h. Representative images are shown from the untreated and treated cells at 0h and 48h*

3.1.2 HCT-116 cells treated using EVOO-enriched and direct addition of EVOO

We repeated the previous experiments using the HCT116 cells line, which is a colorectal cancer cell line with different mutations in PI3KCA and TP53 genes (as described in MATERIALS AND METHODS/2.1.3 Differences between DLD-1 & HCT-116 cells, pg49). HCT116 cells were cultured in a 96-well plate before EVOOs or EVOO-enriched medium was added. Cell growth was monitored using IncuCyte. As shown in **Figure 16**, when HCT-116 cells were treated with the EVOO enriched media in a ratio EVOO:plain culture medium 1:25, cell growth was significantly inhibited, similar to the inhibition of DLD-1 cells.

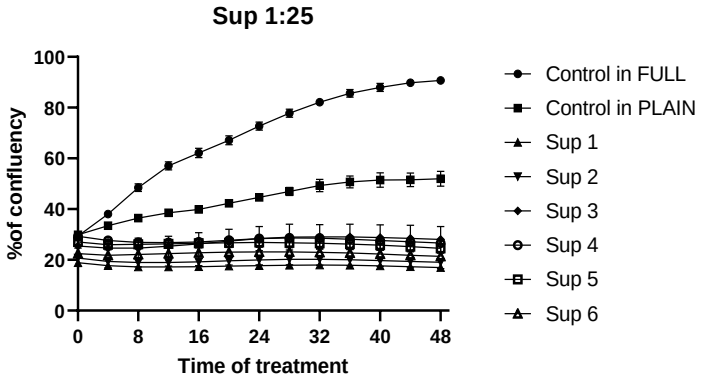


Figure 16*:*Effect of EVOO-enriched medium on HCT-116 cell growth. *HCT-116 cells were treated with 6 different EVOO-enriched plain culture medium in a ratio EVOO:medium 1:25, Cell growth was monitored using IncuCyte for 48h taking images evert 4h. Percentage (%) of confluency is calculated using IncuCyte software*

As for the direct treatment of HCT-116 cells grown in full medium (10% FCS) using EVOO in a ratio 1:25, inhibition was also significant (**Figure 17**).

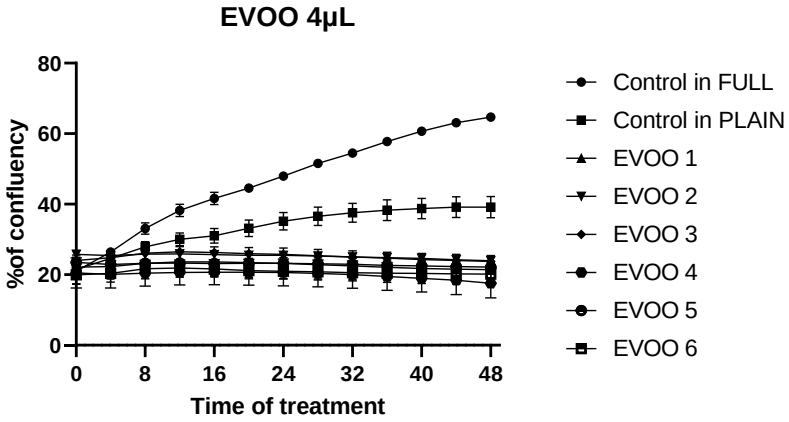


***Figure 17**: **Effect of EVOOs on HCT-116 cell growth after direct addition in culture medium.** HCT-116 cells were treated directly with 6 different EVOOs, Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*

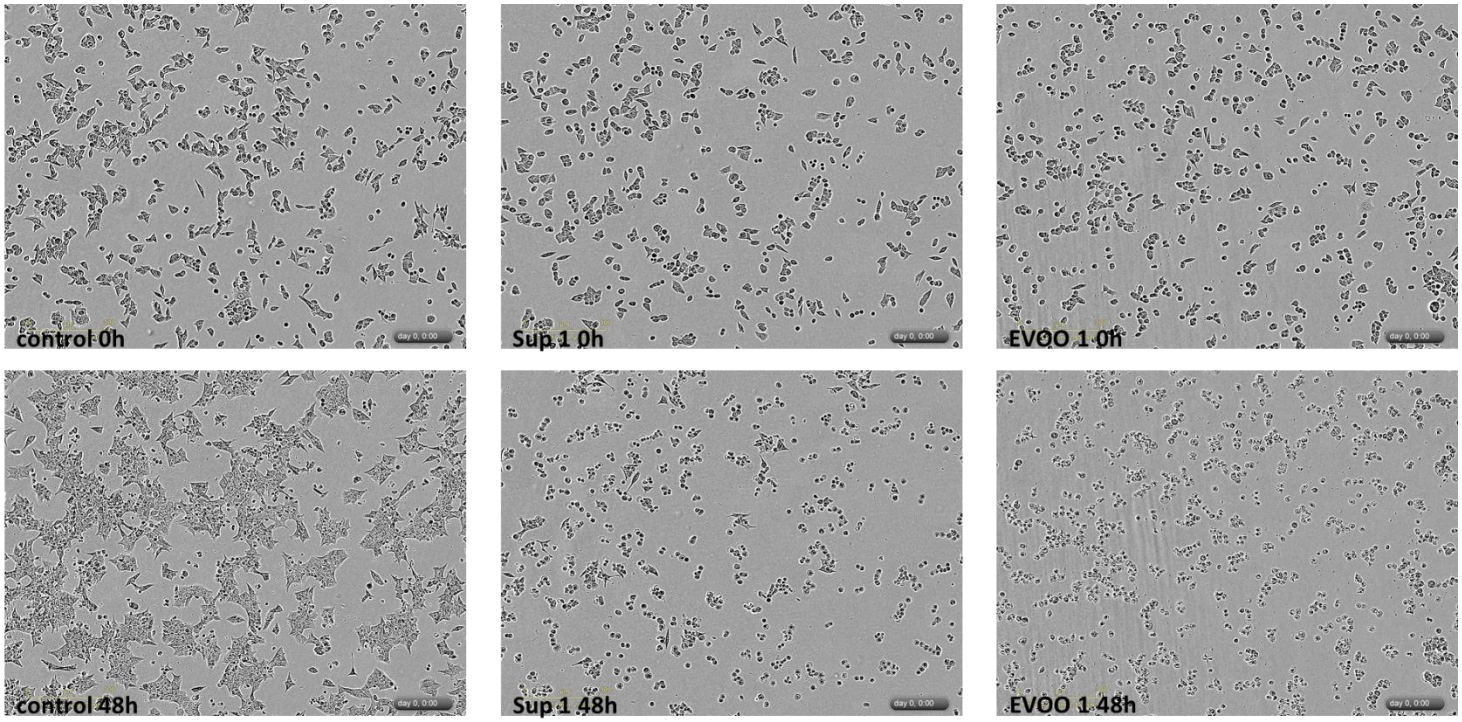


***Figure 18**: **Effect of EVOO1 on HCT-116 cell growth.** HCT-116 cells were treated or not with EVOO1-enriched medium (Sup1) at a ratio EVOO1:medium 1:25 or treated directly with EVOO1. Cell growth was monitored using IncuCyte for 48h, taking image every 4h. Representative images are shown from the untreated and treated cells at 0h and 48h*

3.2 Dose-dependent inhibition

As we examined the effect of EVOO in cancer cell growth (DLD-1 and HCT-116) following 2 different ways of treatment, EVOO enriched media and direct addition of

EVOO, we concluded that either addition of 4ul directly in cells cultured in a 96-well plate (100ul final volume) or treatment of cells with EVOO-enriched medium at a ratio of EVOO-medium 1:25 completely inhibited cell growth. Thus, we decided to examine EVOO-effect on cell growth using three different amounts or dilutions for each EVOO.

To accomplish that, we examined the effect of each EVOO enriched media in dilutions, 1:25, 1:50 and 1:100. We used these specific dilutions in order to establish a baseline for their effect on cell growth inhibition.

3.2.1 DLD-1 cells

As shown in **Figure 13**, we have validated the complete inhibitory effect on cell growth by EVOO-enriched culture medium at a ratio 1:25. Interestingly, at the same ratio, neither corn oil (Sup corn)- nor olive oil poor in phenolic compounds (Sup Ctl)-enriched culture medium presented any inhibition on cell growth (**Figure 20**).

Using the EVOO-enriched medium at a dilution of 1:50 also resulted in cell growth inhibition, in most cases. However, EVOO5- and EVOO8-enriched medium did not have any effect on % of confluency according to IncuCyte software. This was also noticeable from the images taken at the end of the 48h-treatment (**Figure 19**). These results could be attributed to the fact that both EVOO5 and EVOO8 were two of the oils with the lower concentration of phenolic compounds with 268mg/kg and 466mg/kg, respectively. However, EVOO3, which is the olive oil with the lower phenolic compound concentration (110mg/Kg) caused a significant cell inhibition when used to enrich culture medium at a dilution 1:25.

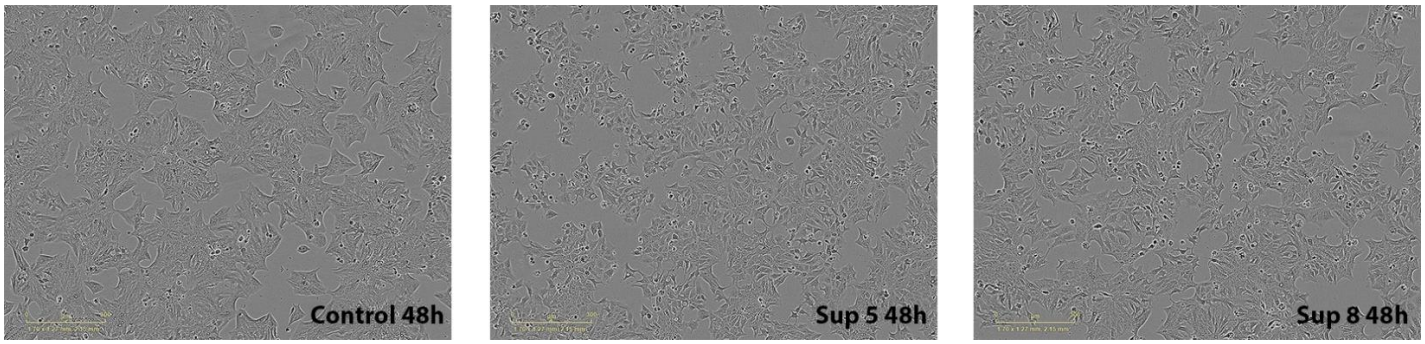
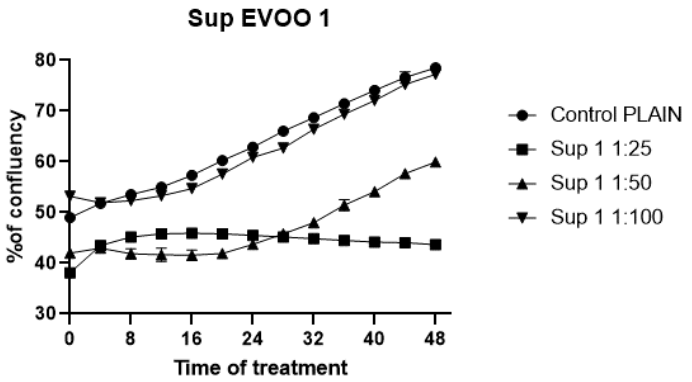
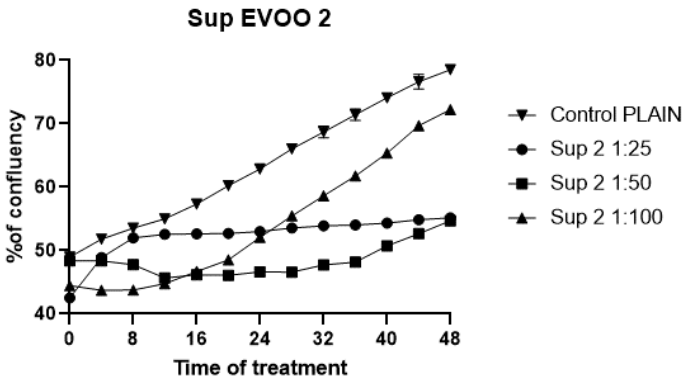


***Figure 19**: **Effect of EVOO5- and EVOO8-enriched medium on DLD-1 cell growth.** DLD-1 cells were treated or not with EVOO5- and EVOO8-enriched medium at a ratio EVOO:medium 1:50 (Sup5 and Sup8 respectively). Cell growth was monitored using IncuCyte for 48h, taking image every 4h. Representative images are shown from the untreated and treated cells at 48h.*

Only EVOO4- and EVOO10 and EVOO11-enriched medium at a dilution 1:100 caused moderate cell growth inhibition, which was expected since these EVOOs have the maximum phenolic concentration of all EVOOs tested.

As negative controls we used corn oil and a commercial EVOO poor in phenolics. It is known that corn oil does not contain any phenolic compounds (Goren et al., 2019). Corn oil and the commercial EVOO do not have any inhibitory effect in DLD-1 growth even when the lowest dilution (1:25) was used.


