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Doutora Andreia Ferreira Castro Gomes
E da
Doutora Maria de Fátima Pereira Duarte Ricardo

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Obrigada a todos!

Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Atividade antitumoral de *Eucalyptus nitens*: abordagem bioquímica ao estado redox em células tumorais e não tumorais de mama

Resumo

Estima-se que, em 2020, o número de novos casos de cancro atinja 22.2 milhões, a nível mundial. O cancro de mama triplo negativo (células não expressam ER, PR e HER2) tem um prognóstico particularmente negativo. Apesar das novas soluções terapêuticas em desenvolvimento, a quimioterapia continua a ser o tratamento de referência, com baixa eficácia e com efeitos nefastos nos tecidos saudáveis. *Eucalyptus nitens* é uma possível fonte de compostos anticancerígenos e antioxidantes, nomeadamente o àcido ursólico (UA) e àcido betulínico (BA). Estes àcidos triterpenóides podem induzir morte celular e inibir a proliferação de células de cancro da mama, com baixa toxicidade em células normais. Neste trabalho testou-se a capacidade do extrato da casca externa de *E. nitens*, UA, BA e da sua combinação (UB), na viabilidade de células humanas de cancro de mama (MDA-MB-231) em comparação com células normais (MCF-10A), com diferentes metodologias: resazurina, MTT, azul tripano e marcação dupla com laranja de acridina e iodeto de propídío. Verificou-se maior citotoxidade em células MDA-MB-231 à incubação com extrato da casca externa de *E. nitens*, UA e BA. No entanto, UB teve resultados contraditórios. O efeito dos àcidos triterpenóides nos níveis intracelulares de ROS foi avaliado com a sonda DCF-DA, com resultados inconclusivos. Foram igualmente testados diferentes protocolos para formar esferóides com ambas as linhas celulares, sendo possível observar que a incubação com o extrato da casca externa de *E. nitens* afetou marcadamente a integridade dos esferóides.

Estes resultados sugerem que o extrato da casca externa do *E. nitens* é uma potencial fonte natural de agentes quimiopreventivos, inibindo ou desacelerando a progressão do cancro da mama, embora os mecanismos de ação não sejam ainda totalmente conhecidos.

Palavras chave: àcido betulínico, àcido ursólico, cancro da mama, esferóides, extrato da casca externa de *Eucalyptus* nitens.

Eucalyptus nitens antitumoral activity: a biochemical approach to assess the redox cellular state on tumor and non-tumor breast cells

Abstract

Cancer incidence is increasing worldwide and it is estimated that, by 2030, the number of new cases will be 22.2 milllion. The most common cancer among women is breast cancer and, in particularly, triple negative breast cancer (no ER, PR and HER2 expression) has poor prognosis. New emerging therapies are being developed but chemotherapy is still the standard treatment, having low effiency and with associated toxicity to normal tissues. *Eucalyptus nitens* can be a source of natural compounds with anticancer and antioxidant activities, namely ursolic (UA) and betulinic (BA) acids. Studies with these triterpenoids showed their capacity to induce cell death and inhibit proliferation in breast cancer cells with low toxicity in normal cells. In the present study, the ability *E. nitens* outer bark extract, UA, BA, and the combination of both (UB), to induce cell death in human breast cancer cells (MDA-MB-231) were tested. For that, the effect of these treatments on tumor and non-tumor breast cells was evaluated with resazurin, MTT, trypan bue and acridine orange and propidium iodide double staining. Our data showed higher cytotoxicity in MDA-MB-231 cells when treated with *E. nitens* outer bark extract, UA and BA. However, the combination of pure acids had contradictory results. The effect of triterpenoids acids in intracellular ROS levels was tested by DCF-DA assay but the results were inconclusive. In this study, we also analyzed different experimental approaches on the capability of forming spheroids using MCF-10A and MDA-MB-231 cell lines. The distinct spheroid formation capabilities of different cell lines stress the need for standardization of spheroid generation protocols for better testing of novel anticancer drugs. Interestingly, it was possible to observe that incubation with *E. nitens* outer bark extract strongly affected spheroid integrity.

Overall, the results suggest that *E. nitens* outer bark extract is a potential natural source of bioactive compounds for the development of chemopreventive agents, to inhibit or slow down the progression of breast cancer, although the mechanisms are yet to be fully elucidated.