Sup EVOO 1
%of confluency
Time of treatment
Control PLAIN
Sup 1 1:25
Sup 1 1:50
Sup 1 1:100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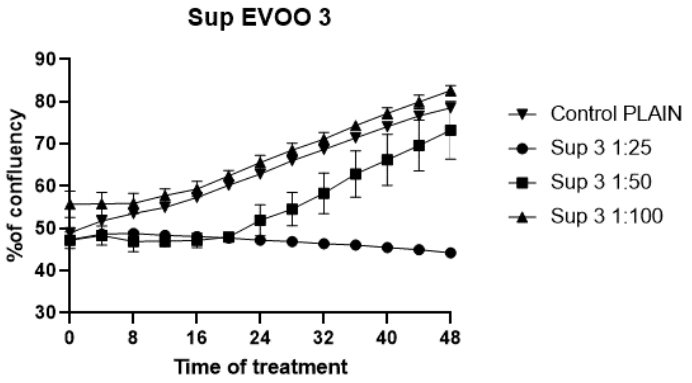
Time of treatment
Control PLAIN
Sup 2 1:25
Sup 2 1:50
Sup 2 1:100


Sup EVOO 3
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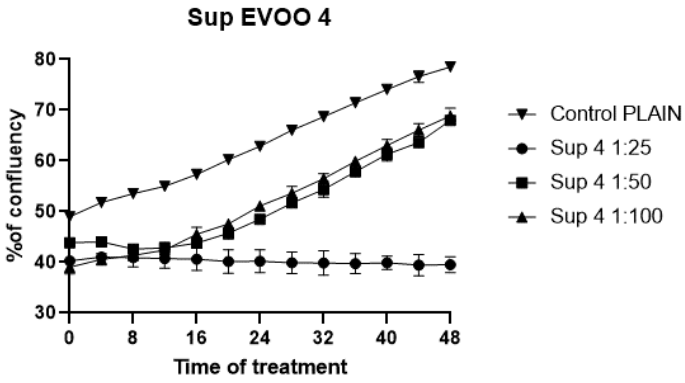
Time of treatment
Control PLAIN
Sup 3 1:25
Sup 3 1:50
Sup 3 1:100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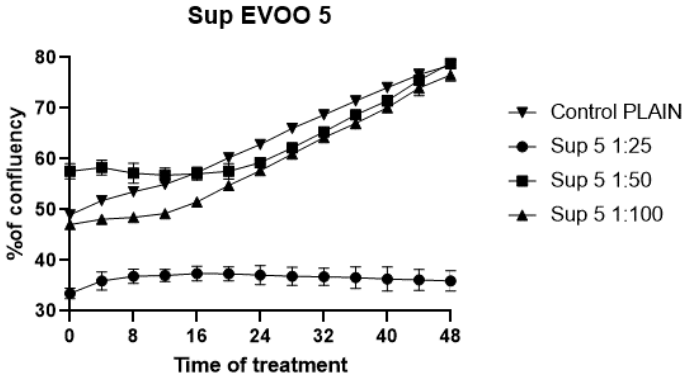
Time of treatment
Control PLAIN
Sup 4 1:25
Sup 4 1:50
Sup 4 1:100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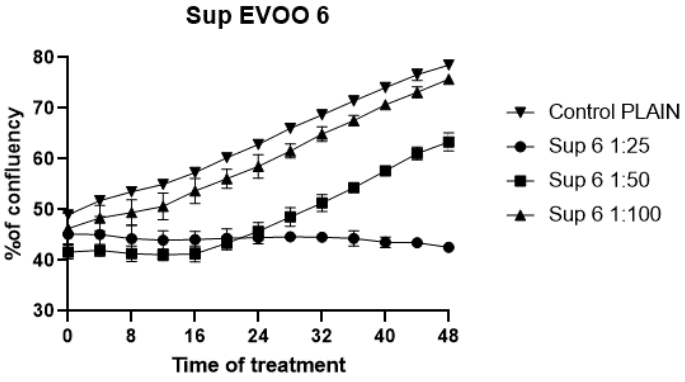
Time of treatment
Control PLAIN
Sup 5 1:25
Sup 5 1:50
Sup 5 1:100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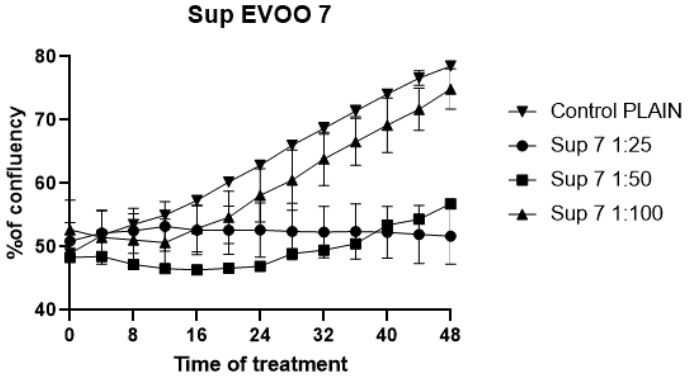
Time of treatment
Control PLAIN
Sup 6 1:25
Sup 6 1:50
Sup 6 1:100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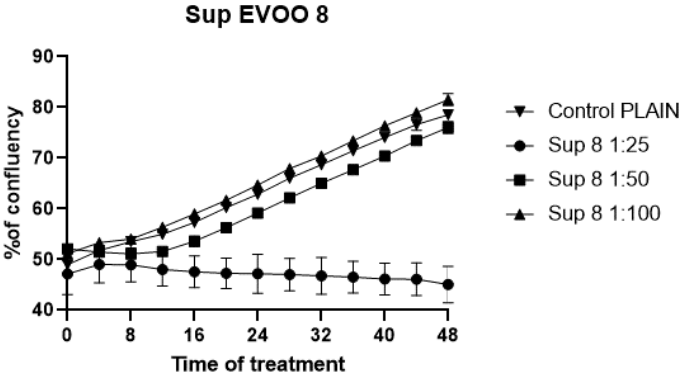
Time of treatment
Control PLAIN
Sup 7 1:25
Sup 7 1:50
Sup 7 1:100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Time of treatment
Control PLAIN
Sup 8 1:25
Sup 8 1:50
Sup 8 1:100

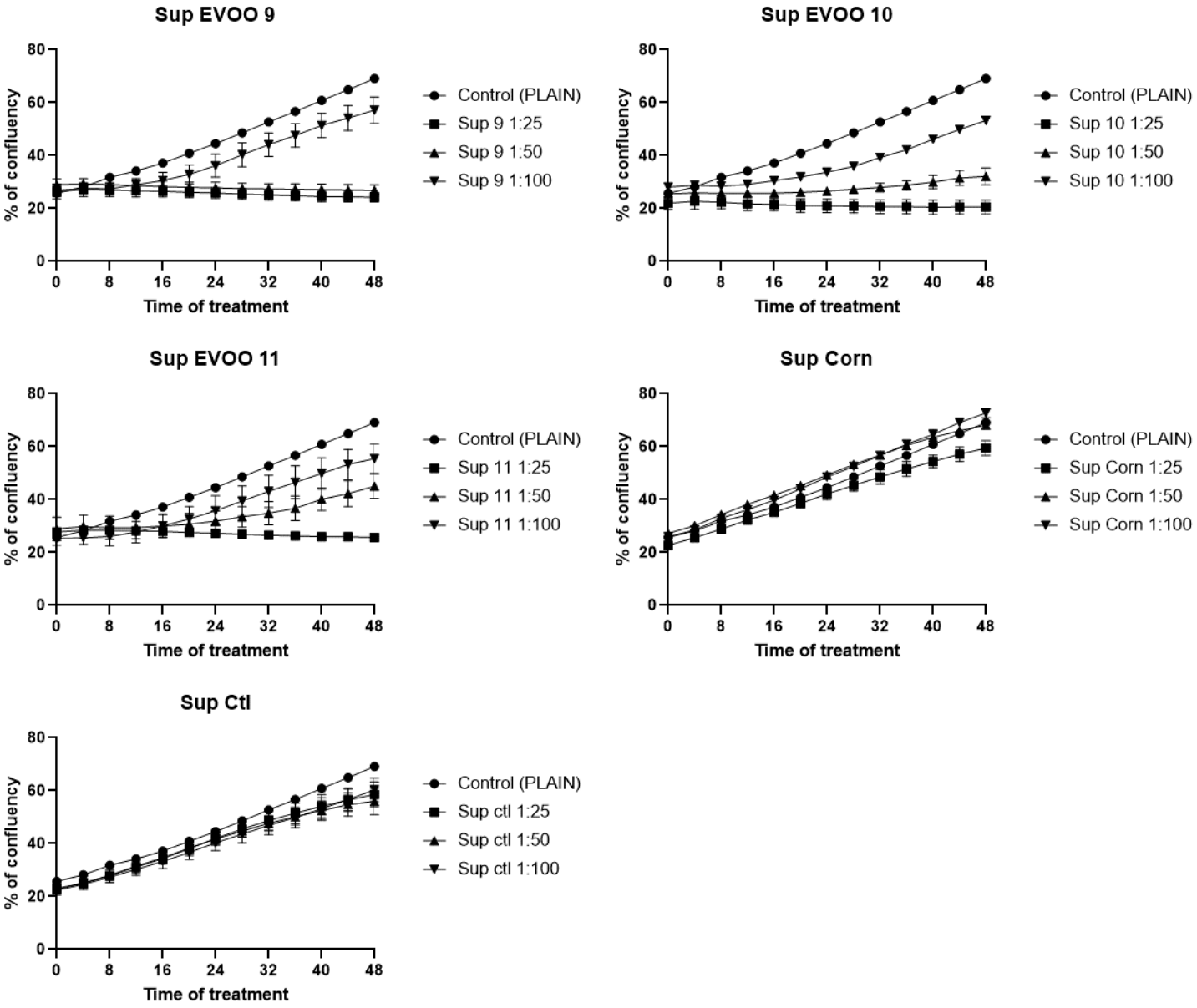
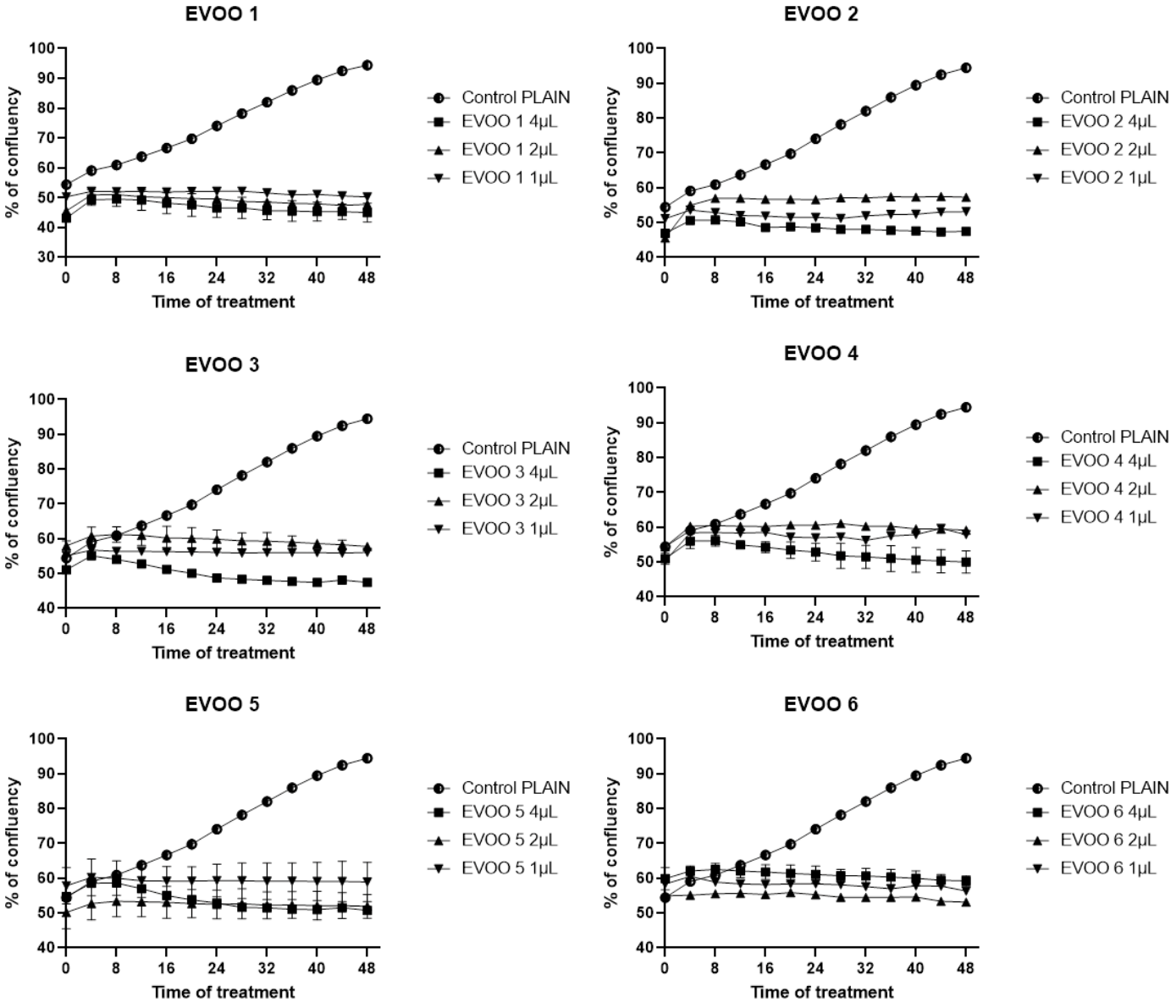


Figure 20: Effect of EVOO-enriched medium on DLD-1 cell growth. *DLD-1 cells were treated with 13 different EVOO-enriched plain culture medium in 3 different ratios EVOO:medium, 1:25, 1:50 and 1:100. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*

In order to examine the effect of direct addition of EVOO on cell growth, we also performed the experiments using three volumes, i.e. 4, 2 and 1µl, of each EVOO in 100 µl of culture medium, resulting in the same dilutions as those of the EVOO-enriched media, 1:25, 1:50 and 1:100. By doing this, we achieved a continuous extraction/presence of phenolic compounds in cell culture media throughout the experiment.

As shown in **Figure 21**, DLD-1 growth was completely inhibited by EVOOs in culture medium, in all three dilutions. Only EVOO 8 had no inhibitory effect in the highest dilution (1:100). Corn oil and commercial EVOO, both poor in phenolic compounds, had no inhibitory effect.



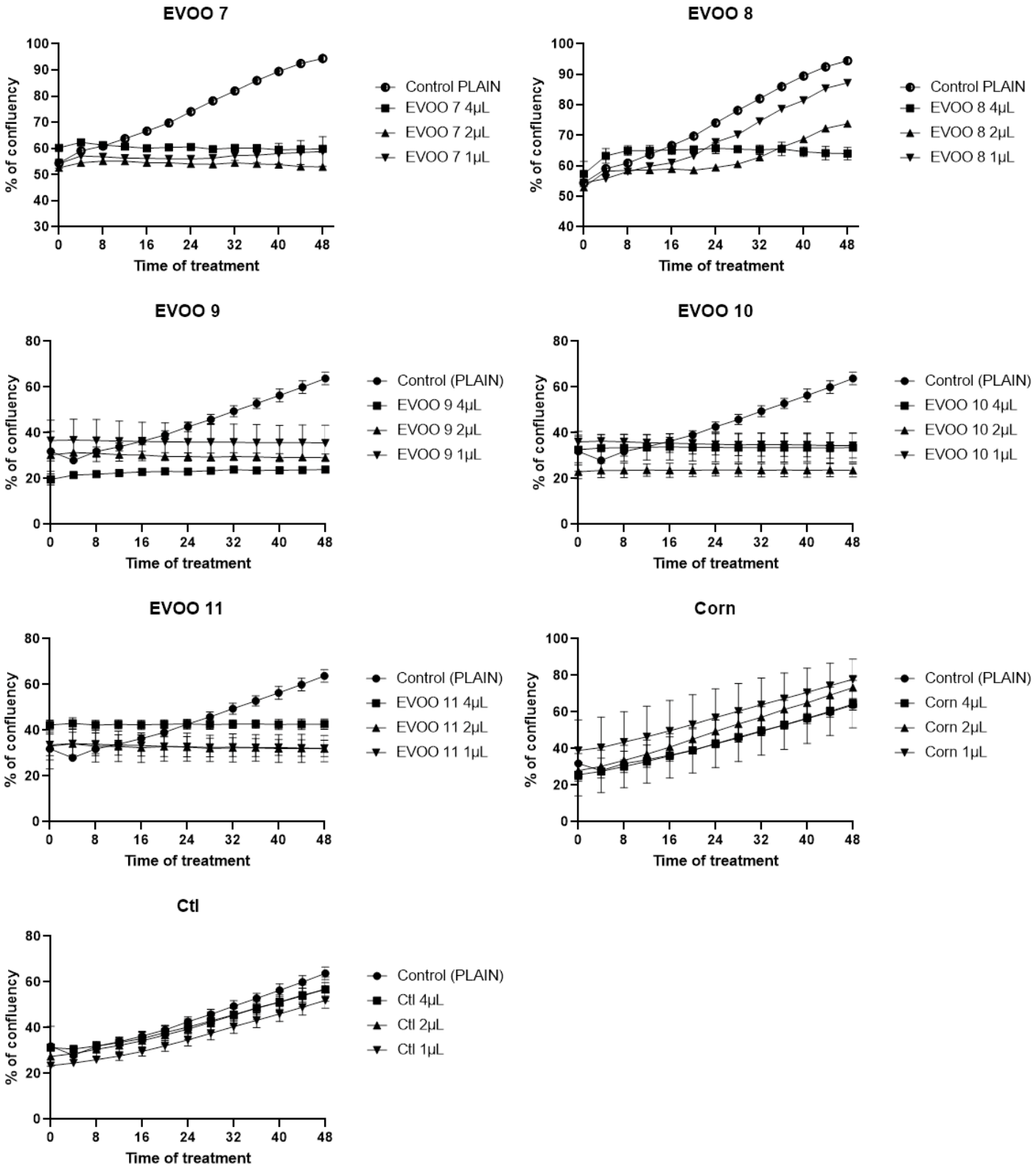
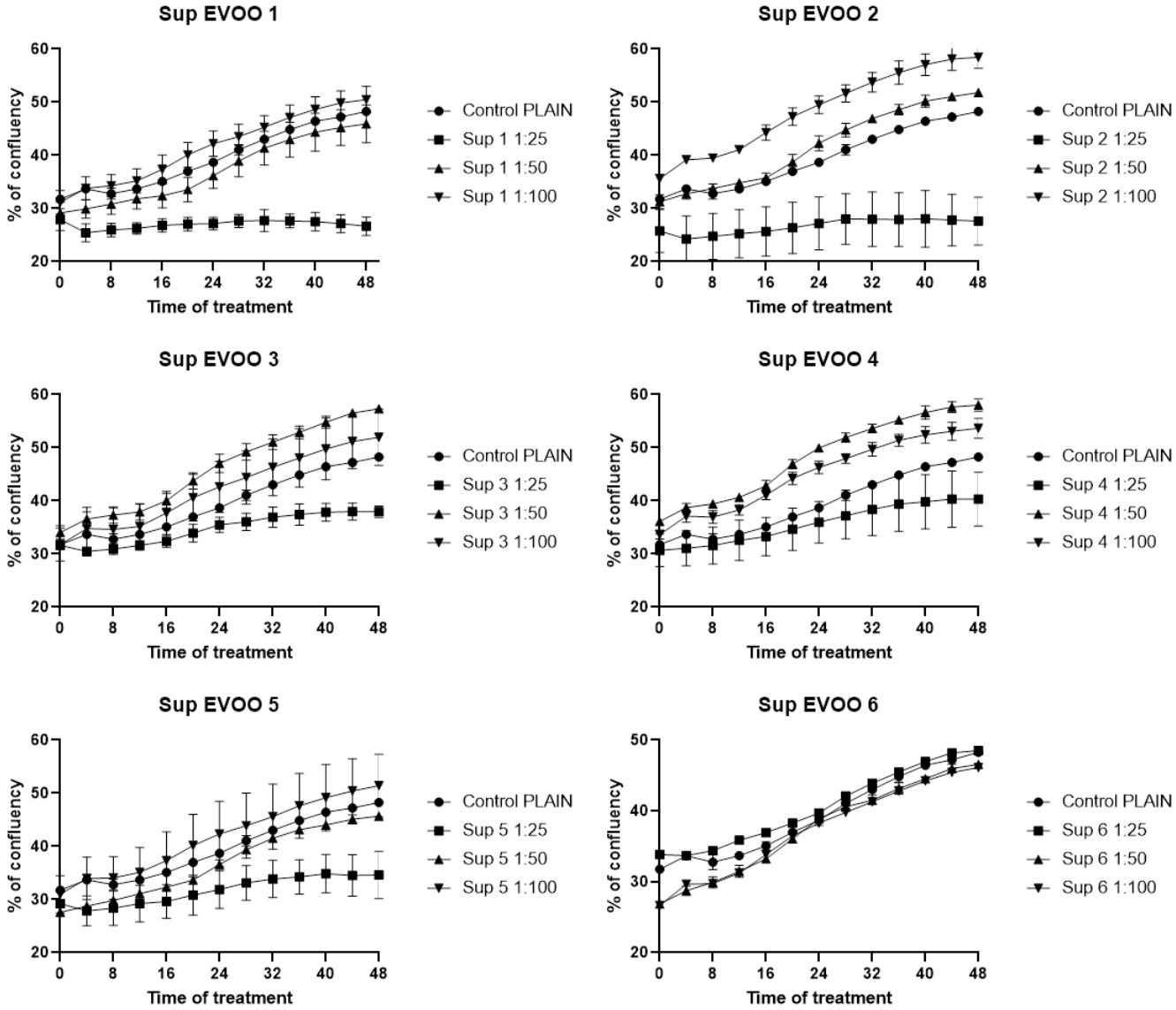


Figure 21: Effect of EVOOs on DLD-1 cell growth after direct addition in culture medium. *DLD-1 cells were treated directly with 13 different EVOOs. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software.*

3.2.2 HCT-116 cells

Next, we examined EVOO effect on HCT-116 cell growth. As shown in **Figure 22**, when HCT-116 were treated with EVOO-enriched media, cell growth inhibition was complete in case of EVOO1, 2 and 7 at the highest concentration. However, some EVOO-enriched culture medium had moderate inhibition when highest concentration was used. In 1:50 or 1:100 dilutions we observed minor or even no effect on cell growth. Compared to DLD-1 cell line, HCT116 are more resistant to growth inhibition when cultured in EVOO-enriched medium.



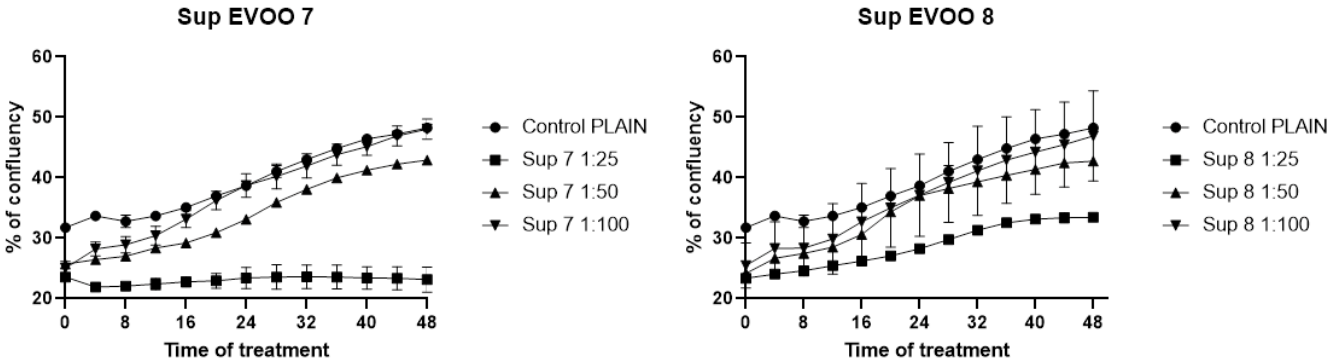
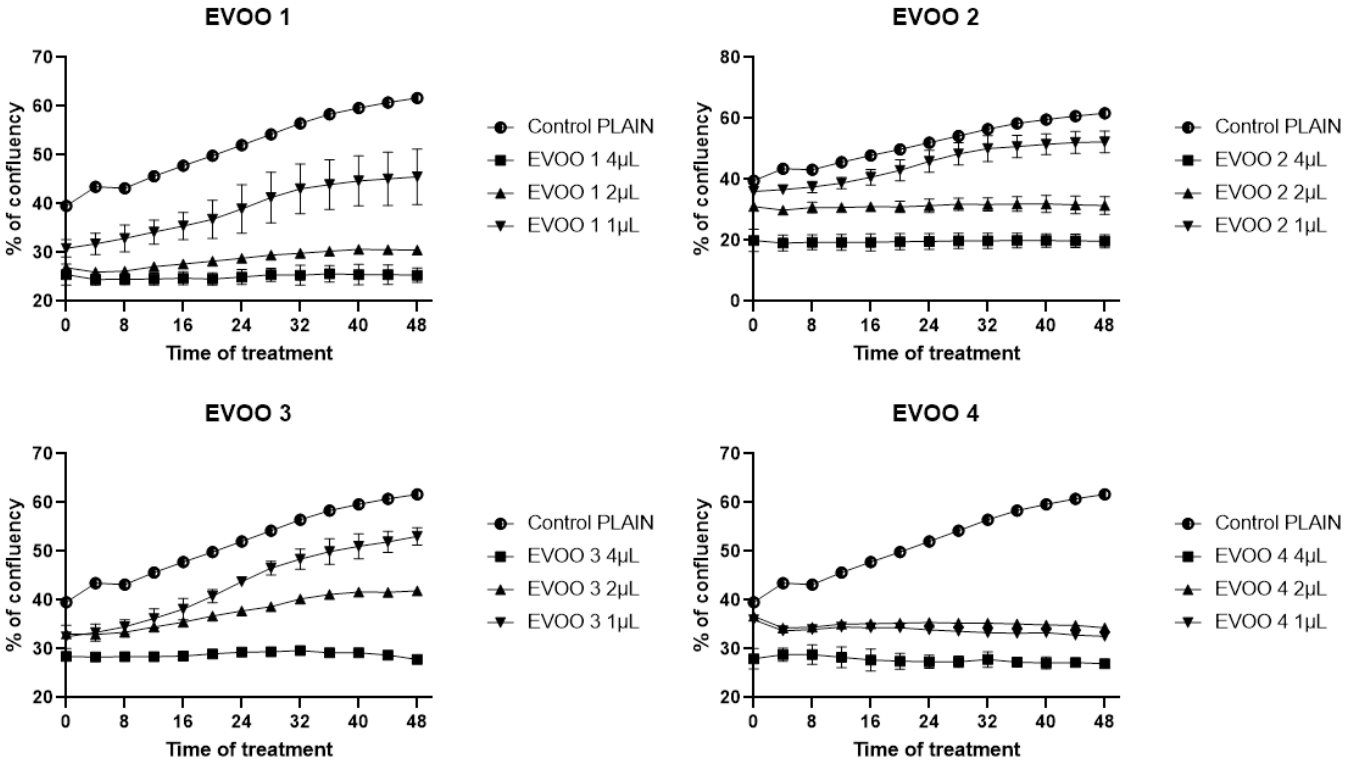


Figure 22: Effect of EVOO-enriched medium on HCT-116 cell growth. *HCT-116 cells were treated with 8 different EVOO-enriched plain culture medium in 3 different ratios EVOO:medium, 1:25, 1:50 and 1:100. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*

Regarding direct treatment of HCT-116 cells using various EVOOs (**Figure 23**), inhibition was stronger compared to EVOO-enriched media, as expected. Among all EVOOs used in HCT-116 for direct treatment, 1:25 dilution exhibited strong growth inhibition in all cases. In most cases, 1:50 dilution had significant growth inhibition, whereas dilution 1:100 had strong effect using EVOO4, 5, 6 and 7.