Keywords: betulinic acid, breast cancer, *Eucalyptus nitens* outer bark extract, spheroids, ursolic acid.

Scientific Output

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List of Abbreviations and Acronyms

8-OH-dG - 8-hydroxy-2-deoxyguanosine
AO - Acridine Orange
ATP - Adenosine Triphosphate
BA - Betulinic acid
CAT - Catalase
CEBAL – Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo
DCF - 2',7'-dichlorofluorescein
DCFDA - 2 ′ ,7 ′ - dicholorofluorescin diacetate
DMEM – Dulbeccos's Modified Eagle Medium
DMSO – Dimethyl sulfoxide
DNA - Deoxyribonucleic Acid
ECM - Extracellular Matrix
EDTA – Ethylenediamine tetraacetic acid
EGF - Epidermal Growth Factor
EGFR - Epidermal Growth Factor Receptor
EMT - Epithelial-Mesenchymal Transition
ER - Estrogen Receptor
FBS - Fetal Bovine Serum
FDA - Food and Drug Administration
GPXs - Glutathione Peroxidases
GR - Glucocorticoid Receptor
HDL - High Density Lipoprotein
HER2 – Human Epidermal growth factor Receptor 2
HS – Horse Serum
JNK - Jun N-terminal kinase
MDR - Multidrug Resistance
MHC – Major Histocompatibility Complex
MTT - 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NADPH - Nicotinamide Adenine Dinucleotide Phosphate, in the reduced form
Nm - Nanometers
OA – Oleanolic acid
PARP-I - Poly-ADP Ribose Polymerase I
PBS – Phosphate buffered saline
PDL1 – Programmed death-ligand 1
PI - Propidium Iodide
PR - Progesterone Receptor
PRXs - Peroxiredoxins
RNA - Ribonucleic Acid
ROS - Reactive Oxygen Species
SEM - Standard Error of the Mean
SOD - Superoxide Dismutase
TAE – Tris-Acetate-EDTA
tBHP - tert-Butyl-hydroperoxide
TIL– Tumor-infiltrating lymphocyte
TNBC - Triple Negative Breast Cancer
TNFR – Tumor necrosis factor receptor
TRX - Thioredoxins
UA - Ursolic acid
UB – ursolic and betulinic acid combination
u-PA - urokinase-type Plasminogen Activator

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1. Introduction

1.1 Cancer

Cancer is currently a major public health problem all over the world. In 2018, there were an estimated 18.08 million new cases of cancer diagnosed worldwide [1]. By 2030, it is estimated that the number of new cases will increase to 22.2 million [2–4].

Among men, the three most common cancers diagnosed in 2018 were lung (14.5% of the total), prostate (13.5%) and colorectum (10.9%) cancers. In women, the three most common cancers were breast (24.3%), colorectum (9.5%) and lung (8.4%) cancers [5].

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation, cell death and, ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing morbidity and, if untreated, death of the host [2,6]. The characteristics that differentiate a malignant cancer from a benign tumor are the abilities to invade locally, to spread to regional lymph nodes, and to metastasize to distant organs in the body [7].

From a molecular and cell biological point of view, cancer may be caused by similar molecular defects in cell function resulting from common types of modifications to a cell's genes. There are many mechanisms by which this altered gene expression occurs. These mechanisms may occur via a direct insult to DNA, such as a gene mutation, translocation, amplification, deletion, loss of heterozygosity, or via a mechanism resulting from abnormal gene transcription or translation [8,9]. Mutations in two broad classes of genes have been implicated in the onset of cancer: proto-oncogenes and tumor-suppressor genes [10].

Proto-oncogenes are activated to become oncogenes by mutations that cause the gene to be excessively active in growth promotion. Tumor-suppressor genes normally restrain growth, so damage to them allows inappropriate growth [7,11].

In many cases, the causes of cancer are not clearly defined, but both external and internal factors play an important role. About 20% of all cancers diagnosed can be prevented, as established cancer causes include a combination of lifestyle factors (external), such as tobacco, alcohol, diet, excess body weight, physical inactivity, exposure to radiation, excessive sunlight exposure, viruses, environmental pollutants and certain infections. Non-modifiable (internal) factors, such as inherited genetic mutations, hormones and immune conditions can also cause cancer [8,12].