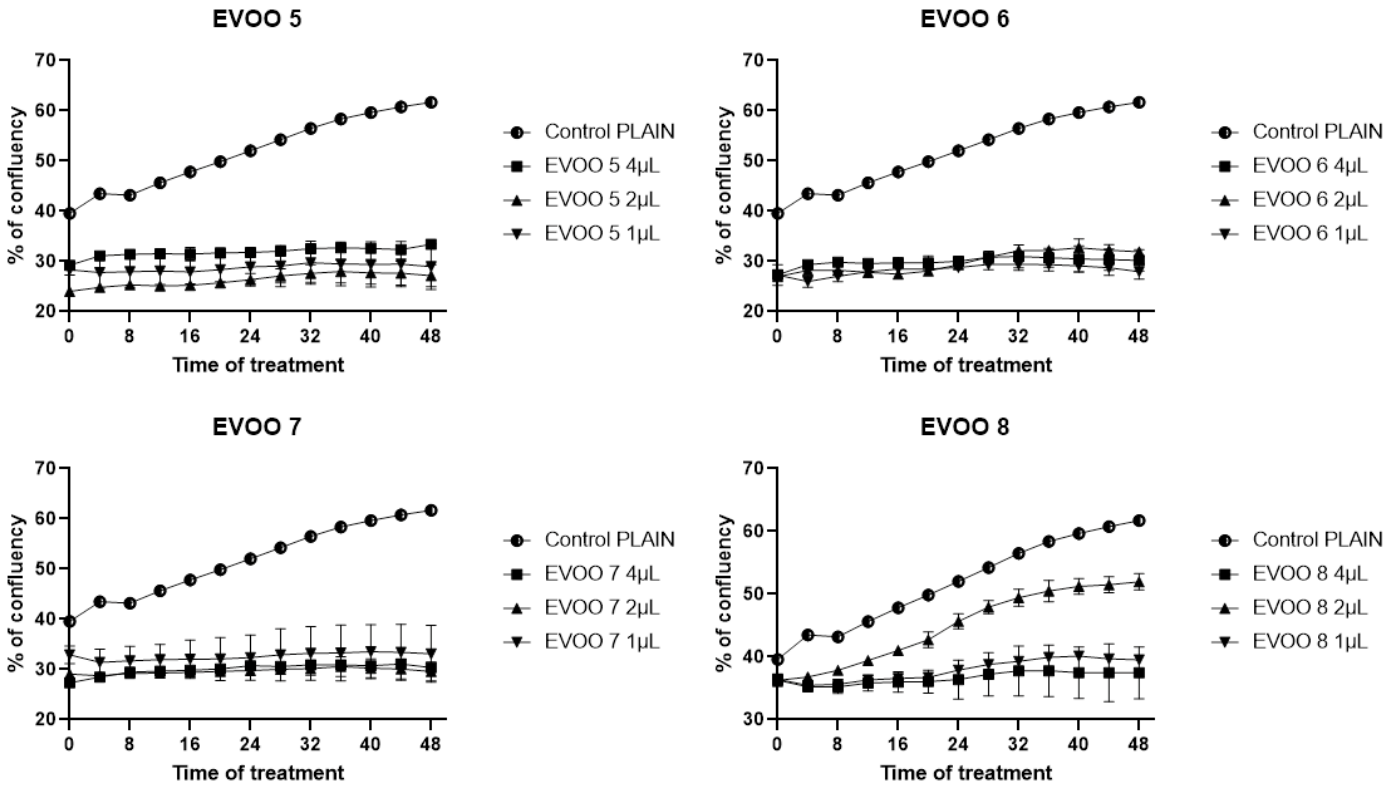


Figure 23: Effect of EVOOs on HCT-116 cell growth *HCT-116 cells were treated directly with 8 EVOOs. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software*

3.2.3 HCT-116 are more resistant to EVOO-enriched media

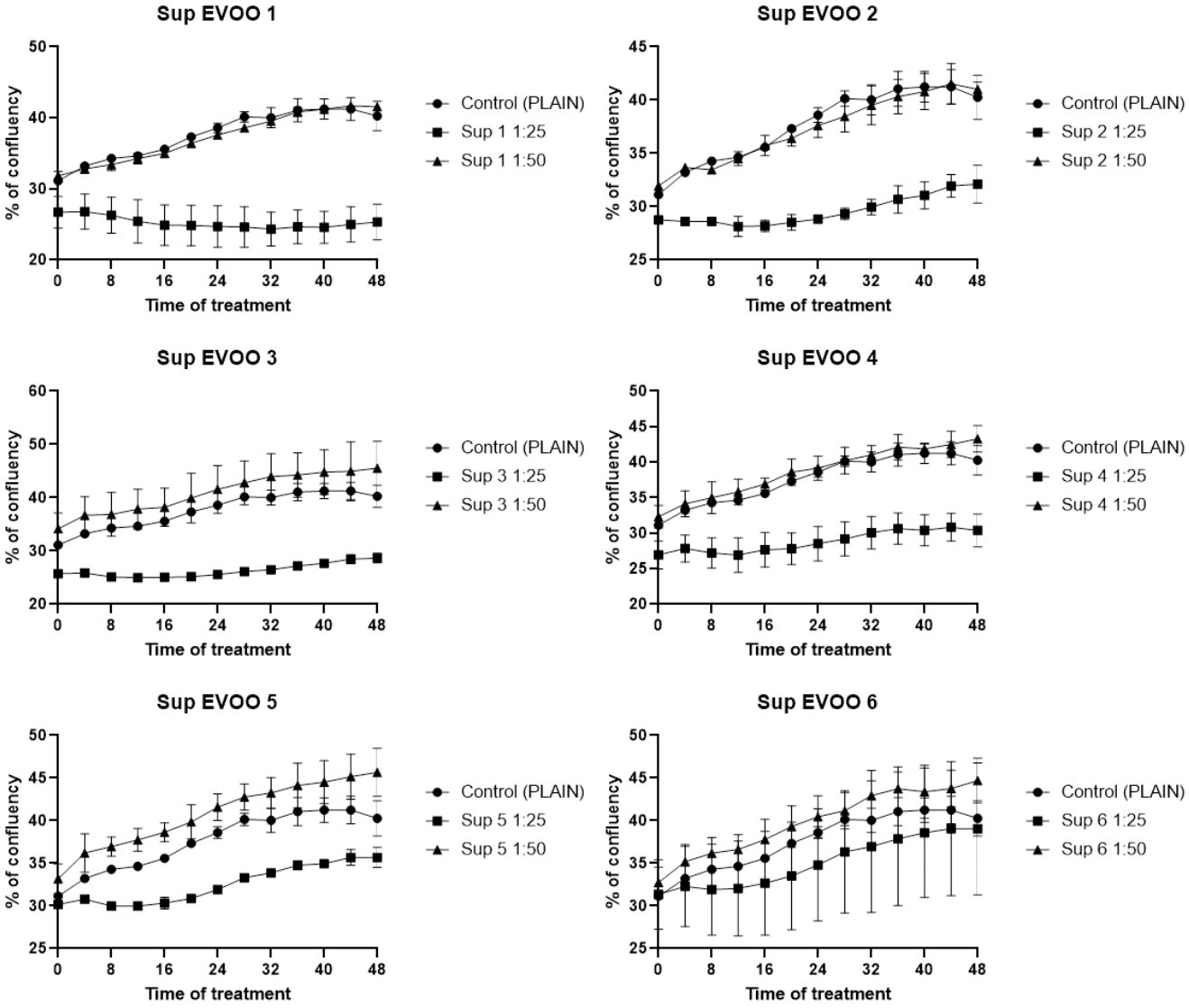
Based on the results above, HCT-116 cells showed some resistance when treated with the EVOO enriched media compared to DLD-1 cells. The same resistance was not observed when HCT-116 cells were treated directly with EVOO.

3.2.4 MCF-7 cells

In addition to colon cancer cell lines, we tested the effect of EVOOs using on MCF-7 breast cancer cell line. We should note that when MCF-7 cells did not exhibit comparable growth to DLD-1 in serum free medium however some initial results could be also obtained.

EVOO enriched medium in a ratio of 1:25 inhibited cell growth in most cases. Dilution 1:50 had a minor inhibition on cell growth. In most cases, cell growth of MCF-7 cells treated with EVOO-enriched medium 1:50 was like cell growth in serum free (Plain) medium.

Medium enriched with corn oil or commercial EVOO (Ctl) had no effect on cell growth.



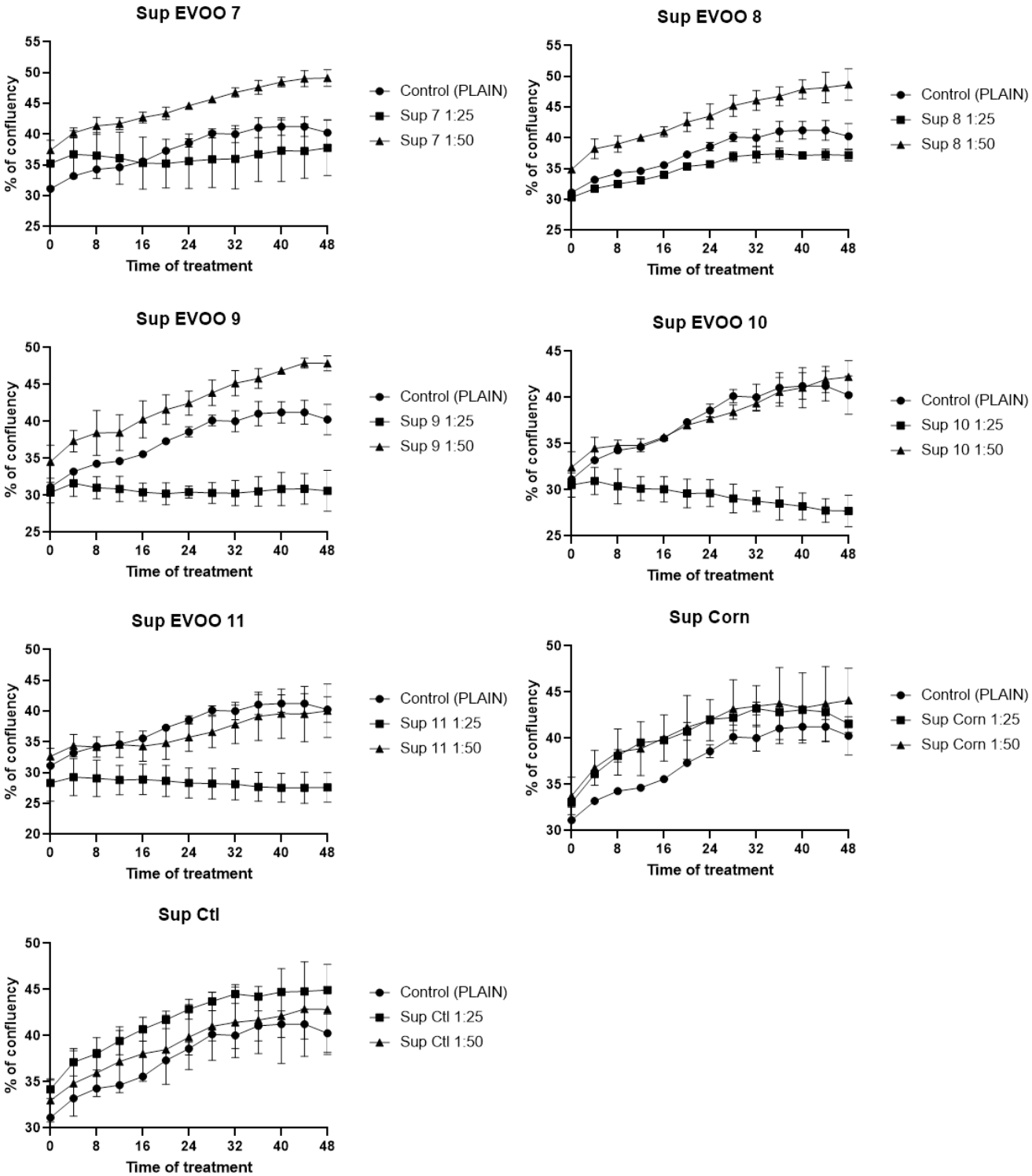
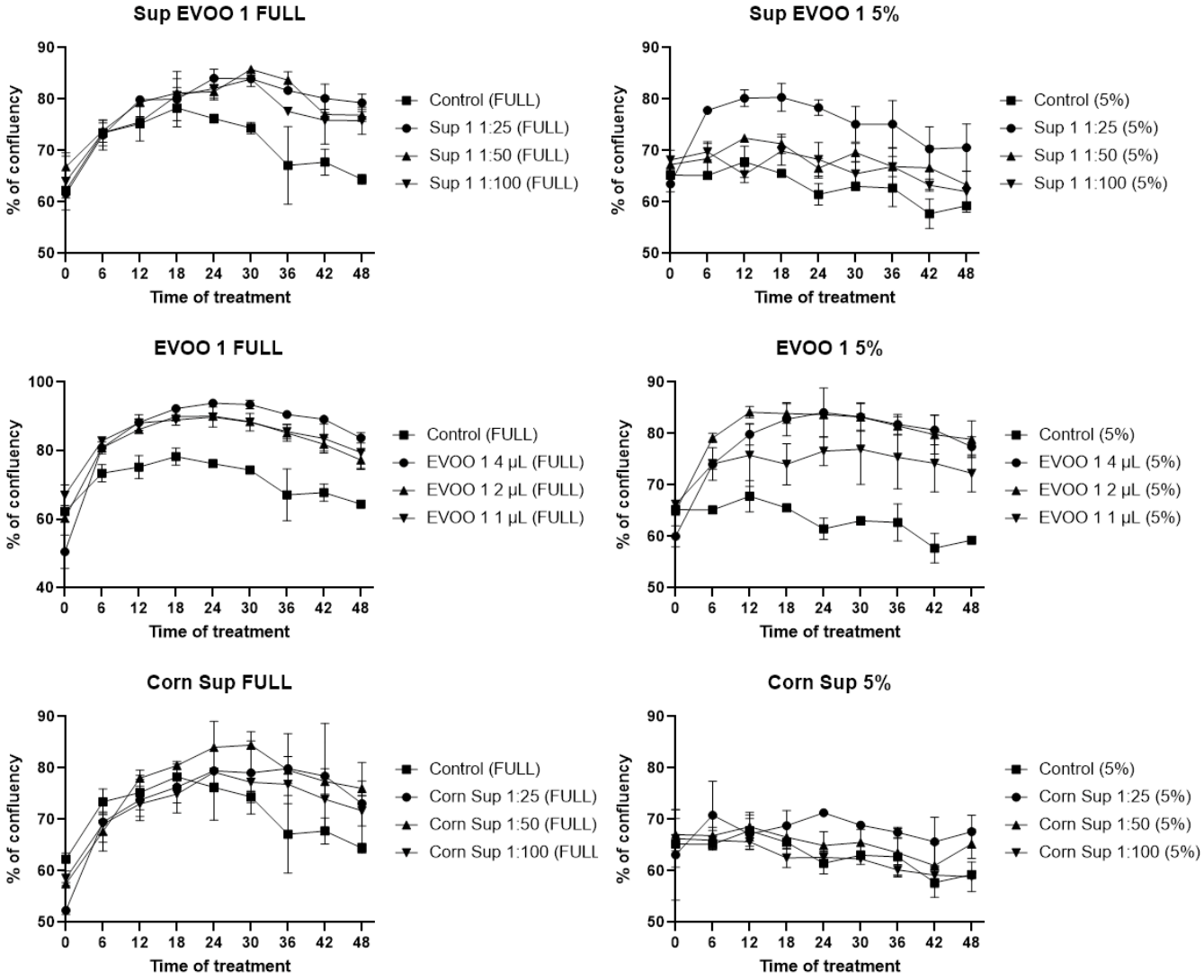


Figure 24: Effect of EVOO-enriched medium on MCF-7 cell growth. *MCF-7 cells were treated with 14 different EVOO-enriched plain culture medium in 2 different ratios EVOO:medium, 1:25 and 1:50. Cell growth was monitored using IncuCyte for 48h taking images every 4h. Percentage (%) of confluency is calculated using IncuCyte software.*

3.2.5 HUVECs showed increased cell growth in serum free conditions

Next, we examined the effect of EVOOs on non-cancer cells, and more specifically on HUVECs. First, we tested EVOO effect on HUVECs when cultured in medium supplemented with 20% FBS. As shown in **Figure 25**, EVOO1 had no effect on cell growth, either in direct treatment or as enriched medium. The presence of EVOO lead cells to preserve a plateau on their growth and not to regress, as in the control cells. Similar results were obtained when we cultured HUVECs in medium supplemented with 5% FBS serum-reduced conditions. We observed that EVOO retained the % of confluency from reduction at later time points.

Corn oil had no effect in cell growth, either in 20% FBS or 5% FBS supplemented culture medium.



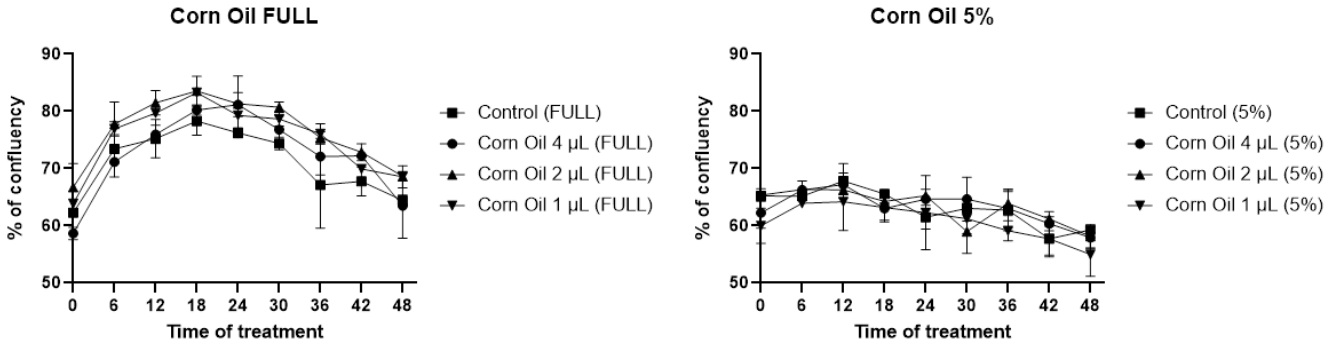


Figure 25: Effect of EVOO1-enriched, Corn oil-enriched medium and direct addition of EVOO1 and Corn oil on HUVECs cell growth. *HUVE cells were treated with EVOO1-enriched, Corn oil-enriched medium, either Full or 5% FBS, in 3 different ratios, 1:25, 1:50 and 1:100 and directly with EVOO1 and Corn oil, either in Full or 5% FBS. Cell growth was monitored using IncuCyte for 48h taking images every 6h. Percentage (%) of confluency is calculated using IncuCyte software.*

3.3 DLD-1 cells undergo late apoptosis-necrosis in the presence of EVOO

We observed that DLD-1 cell growth is inhibited by the presence of both EVOO enriched medium or direct treatment using EVOO. Also, during cell imaging with IncuCyte we observed that cells became round after treatment. For this reason, we investigated whether propidium iodide (PI) could enter the cells in the presence or absence of EVOOs, indicating membrane permeabilization and cell death. Propidium Iodide (PI) is a red-fluorescent nuclear and chromosome counterstain. It is not permeant to live cells, so it is commonly used to detect dead cells in a population. PI binds to DNA by intercalating between the bases with little or no sequence preference. Once the dye is bound, its excitation maximum is at 535nm and fluorescence emission maximum at 617nm

EVOO-enriched culture medium and direct addition of EVOO occurred in three different induction times (24h, 48h and 72h of induction) so to elucidate at which timepoint necrosis happens a when is the highest rate. After PI treatment at the end of the three different induction times, we observed that most necrotic cells were evident at 72 hours of induction using either EVOO enriched medium or direct treatment using EVOO. At 24 hours induction, the necrotic cells were not that much. Direct treatment using EVOO showed a high number of necrotic cells.

Corn oil had no effect on cell growth and necrosis occurred under physiological conditions.

24h induction

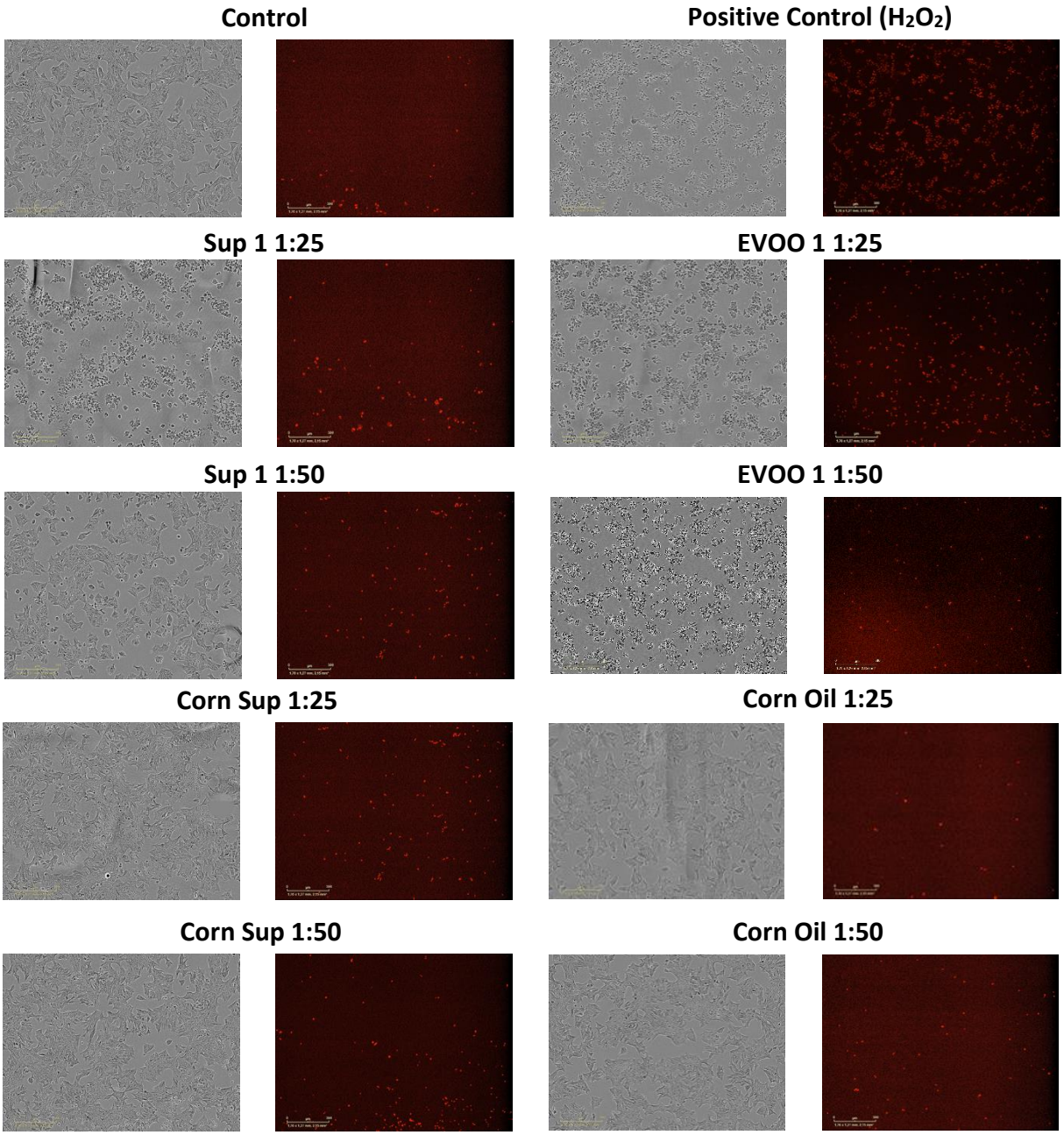


***Figure 26**: **Effect of EVOO1-enriched, Corn oil-enriched medium and direct addition of EVOO1 and Corn oil on DLD-1 apoptosis-necrosis.** DLD-1 cells were treated with EVOO1-enriched and Corn oil-enriched medium and directly with EVOO1 and Corn oil for 24h. 1ug/mL of Propidium Iodide (PI) was added 24h after induction and images were taken after 2h of PI addition using IncuCyte.*