Carcinogenesis, is the process by which a normal cell in the body turn into a cancer cell, and it is a complex process divided in three phases: initiation, promotion and progression [13]. A single cell

suffers damage, causing mutation in DNA which is not repaired, or the repair is defective resulting in unrestrained proliferation (initiation). The continuous proliferation facilitates more mutations originating an abnormal mass of cell – a tumor (promotion). The growth of the tumor is aided by angiogenesis, which not only provides nutrients to the tumor, but also allows malignant cells to migrate to other tissues via the circulatory system, metastasizing the tumor (progression), which normally is lethal [14,15].

Besides the resistance to apoptosis, sustained proliferative signaling, promotion of angiogenesis as well the ability to tissue invasion and establishment of metastasis, other characteristics ("hallmarks") of cancer include avoidance of immune destruction, deregulation of cellular energetics, tumor-promotion inflammation and replicative immortality [16].

One of the most threatening problem associated with cancer therapy is multidrug resistance (MDR), which is common cause of chemotherapy failure and recurrence due to which the survival rate of many cancers is very poor [17]. Combined therapies may be required to overcome cancer. Cancer therapy has been mainly focused on the use of different anticancer drugs, from natural to natural-derived products, attempting to set aside the standard protocols such as surgery, chemotherapy, radiotherapy and hormones. These conventional treatments are costly, causes many dreadful side effects and can only extend the patient's lifespan by a few years [18].

Therefore, there has been a continuous research for less expensive and non-toxic natural drugs [18,19]. In fact, in the last years, 75% of the drugs approved by the Food and Drug Administration (FDA) are derived from natural sources, from which 49% are currently in use in cancer therapy, including lung, prostate, colorectal and stomach, breast, brain tumors and others [20].

1.1.1 Breast Cancer

Breast cancer is the most prevalent cancer in women worldwide, with more than 1 million new cases per year and nearly half million related deaths [2,21]. It has been predicted that the worldwide incidence of female breast cancer will reach, approximately, 3.2 million new cases per year, by 2050 [3,22]. While improvements in screening have contributed to a steadily increasing number of breast cancer diagnoses, advances in characterizing and personalizing treatment have aided in decreasing breast cancer mortality rate [4,22,23]. Nevertheless, breast cancer mortality rate remains high, affirming the ongoing need for improvements in understanding breast cancer progression and how to effectively treat it [24].

1.1.1.1 Physiology of breast tissue

Healthy breast tissue is essentially composed of cells organized into branching ducts within an extracellular matrix (ECM) of connective tissue, collagen I, basement membrane, and fat. Each mammary gland contains 11-58 lobes, with each lobe containing many smaller lobules [25]. Lobules are clusters of 10-100 sacs called acini, where milk production occurs. The acini and connecting ducts consist of luminal epithelial cells lining the inner surfaces of breast ducts and glands and myoepithelial cells forming a thin layer between luminal cells and basement membrane [25,26].

Throughout a woman's lifetime, breast composition undergoes several phases of change and development, as well as periods of regulation and maintenance. Broadly, the ductal system within the breasts are maintained by epithelial cells, which are cuboidal in shape and organize into single-layered sheets that line acini and ducts, and a surrounding layer of stellate-shaped myoepithelial cells **(Figure 1)**. Growth during embryo development, changes to milk glands during menstrual cycles, extensive lobule formation and lactation during pregnancy, and gland involution during menopause are all regulated modifications to breast structures that necessitate changes in cell shape, during cell division and differentiation [26–29].