48h induction

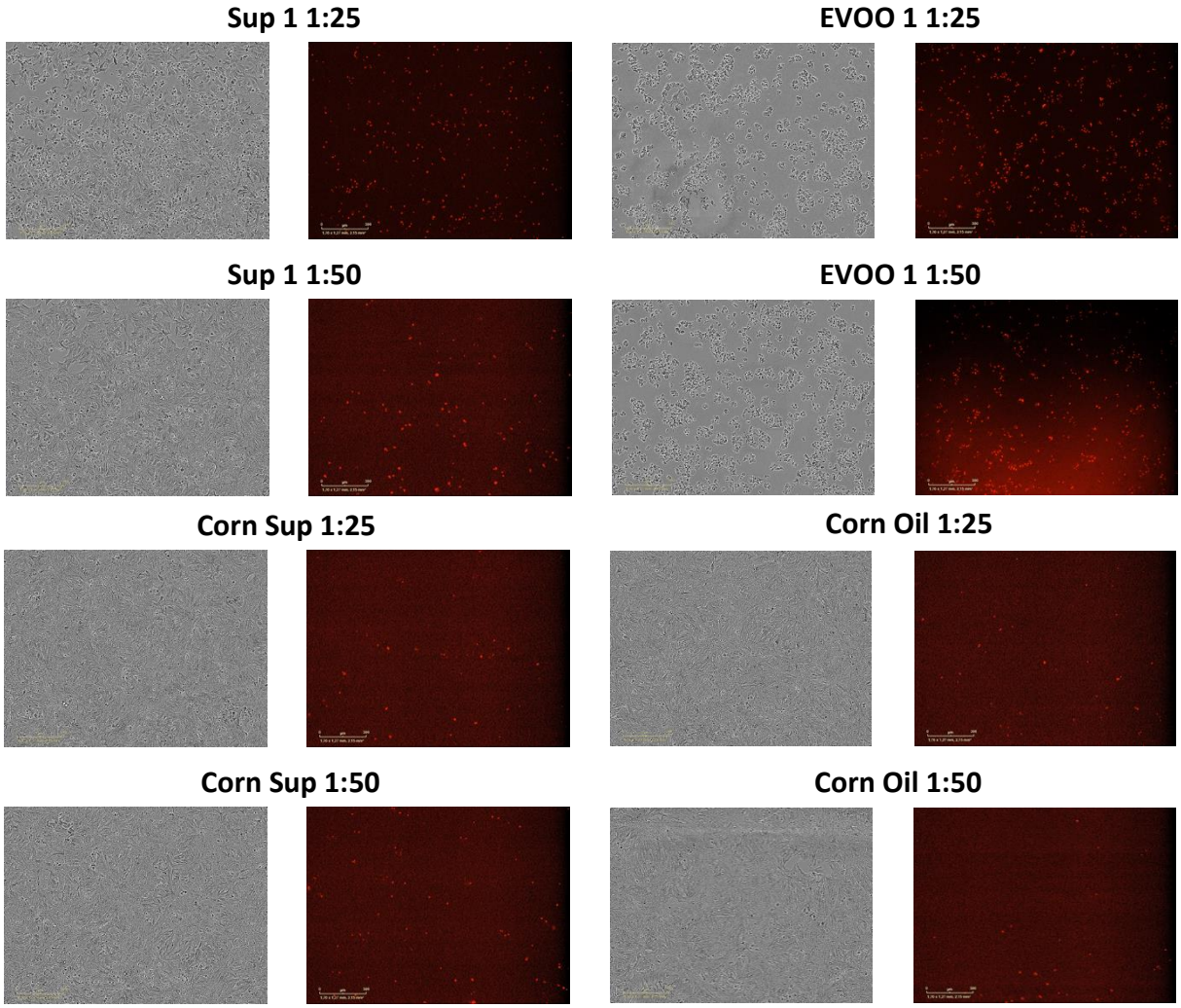


***Figure 27*: Effect of EVOO1-enriched, Corn oil-enriched medium and direct addition of EVOO1 and Corn oil on DLD-1 apoptosis-necrosis.** *DLD-1 cells were treated with EVOO1-enriched and Corn oil-enriched medium and directly with EVOO1 and Corn oil for 48h. 1ug/mL of Propidium Iodide (PI) was added 48h after induction and images were taken after 2h of PI addition using IncuCyte.*

72h induction

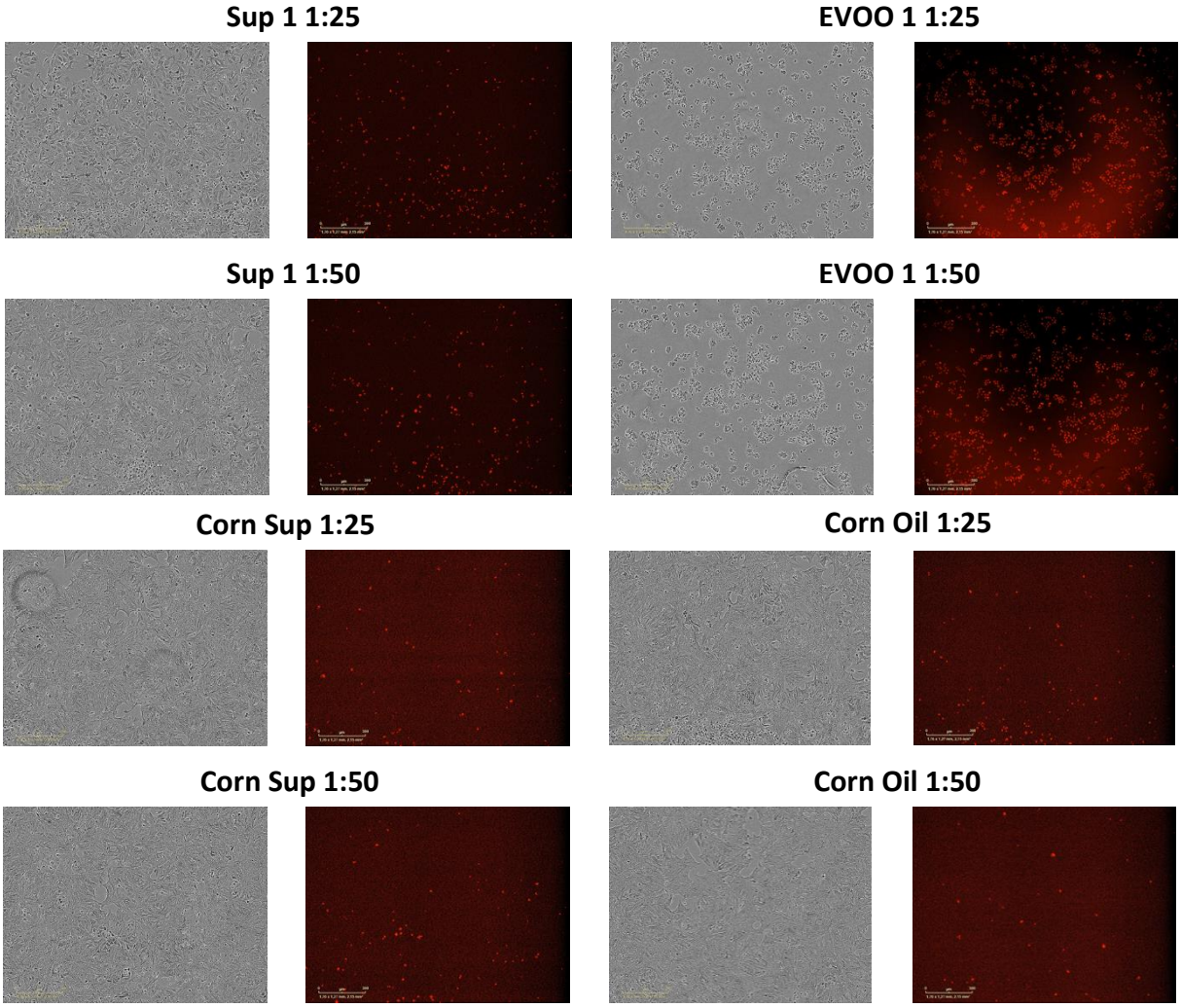


***Figure 28**: Effect of EVOO1-enriched, Corn oil-enriched medium and direct addition of EVOO1 and Corn oil on DLD-1 apoptosis-necrosis. DLD-1 cells were treated with EVOO1-enriched and Corn oil-enriched medium and directly with EVOO1 and Corn oil for 72h. 1ug/mL of Propidium Iodide (PI) was added 72h after induction and images were taken after 2h of PI addition using IncuCyte.*

3.4 Apoptotic assay using IncuCyte® green Caspase reagent

As described above we observed that DLD-1 cells undergo necrosis during EVOO treatment, we decided to further examine whether they undergo apoptosis through caspase-3/7 or not. In order to address this, we used the IncuCyte Green caspase-3/7 Reagent as described in 2.3.4 Apoptosis assay.

Observing that in some cases cell growth inhibition was not linked to phenolic compound concentration, we used EVOO 3, EVOO 4 and EVOO 5 which differ in their composition (2.5 Extra-Virgin Olive Oil chemical analysis). EVOO was directly added in cell culture media in a ratio EVOO:medium 1:50 (2ul in 100ul culture media for a 96-well plate) and live cell imaging followed

As a positive control, we added TRAIL, a TNF family ligand capable of inducing apoptosis (Wang & El-Deiry, 2003). Interestingly, EVOO4, which has the highest phenolic concentration of all EVOOs tested, did not exhibit an increased apoptotic effect compared to EVOO5, which has lower phenolic concentration. In addition, EVOO 3, with the minimum phenolic concentration of all EVOOs tested had comparable effect to EVOO 5. Corn oil does not cause apoptosis in DLD-1 cancer cells as shown in **Figure 29**. On the contrary, Ctl oil has higher apoptotic rate compared to Corn oil despite the fact that is has no phenolic compounds. Representative images of cells are shown in **Figure 30**.

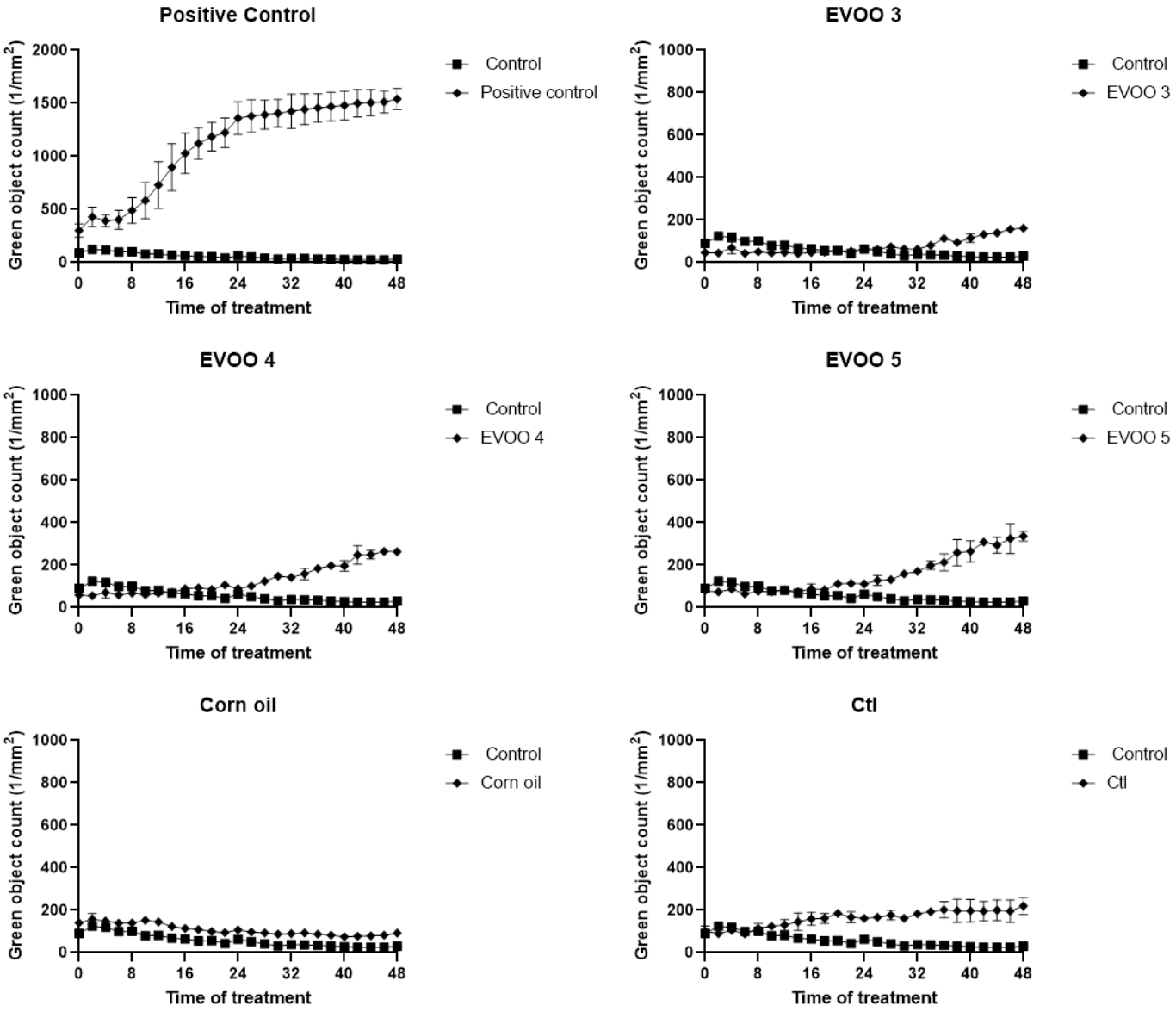


Figure 29: Effect of EVOOs directly added on DLD-1 apoptosis assay using IncuCyte Green Caspase 3/7 Reagent.
DLD-1 cells were treated directly with EVOOs, Corn oil and Ctl oil in plain culture medium. IncuCyte Green Caspase 3/7 Reagent is added according to manufacturer's protocol. Cell growth and fluorescence is monitored using IncuCyte for 48h taking images every 2h. Green object count /mm² is calculated using IncuCyte software.