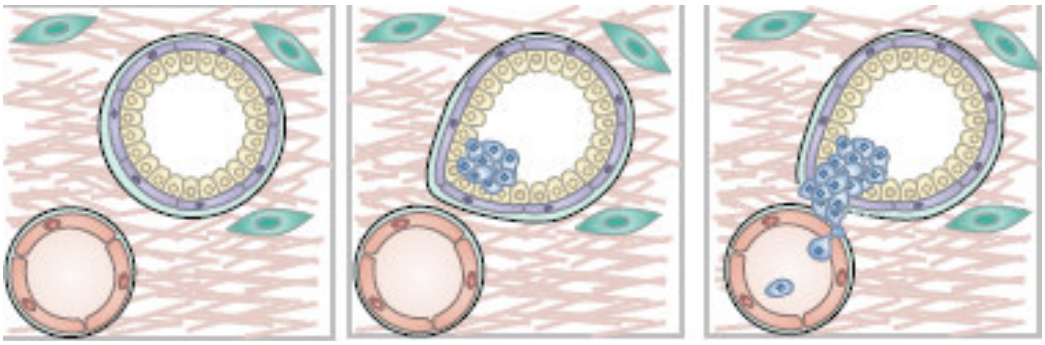

Figure 1 - Progression of breast cancer metastasis. Cancer cells are presented in blue, epithelial cells yellow, myoepithelial cells purple, basement membrane is light blue, fibroblasts green, blood vessel is red, and surrounding pink fibers are collagen I-rich ECM. Healthy breast tissue is shown before cancer cells form, blue cells indicate initial tumor growth in the breast duct, and finally, invasive tumor cells breach the basement membrane and intravasate into a nearby blood vessel. Extracted from [28].

1.1.1.2 Breast cancer classification

Molecular subgrouping can be provided by using a few immunohistochemical markers. A panel including Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal factor Receptor 2 (HER2), Ki67, Epidermal Growth Factor Receptor (EGFR) and basal cytokeratins (CK14 and CK5 /6) can be used to distinguish between "luminal", HER2 and triple negative tumors **(Table 1)**. Proliferation

markers are also very important in molecular subgrouping besides ER and HER2, especially in ER-positive tumors [30,31].

Around 70% of breast cancers are ER positive [32] and is divided into two subtypes: luminal A and luminal B. Separation of luminal tumors into subgroups is mainly based on proliferation intensity. Luminal A subtypes are ER or progesterone receptor (PR) positive and express high level of ER-associated genes but are HER2 negative. Luminal B subtypes identify as ER or PR positive and HER2 positive [33]. Luminal A subtype has better prognosis than luminal B because this last express low levels of ER and has a higher cellular proliferation rate [34,35]. Luminal A typically responds more effectively to selective estrogen receptor modulators such as tamoxifen [32,33,36].

HER2 positive breast cancer presents the overexpression or amplification of the ErbB-2 oncogene and exhibits 15% of breast cancer cases [37]. HER2 is a transmembrane receptor tyrosine kinase, a member of the epidermal growth factor family that is a potent mediator of normal cell growth and development [38]. This subtype presents HER2 positive but ER negative with poor differentiation [39]. The first-line treatment agent for HER2 positive breast cancer patients is trastuzumab, a recombinant monoclonal antibody. Trastuzumab down-regulates HER2 expression by accelerating its receptor endocytosis and inhibits cell cycle progression by inducing the formation of p27Kip1/Cdk2 complex [40,41].

Breast cancers that lack the expression of ER, PR and HER2 are known as triple negative breast cancer (TNBC), which represents 15% of breast cancer cases [35,42,43]. Although TNBC is not the most common breast cancer in women worldwide, it is the most aggressive subtype of breast cancer. TNBC has the highest percentage of deaths from breast cancer [44,45]. In addition, patients with TNBC have high recurrence and treatments are limited [44,46], as TNBC is resistant to the treatments for ER positive and HER2 positive breast cancer [47].

Table 1 - Major molecular subtypes of breast cancer. Adapted from [31].

	Molecular subtype			
	Luminal A	**Luminal B**	**HER2**	**Triple negative**
Gene expression pattern	Expression of luminal cytokeratins High expression of hormone receptors and related genes	Expression of luminal cytokeratins Moderate expression of hormone receptors and related genes	High expression of HER2 Low expression of ER and related genes	High expression of basal epithelial genes and basal cytokeratins Low expression of ER and related genes, low expression of HER2
Clinical and biologic features	50% of invasive breast cancer ER/PR positive, HER2 negative	20% of invasive breast cancer ER/PR positive, HER2 expression variable Higher proliferation than Luminal A, higher histologic grade than Luminal A	15% of invasive breast cancer ER/PR negative, HER2 positive High proliferation, diffuse TP53 mutation, high histologic grade and nodal positivity	15% of invasive breast cancer ER/PR/HER2 negative High proliferation, diffuse TP52 mutation, BRAC1 dysfunction (germline, sporadic)
Response to treatment and prognosis	Response to endocrine therapy Variable response to chemotherapy Good prognosis	Response to endocrine therapy (tamoxifene and aromatase inhibitors) not as good as Luminal A Variable response to chemotherapy (better than Luminal A) Prognosis not as good as Luminal A	Response to trastuzumab Response to chemotherapy with antracyclins Usually unfavorable prognosis	No response to endocrine therapy or trastuzumab Sensitive to platinum group chemotherapy and PARP inhibitors Worst prognosis

1.1.1.2.1 Risk factors for triple negative breast cancer

TNBC is highly invasive and has a high incidence of lymph node involvement [37]. Clinically, they have high nuclear mitotic grade and large tumor size. Taken together, these factors may be a reason for patients to have poor overall survival and high risk of early recurrence with most deaths occurring in the first 5 years of diagnosis [44]. There is also rapid progression from the onset of metastasis to death and strong correlation between obesity and chemosensitivity [37].

TNBC develops early in life and more often in pre-menopausal women [48,49]. Individuals with metabolic syndrome such as high blood glucose, triglyceride or low high density lipoprotein (HDL) [50], increased body weight [51] and younger age, which is identified as less than 50 years old at diagnosis

[52,53], are considered as high risk groups of TNBC. In addition, individuals who are of Hispanic or African-American ethnicity [48,54], have never breast-fed or used oral contraceptives [55,56] have higher chance to develop TNBC. Patients with hereditary breast and ovarian cancer syndromes have a 50–85 % risk of developing breast cancer during their lifetime [57]. Germline mutations in the BRCA1 breast and ovarian cancer susceptibility gene have been associated with TNBC, with 60–80 % of breast tumors from BRCA1 mutation carriers displaying a TNBC phenotype [58,59].

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1.1.1.2.2 Current therapies for triple negative breast cancer

Effective therapies are very limited for this aggressive subtype when compared to hormone receptor and HER2-positive breast carcinomas [60].The standard therapy for TNBC is chemotherapy [61]. Chemotherapies are divided in two groups: neoadjuvant and adjuvant chemotherapy. Neoadjuvant chemotherapy is used in the treatment of localized early-stage breast cancer with a goal of breast-conserving surgery, or for patients for whom surgery is temporarily contraindicated [47,61]. The optimal chemotherapy regimen for the neoadjuvant treatment of TNBC has not yet been established. However, platinum-based regimens have been suggested to possibly be more active in TNBC.

Adjuvant therapy can further be divided into two groups: cytotoxic agents, which have DNA damaging effect to rapidly dividing cells and targeted therapies such as inhibition of poly-ADP ribose polymerase I (PARP-I) and EGFR [47,61,62]. The optimal adjuvant regimen for TNBC has not been established, but current guidelines support the use of regimens that contain an anthracycline and taxane [63].

As molecular research advances, there has been evidence of the primary role of the immune system in influencing the disease progression of TNBC [64]. Immunohistochemical studies recognized the presence of Tumor-Infiltrating Lymphocytes (TILs), as a predictor of good prognosis in both adjuvant and neoadjuvant regimens [65]. Also, the expression of immune evasion molecules in the tumor microenvironment, as Programmed Death-Ligand 1 (PD-L1), has been shown to influence TNBC prognosis. These molecules can function as targets and from these data, new immunotherapeutic agents can be developed to directly target immune checkpoint molecules, such as anti-PD-1 and anti-PD-L1 monoclonal antibodies, in TNBC patients [66,67].

Chemotherapy, that was initially thought to be exclusively immunosuppressive, can induce multiple immunomodulatory changes in the tumor microenvironment, including increased antigen release by tumor cells, PD-L1 upregulation, and hyperexpression of immunogenic cell surface markers (e.g., MHC

class I molecules). Collectively, these modifications might positively influence the effectiveness of immunotherapy [68].

Previous studies suggest that early TNBC has a reduced immunosuppressive phenotype compared to metastatic cases, thus immunotherapy could be implemented as first-line strategy [69]. Therefore, there is increasing interest in testing immunotherapeutic strategies in both neoadjuvant and adjuvant settings. Neoadjuvant studies for TNBC are currently ongoing [70].

1.1.1.3 Natural compounds in treatment of breast cancer

Traditional medicine and diet have been serving humanity throughout the centuries as a means of preventing and treating most of chronic diseases, either in its natural form or as models to synthetic modification [71–73].

Natural compounds exhibit a great chemical diversity and therefore play an important role in the efforts of discovering new drugs [71–74]. The interest in natural compounds has increased over the last few years, due to concerns about drug costs and, mainly, due to their security, as natural compounds have usually low toxicity [19]. Furthermore, some of these compounds have a high extraction yield, are easy to obtain, have promising physiological functions and are generally accepted as dietetic supplements [72,73,75].