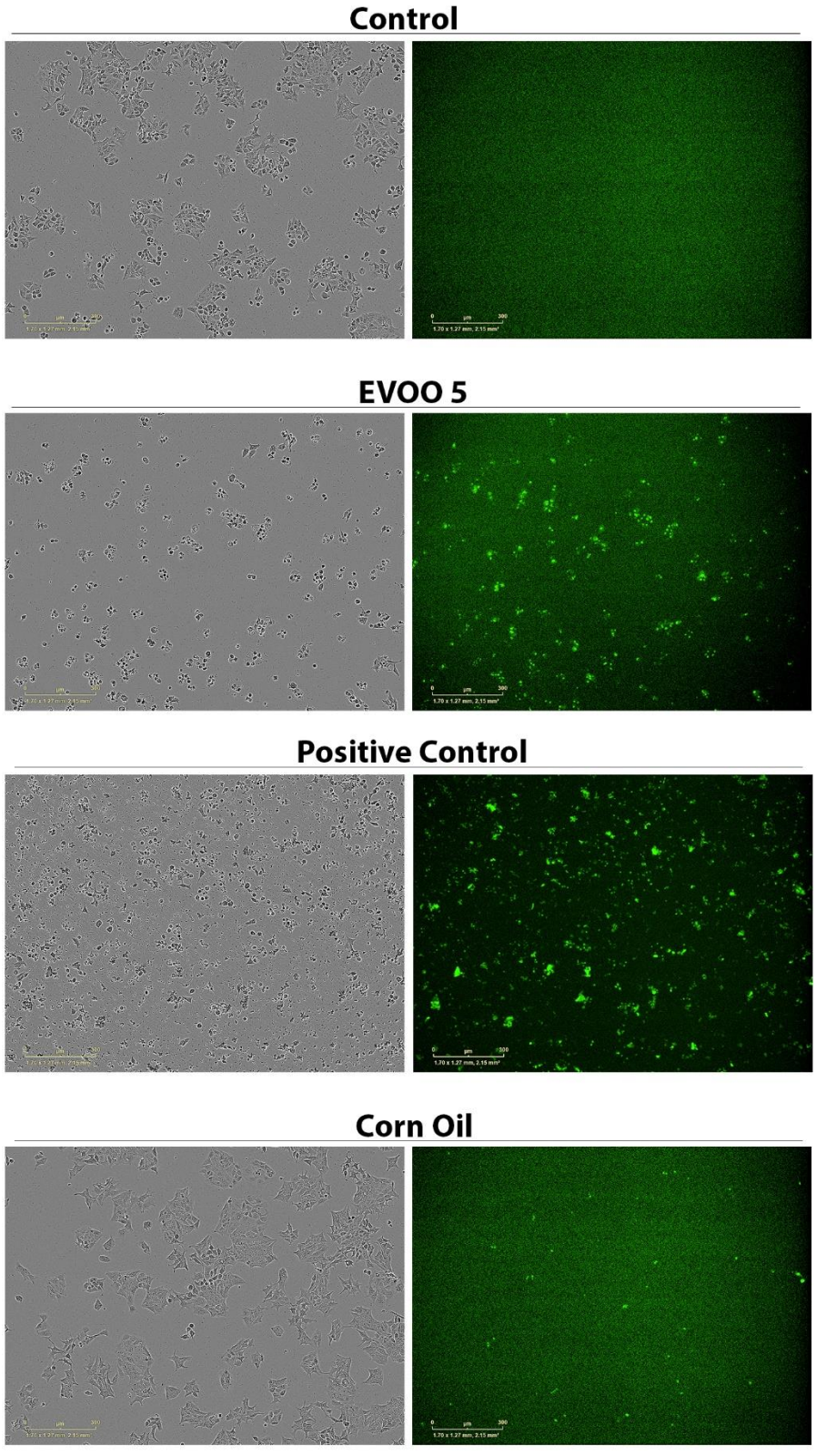


Figure 30: Effect of EVOOs directly added on DLD-1 apoptosis assay using Incucyte Green caspase-3/7 Reagent.*DLD-1 cells were treated direclty with EVOOs at a ratio EVOO:medium 1:50. Cell growth and fluorescence were monitored using IncuCyte for 48h taking images every 2h. Representative images are shown from untreated and treated cells at 48h. Each duplicate shows phase/green phase*

3.5 Migration of DLD-1 cells

As some EVOOs were found to have anti-proliferative effect on DLD-1 cells, we examined the effect of EVOOs on migration of cancer cells.

As shown in **Figure 31**, EVOO 1 completely inhibited migration of DLD-1 cells, whereas the presence of corn oil, an oil poor in phenolic compounds, did not have any anti-migratory effects on DLD-1 cells, but rather increased cell migration.

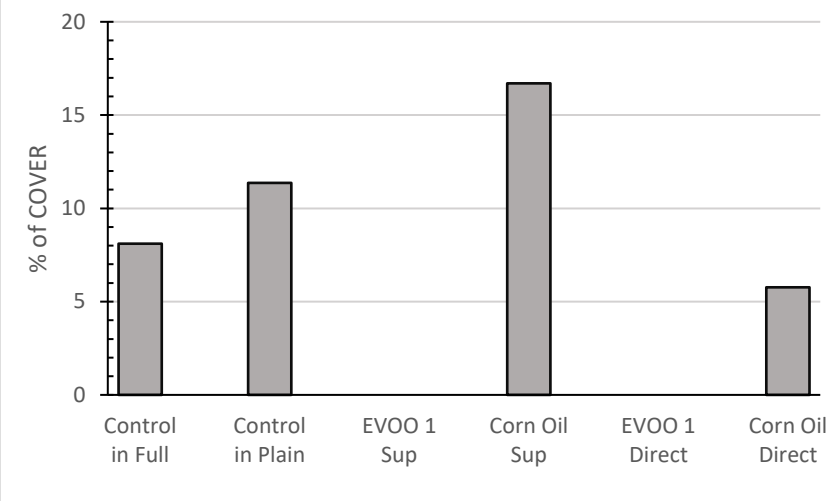


***Figure 31**: **Effect of EVOO1-enriched, Corn oil-enriched medium and direct addition of EVOO1 and Corn oil in DLD-1 cell migration.** Cells were treated with EVOO1-enriched, Corn oil-enriched plain culture medium and directly with EVOO1 and Corn oil after scratch wound in each well. Images Images were taken right after scratch wound using IncuCyte and 16h after treatment with each condition. Percentage (%) of confluency is calculated using IncuCyte software and images are analyzed using Fiji-ImageJ.*

3.6 Migration HUVECs

Next, we examined the effect of EVOOs on VEGF- induced migration of endothelial cells, an experimental approach which resembles a wound healing process.

As shown in **Figure 32**, EVVO 1 and 3, two oils with different phenolic compound concentrations, had comparable effect on HUVEC-migration, about 20% inhibition. However, EVOO 9, an olive oil rich in phenolic compounds, eliminated migration of HUVECs in the presence of VEGF. Corn oil, as expected, did not affect endothelial migration.

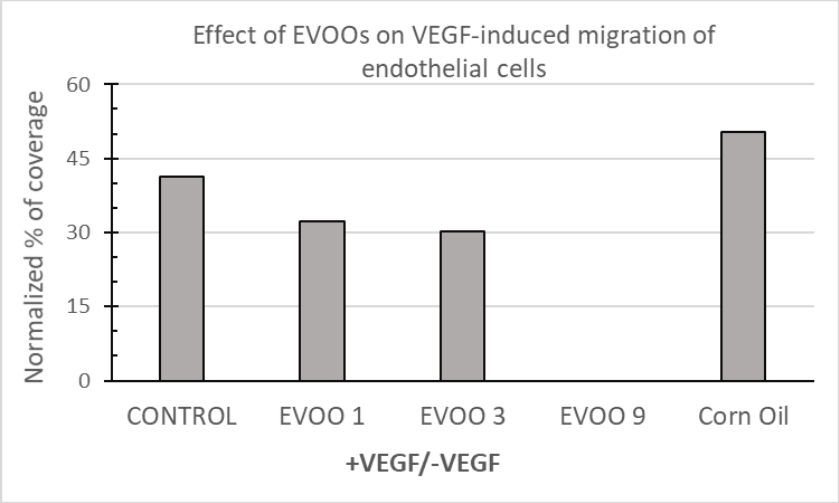


Figure 32 Effect of EVOO-enriched medium and direct addition of EVOOs in HUVECs cell migration.*HUEVCs were treated with EVOO-enriched 5% FBS culture medium in a ratio EVOO:medium 1:50 after scratch wound in each well. VEGF was added in final concentration of 10ng. Images were taken right after scratch wound using IncuCyte and 24h after treatment with each condition. Percentage (%) of confluency is calculated using IncuCyte software and iamges are analyzed using Fiji-ImageJ.*

3.7 Phalloidin for actin cytoskeleton visualisation

In addition to cancer cell growth inhibition upon EVOO treatment, during live cell imaging with IncuCyte (see **Figure 14**) we also observed that cell shape is altered. To investigate further this change of shape we stained DLD-1 cells using Phalloidin (2.2.1 Staining using Phalloidin), which bind to actin filaments.

In **Figure 33** A&B, phalloidin staining in DLD-1 cell in the absence of EVOO is shown. Untreated cells revealed a fine, regular meshwork and filamentous actin network. Observing cells treated with control oil (ctl oil), which is an EVOO poor in phenolics, revealed that actin filaments are not disrupted but acquire a more threadlike shape compared to their normal form (Figure 36C). Cells treated directly with EVOO1 in a ratio EVOO:medium 1:50 (D and E) show actin-rich foci near the plasma membrane. In **Figure 33** D, we also observed diffuse fluorescence along with actin-rich foci. Cells treated directly with EVOO1 in a ratio 1:125 show actin-rich foci near the plasma membrane without any diffuse fluorescence inside the cell. Cells treated directly with EVOO3 in a ratio 1:50 (G, H, and I) exhibit mostly actin-rich foci, especially in Figure 36H, while fluorescent diffusion is not much observed. Furthermore, we observed that the EVOO treated cells have many actin-rich filopodia (see arrows in **Figure 33** F).

Comparing EVOO1 and EVOO3 direct treatment in a ratio 1:50, EVOO3 caused a more significant cell shape change than EVOO1, despite that EVOO1 has higher concentration in phenolics than EVOO3 (2.5 Extra-Virgin Olive Oil chemical analysis).

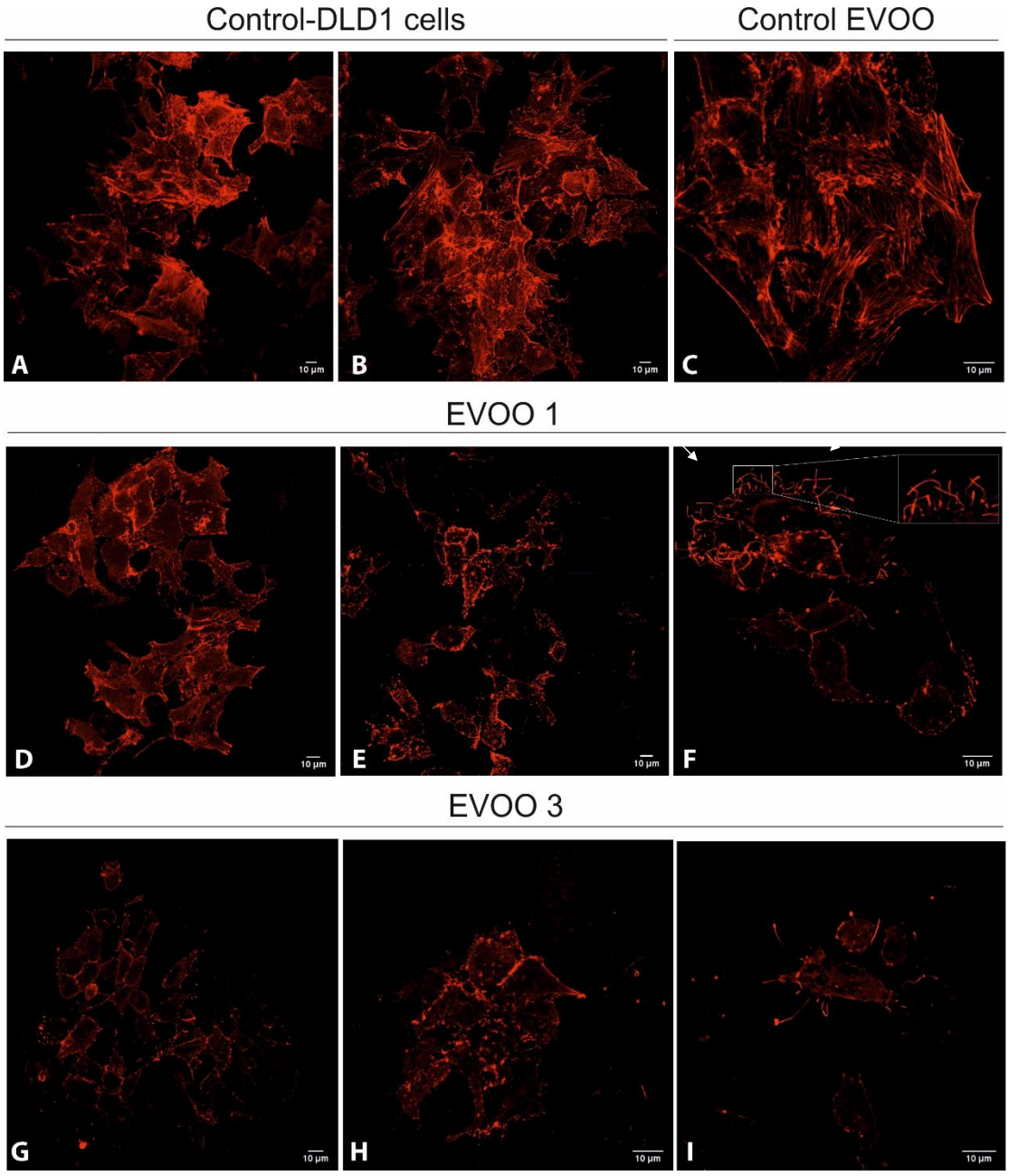


***Figure 33*: Effect of EVOO1-enriched and Corn oil-enriched medium and direct addition of EVOO1 and Corn oil in DLD-1 cell morphology.** *DLD-1 cells were treated with EVOO-1, EVOO-3 -enriched and Corn oil-enriched plain culture medium and directly with EVOO1, EVOO3 and Corn oil for 24h. 200uM of Phalloidin was added for 20min. A & B. Cells in plain culture medium, C. Control EVOO (ctl oil) direct addition in a ratio EVOO:medium 1:50, D and E. EVOO1 direct addition 1:50, F. EVOO1 direct addition 1:125, G, H & I. EVOO3 direct addition 1:50. Arrows indicate actin-rich filopodia. Images were taken using Leica SP5 confocal microscope*

Chapter 4 DISCUSSION

Olive oil certification is a procedure that is used to monitor the quality of olive oils. Indeed, olive oil contains many of the phytochemical classes that have been identified in plant-based diets and shown to have biological activity *in vitro* and in experimental animal models. Certification is currently based on chemical analysis by determining the level of the predominant metabolites of phytochemicals. Then, depending on the concentration levels of selected metabolites, indirect conclusion/prediction of the expected health benefit of the said olive oil is deduced, based on the published effects of these metabolites. However, estimation of biological activity relying upon the effect of few, selected (often the most abundant) metabolites from certain phytochemical classes cannot be accurate (as explained in the aims of the study)

Predicting accurately and reliably the biological activity of olive oil samples would definitely constitute a step forward development in olive oil certification. Especially if the method is easy and automated. Developing such an olive oil certification approach is the aim of the present research work. Successful accomplishment of this endeavour has two prerequisites, i) the development of *in vitro* assays that reconstitute the biological activity to be tested, and ii) the extraction method should not be biased or discriminate between phytoestrogen classes and metabolites thereof.