Over the last decade, many bioactive compounds have been identified in plants and in human diet and are being developed as chemopreventive agents to inhibit, slow down or even reverse the progression of several cancers [20]. The anticancer activity of natural compounds has been partially explained by their ability to trigger cellular death pathways, including apoptosis [71].

1.1.2 Oxidative Stress and Cancer

Aerobic respiration generates energy in the mitochondria of eukaryotic cells, and as a result of this oxidative metabolism, several compounds are produced. Most of these compounds are beneficial, yet, less than 5% of them can be toxic for the cell if their concentration increases [76]. These normally low-concentration products, that are derived from oxidative metabolism, are necessary for certain subcellular events, including signal transduction, enzyme activation, gene expression, disulfide bond formation during the folding of new proteins in the endoplasmic reticulum, and the control of the caspase activity that is activated during the apoptotic process [77,78].

Sources of internal oxidative stress include peroxisomes and enzymes, particularly the detoxifying enzymes from the P450 complex, xanthine oxidase, and the nicotinamide adenine dinucleotide (NADPH)

oxidase complexes, which include the NOX family [79]. Most of these enzymes act in the mitochondria, which is the main source of oxidative stress, during intracellular metabolic processes, such as the electron transport chain [80]. External sources of oxidative stress include UV radiation, chemical compounds from environmental pollutants, smoking and alcohol, exercise and therapies, as radiotherapy and chemotherapy [81].

Reactive oxygen species (ROS) include superoxide anion (O_2 –), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), singlet oxygen ($1O_2$) and ozone (O_3). The damage that these ROS can cause to the cell, not only depends on their intracellular concentration but also, on the equilibrium between the ROS and the endogenous antioxidant species. When the pro-oxidant/antioxidant equilibrium is lost, oxidative stress is generated, altering and damaging many intracellular molecules, including DNA, RNA, lipids and proteins [82].

Natural antioxidants are the cell's defense mechanisms that scavenge reactive species, and they can be classified into different groups according to their properties: endogenous antioxidants, natural antioxidants and synthetic antioxidants. Endogenous antioxidants include glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, l-carnitine, melatonin, enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRXs) [83,84].

The cell membrane is rich in polyunsaturated lipids that are susceptible to oxidation by reactive species. ROS liberate lipid peroxidation reactions and consequently increase the permeability of the cell membrane, which could lead to cell death. Moreover, ROS cause nicks in the DNA and malfunctions in the DNA repair mechanism. DNA oxidation by these reactive species generates 8-hydroxy-2-deoxyguanosine (8-OH-dG), which is a product that is able to generate mutations in DNA, in a process that enhances aging and carcinogenesis [85,86]. Besides, ROS can promote many aspects of tumor development and progression, which can be classified into the following biological processes: cellular proliferation, evasion of apoptosis, tissue invasion and metastasis, and angiogenesis [87].

Biomarkers of oxidative stress have been investigated for their association with the development and progression of several cancer types, and in particular breast cancer **(Figure 2)** [88].

Direct measurement of ROS is almost impossible due to the relatively short-term presence of the reactive intermediates [81]. Thus, measurement of oxidative damage is a more common way to monitor oxidative stress. DNA adducts and 8-OH-dG, and malondialdehyde (MDA) metabolites and adducts, caused by lipid peroxidation, are biomarkers commonly used in studies of breast cancer, as oxidative

stress mechanisms may be involved in several known breast cancer risk factors, including obesity and daily alcohol intake, and circulating estrogen levels [79,88].

An increased level 8-OHdG was observed in ER-positive malignant tissues. The 8-OHdG levels in ER-positive MCF-7 cell line were significantly higher than in the ER negative MDA-MB 231 cell line [85].

The epithelial-mesenchymal transition (EMT) is a physiological process that is closely related to the acquisition of an invasive and metastic phenotype, and occurs during embryonic development, tissue repair, and fibrosis. In this process, morphological changes lead to increased mobility, invasiveness, loss of polarity, cytoskeletal reorganization, loss of adherence junctions, and changes in the production of proteins of the extracellular matrix (ECM) [89]. Several authors claim that EMT and oxidative stress configure a bidirectional interplay that directly affects tumor malignancy [87]. It was shown that in breast epithelial normal (MCF-10A) and tumoral (MDA-MB-231) cells, NOX4-derived ROS production is the pathway through which TGF-β drives the EMT and cellular migration, but not proliferation [90,91]. Furthermore, the tumor suppressor p53 seems to have repressive functions in the induction of NOX4 by TGF-β, what is abolished in breast cancer cells expressing the mutant forms of p53 [92].

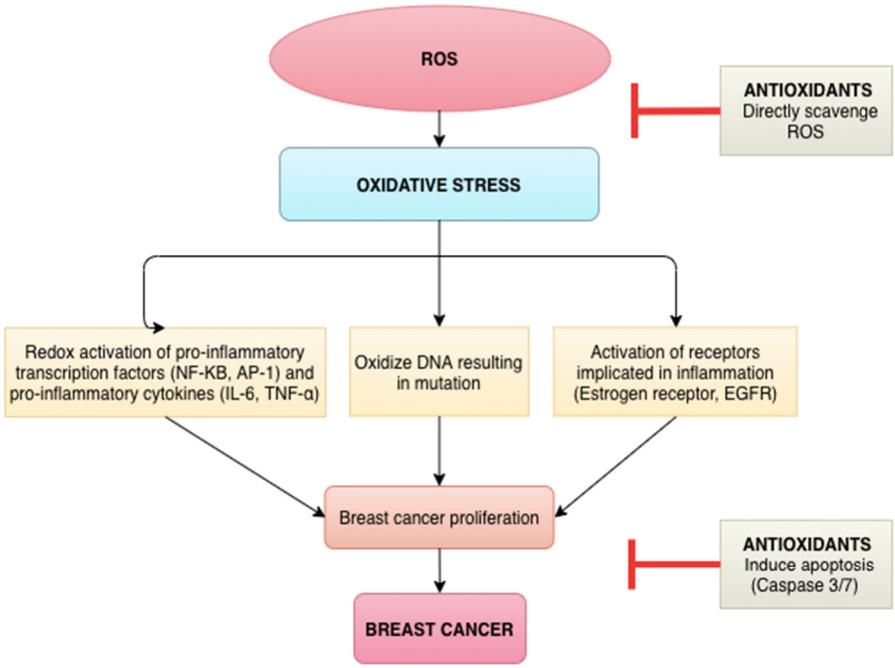


Figure 2 – ROS-mediated oxidative stress plays a role in carcinogenesis at many levels. It can alter pro-inflammatory transcription factors (NF-kB, AP-1) and pro-inflammatory cytokines (IL-6, TNF-α), and promotes DNA oxidation, causing changes in the coding region of key regulatory proteins. Cysteine residues oxidation in key receptors that get autoactivated without ligand binding, benefits cell proliferation, due to oxidative stress products. Adapted from [81].

ROS-generating and scavenging systems are potential targets for cancer therapy [78]. An important feature of the pro-oxidant therapy is that the additional ROS might lead to the preferential killing of cancer cells sparing non-tumoral cells, since normal cells normally have lower oxidant levels and would be able to cope with the rise without undergoing oxidative damage and death [79,87,92]. In this context, previous reports have suggested that high vitamin C doses are more cytotoxic to cancer cells than to normal ones, due to its prooxidant effects. A high-dose vitamin C increased the levels of upstream metabolites of glycolysis pathway and tricarboxylic acid cycle, while adenosine triphosphate (ATP) levels and adenylate energy charges were decreased in MCF-7 cells [93].

Some antioxidants and polyphenolic compounds have been reported to exert antiproliferative effects on breast cancer cells [20,94–96]. Antioxidants can also induce apoptosis in cancer cells, by binding to transmembrane lipids, causing loss of the mitochondrial transmembrane potential and release of cytochrome c, thus activating the intrinsic caspase-dependent apoptotic pathway [97].

1.2 *Eucalyptus spp.* and *Eucalyptus nitens*

Eucalypts comprise the greatest area of non-native forests in Europe and are located mostly in the Iberian Peninsula, where they cover about 1.5 million hectares [98]. This increase in *Eucalyptus* production areas is associated with its high yield, short production cycle (10-12 years), diverse uses of its wood (e.g. fiber, sawtimber, construction, etc.), high capacity to tolerate variable climatic conditions, and ability to be manipulated to produce hybrids and clones with higher wood quality [99–101].