In the present diploma work, we decided to focus on the protective effect of olive oil on cancer incidence. Cancer is a multistage process in which genetic changes accumulate in normal cells eventually transforming them to cancer cells. The accumulated genetic changes equip the cancer cell with acquired capabilities that allow it to dominate, constituting the hallmarks of cancer that include proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, and activation of invasion and metastasis (Hanahan & Weinberg, Cell 2011, 144: 646-674).

Phytochemicals may have an impact on any of these processes, which can be detected using an appropriate *in vitro* assay. For instance, the hallmarks of cancer proliferative

signalling, evasion of growth suppressors and resistance to cell death all lead to increased cell numbers in proliferation assays with cancer cells (Hanahan & Weinberg, 2011). Protective phytochemicals inhibit the increase in cell numbers of cancer cells (Goulas et al., 2009). An additional important process that is critical for further expansion of tumours beyond a diameter of 2 mm is their ability to induce angiogenesis, the generation of new vessels, to provide oxygen and nutrients to the fast proliferating tumor cells (Folkman & Cotran, 1976). Angiogenesis is a complex process requiring the coordinated, sequential involvement of a number of cellular events. Indeed, formation of new capillaries begins with a localized breakdown of the basement membrane of the parent vessel, through the finely tuned elaboration of proteolytic enzymes and their inhibitors, followed by migration and proliferation of endothelial cells, invasion of the surrounding matrix and new lumen formation. Proliferation, migration, and survival of endothelial cells are all regulated by VEGF in endothelial cells. All these assays are partial steps of angiogenesis and their inhibition *in vitro* by a molecule render this compound a candidate for testing in *in vivo* models of angiogenesis (Fotsis et al., 1998). Thus, to fulfil the first prerequisite we have used the following *in vitro* assays to reconstitute the protective effect of olive oil against cancer incidence:

i. Antimitotic (anti-proliferative) effect on DLD-1, HCT-116, MCF-7 and HUVECs

ii. Anti-migratory effect on DLD-1 and HUVECs

iii. Apoptosis and Necrosis induction on DLD-1

These *in vitro* assays cover extensively the protective effect of olive oil in cancer incidence. The assays may be changed depending on the anti-cancer activity to be tested. Moreover, their number could be reduced should some of them do not offer additional improvement to the comparative statistics.

The other prerequisite for developing a proper certification procedure for olive oils was the employment of an extraction method that did not discriminate between the different phytochemical classes and their metabolites. For instance, olive oil contains biologically active phytochemicals that are either hydrophilic (phenolic acids) or

lipophilic (flavonoids). Consequently, a single extraction method would not result into a quantitative yield of both phytochemical classes. Thus, depending on the extraction approach a bias towards a certain phytochemicals was introduced. In one very common extraction protocol, a mixture of EVOO with 80% methanol in a ratio of 2: 5 is incubated for 10 minutes in an ultrasonic bath and after centrifugation at 4000 rpm for 20 minutes, the phenolic compounds can be extracted in the hydroalcoholic phase (Karkoula et al., 2012).

We have used a fast protocol of extraction of phenolic compounds using cell culture medium without FBS. In short, EVOO was added to cell culture medium in a ratio of 1:25. After homogenization by vortexing for 20 seconds the mixture was allowed to stand for 5 minutes with the phenolic compounds found in EVOO, this medium was used for culture the cell lines mentioned above (**Figure 12**). Whereas this was a fast extraction approach for phenolic compounds, it did not provide any extraction method that could facilitate developing an unbiased certification assay of olive oil.

Towards this purpose, we decided to use directly EVOO at 4% v/v (v/v) motivated by our previous experience with flavonoids that though lipophilic were active in culture medium (Bellou et al., 2012). Specifically, we added EVOO in the same ratio (1:25) as we prepared the enriched cell culture medium (**Figure 12** & **Figure 21**). From the observation of the results of **Figure 13** in comparison with those of **Figure 14** it is obvious that the inhibitory effect of EVOO on the proliferation of DLD1 cancer cells is the maximum possible in the immediate administration of EVOO 1-7 (**Figure 21Error! Reference source not found.**). Indeed, all three doses (1, 2 and 4 uL) of EVOO 1-7 completely inhibit DLD1 cell proliferation, while only the corresponding doses of EVOO 8 showed dose-dependent inhibition (**Figure 21Error! Reference source not found.**). In contrast, all EVOOs (1-11), using the rapid extraction protocol in FBS-free mediums, did not show maximum inhibitory activity in all three doses (**Figure 20**). It is clear that either the 1:50 or 1:100 dose or both did not have a strong inhibitory effect on colon cell proliferation. It seems that the extraction of phenolic compounds in the nutrient medium of the cultures within 5 min is not quantitatively high. On the contrary, the direct addition of EVOO to the nutrient material leads to the "utilization" of all the potential of phenolics and other phytochemicals contained in it.

Exactly the same comments apply to the activity of EVOOs in the proliferation of the human colon HCT116 carcinoma cell line (**Figure 22** & **Figure 23**). The only difference is that proliferation inhibitory activity is lower in these cells compared to that in DLD1 cells. Indeed, the three doses (1, 2 and 4 uL) of immediate administration of EVOO 4, 5, 6 and 7 had maximum inhibitory activity on HCT116 cells (**Figure 23**) compared to the maximum inhibition of the corresponding doses of EVOO 1-7 in DLD1 cells (**Figure 21Error! Reference source not found.**).

Finally, corn oil, which is poor in phenolic compounds, did not inhibit the proliferation of DLD1 cells by either rapid extraction or direct culture, at any of the doses. (**Figure 20** & **Figure 21**). This works as a good negative control, but it would be good to have olive oils with low phenolics.

When we treated 3 different cancer cell lines (DLD-1, HCT116 and MCF-7) with EVOO-enriched plain culture medium in ratio EVOO:medium 1:25, we observed significant cell growth inhibition. Corn oil and commercial (ctl) oil, which have no phenolic compounds, showed no effect on cell growth. Direct addition of EVOO in cells had comparable inhibitory effect. Thus, it can be concluded that inhibition is attributed to phenolic compounds, in agreement with the literature (Fabiani, 2016). DLD-1 cells were more susceptible to EVOO than HCT-116, despite both being colorectal cancer with genetic differences.

As the inhibitory effect was significant following two different protocols for cell-treatment with EVOO, i.e. EVOO-enriched culture medium and direct EVOO addition in cells, we performed dose dependent experiments to identify the range of the EVOO effect. These experiments revealed that the inhibitory effect varies among different EVOOs. EVOO 5 and EVOO 8 using EVOO-enriched medium in a ratio EVOO:medium 1:50 had no significant effect on % of confluency according to IncuCyte software. These two EVOOs have low concentration of phenolic compounds, with 268mg/kg and 466 mg/kg, respectively. EVOOs with high phenolic concentration (EVOO4, EVOO10 and EVOO11) showed significant cell growth inhibition even at the maximum dilution EVOO: medium 1:100. Surprisingly, EVOO 3, which has the lowest concentration in phenolics (110mg/kg), exhibited significant cell growth inhibition in 1:25 dilution. These findings led us to the conclusion that cell growth inhibition could be

independent of the concentration of the known phenolic compounds that were analyzed. Despite cancer cells, primary cells were also used. Isolated HUVECs (Bellou et al., 2009) treated with EVOO1-enriched culture medium in two culture conditions, 20% FBS and serum starved (5%), did not exhibit cell growth inhibition but, on the contrary, their growth reserved a plateau without regression. Corn oil and ctl oil had no effect on cell growth in the three dilution, 1:25, 1:50 and 1:100 used. According to the literature, oleocanthal extracted from EVOO has anti-proliferative effect in human multiple myeloma cells by activating apoptosis and down-regulating ERK1/2 and AKT signaling pathways (Scotece et al., 2013), cytotoxic activity on human melanoma cells by decreasing the expression of the anti-apoptotic protein Bcl-2 without affecting the expression of BAX (Fogli et al., 2016a), c-Met inhibitory effect in breast cancer models (Akl et al., 2014) and cell growth inhibition in human hepatocellular carcinoma by blocking STAT3 activation (Pei et al., 2016). Also, it is revealed that phenols extracted from EVOO are capable of block cell cycle progression at G2/M phase in bladder cancer cells (Coccia et al., 2016)

Concerning the direct addition of EVOO in cells, we followed the same pattern of dilutions as in EVOO-enriched culture media. Inhibition was much stronger when direct addition was used since the extraction of phenolic compounds was continuous in culture medium throughout the experiment. Only EVOO8 had no inhibitory effect on cancer cell growth at the highest dilution (1:100), in accordance with its poor phenolic compound concentration. In HUVECs treated with EVOO 1, there was no inhibitory effect on cell growth. Thus, treated cells showed a preservation of plateau in their growth without regression, in two culture condition of 20% FBS and serum reduced (5%). Corn oil and ctl oil again had no inhibitory effect either on cancer cell growth or on HUVECs. From all the above experiments, we conclude that cell growth inhibition is specific for cancer cells.

In addition to EVOO effect on proliferation of cancer cells, we also examined their effect on cell survival. Both Propidium Iodide (PI) and IncuCyte green caspase-3/7 Reagent showed elevated levels of apoptotic cells, either through late-apoptosis or activation of Caspases 3/7. However, we should point out that elucidating the exact signalling cascade that is activated by EVOOs is the next step to decipher the

mechanisms underlying the effect of EVOO in cancer cells survival. Research revealed that oleocanthal extracted from EVOO rapidly induces lysosomal membrane permeabilization resulting in cell death selectively in cancer cells, like prostate cancer cell line PC3 and human breast cancer cell line MDA-MB-231, while non-cancer cells, like BJ human fibroblasts, are reversibly inhibited without cell death induction (LeGendre et al., 2015). Evidence show that oleocanthal has cancer-specific effects without inhibiting cell growth of non-cancer cells (Fogli et al., 2016a; Goren et al., 2019; LeGendre et al., 2015). We also concluded this cancer cell specificity of EVOO. HUVECs treated with EVOO did not exhibit cell growth inhibition or cell death when cell growth was monitored using the IncuCyte.

EVOO treated DLD-1 cells were found less migratory than non-treated an effect specific for cancer cells, since it was not observed in HUVE cells. As it has been shown, phenolic fraction of EVOO can impede fibrogenic and oncogenic Epithelial-to-Mesenchymal Transition (EMT) by preventing TGF-β1-induced activation of the EMT program in MDCK and MCF-7 cell lines respectively (Vazquez-Martin et al., 2012). Moreover, it is observed that oleocanthal has the ability to inhibit migration and invasion of human hepatocellular carcinoma both *in vitro* and *in vitro* (Pei et al., 2016), while also inhibits c-Met in metastatic breast and prostate cancer resulting in anti-proliferative, anti-migratory and anti-metastatic capabilities with potential for therapeutic applications (Elnagar et al., 2011)

During EVOO treatment we observed alteration in DLD-1 cells morphology. Thus, we performed phalloidin staining of DLD-1 cells after EVOO-addition that revealed actin granules near the plasma membrane and disruption of actin filaments. According to literature, during the execution phase of apoptosis major cytoskeletal filaments, including microtubules, cytokeratin, and actin, are degraded (Janmey, 1998). Actin is a substrate for caspases and a fragment of this cleavage (tActin) is capable of initiating the morphological hallmarks of apoptosis, such as cell rounding and chromatin condensation (Mashima et al., 1999). According to these findings, we can wonder whether phenolic compounds in EVOO initiate apoptosis by directly disrupting actin filaments or not.

As a summary, EVOO phenolic compounds affect cell growth of cancer cells by inhibiting their growth, changing their morphology, and enabling apoptosis where on primary endothelial cells, they had no inhibitory effect.

According to literature, oleocanthal and oleacein account for the inhibitory and cancer specific cytostatic effect (El Riachy et al., 2019; Elnagar et al., 2011; Goren et al., 2019; Gu et al., 2017; Romani et al., 2019). Our findings indicate that cell growth inhibition is not only dependent on the known phenolic compounds that were analyzed. In our experiments, EVOOs with low concentrations of oleocanthal and oleacein, EVOO 3, EVOO 5 and EVOO 8, showed significant cell growth inhibition at a dilution of 1:25, especially EVOO 3, showing that probably cancer-specific cell growth inhibition is not only dependent on oleocanthal and oleacein concentration in our samples but is also associated with other unidentified phenolic compounds.

LIST OF REFERENCES

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