The fast-growing commercial plantations of *Eucalyptus* species have nowadays an important role in the fulfillment of the worldwide increasing demand for pulpwood [102]. In fact, the *Eucalyptus* species are the most important fiber sources for pulp and paper production in South-West Europe (Portugal and Spain), South America (Brazil and Chile), South Africa, Japan and other countries, and in the temperate and Mediterranean zones, *E. globulus* and *E. nitens* are the most common planted species [102,103]. Specifically, *Eucalyptus nitens* is adapted to cold temperate climates, as it is less susceptible to low temperatures and frost [103]. In Portugal, *Eucalyptus spp*. expanded extensively from the mid-twentieth century onwards, and are already the most important forestry species, covering 26% of Portuguese forests [104].

During the last decade, a re-emerging interest for the integrated exploitation of plant biomass as sources of materials, chemicals, fuels and energy has been emerging, within the biorefinery concept [105]. This renewed interest has drawn the attention of agro-forest industries, concerned with taking maximum values out of their crops [102,106,107][102,106,107][102,106,107]. The biomass residues

resulting from *Eucalyptus* pulp mill operations are mainly bark, normally removed in the mills and burned for energy production, but also leaves, branches and fruits from harvesting and logging operations, which are either left in the forest for nutrition, or burned for energy [105]. Some of these residues and by-products can be sources of valuable compounds with antioxidant activity **(Table 2)**, among other bioactivities – such as polyphenols phytosterols, lignans and triterpenoids [102,106–109].

Table 2 - Experimental values of yield bark extraction, total phenol concentration and antioxidant capacity of *E. globulus* and *E. nitens*, at optimal conditions. Adapted from [98].

Species	*E. globulus*	*E.nitens*
Yield (% g extract/g dry bark)	10.41 ± 1.21	15.11 ± 1.03
Total phenol concentration (mg gallic acid equivalent/g dry bark)	36.90 ± 1.26	46.76 ± 2.07
Antioxidant capacity (mg ascorbic acid/g dry bark)	35.09 ± 1.51	43.11 ± 1.22

However, a fundamental step in the production processes for obtaining active substances is the extraction from the source material. This involves contacting the material containing the substance of interest (or solute) with a specific solvent, occurring a transfer of the active compound from one phase to another, for example, by Soxhlet extraction, as described by R.M.A Domingues *et al.* [110,111].

Previous studies devoted to the lipophilic composition of bark in some of the most important *Eucalyptus* species used by pulp industry worldwide showed that, for technical and economic reasons, bark is among the most interesting residues for possible exploitation in an integrated way [102].

Furthermore, it has been reported that the lipophilic extracts present in the outer barks of many *Eucalyptus* species contain high amounts of triterpenoid acids with lupane, ursane and oleanane skeletons, namely betulonic, betulinic, ursolic, 3- acetylursolic, oleanolic and 3-acetyloleanolic acids **(Table 3)**, making this fraction of bark the residue from which these compounds can be efficiently extracted, in an integrated way with the existing kraft pulp mills [102,112].

Principally, the outer layer of *E. nitens* bark contains a small, but higher quantity of triterpenoids and acetyl derivatives, compared to *E. globulus* [102,108].

E. nitens is not the most common eucalypt species in Portugal, nonetheless, *E. nitens* shows great features, as above mentioned, indicating that this could be a good candidate to an eventual natural drug source.

Table 3 – Major triterpenic components identified in *Eucalyptus* species outer bark (g/kg). Adapted from [101].

Component	World regions usage for pulpwood				
	Temperate and Mediterranean			Sub-tropical and tropical	
	E. globulus	*E. nitens*	*E. maidenii*	*E. urograndis*	*E. grandis*
Main triterpenic acids	21.3	21.6	8.4	4.5	5.1
Betulonic acid	2.6	2.4	1.0	-	-
Oleanolic acid	4.1	8.4	1.7	1.2	0.7
Betulinic acid	2.6	6.6	2.0	1.4	2.1
Ursolic acid	12.1	4.2	3.6	1.9	2.4
Other triterpenoids	1.5	3.0	0.2	-	0.1
Total triterpenoids	22.8	24.6	8.5	4.5	5.2

1.2.1 Triterpenoid acids

Terpenes are an important group of aromatic compounds that can be divided according to the number of isoprenes units into mono-, sesqui-, di-, sester-, tri-, tetra-, and poly-terpenes and, in association with sterols, these create extensive groups of isoprenoids [113].

Triterpenoids represent the largest group of phytochemicals. It has been estimated that more than 20 000 triterpenoids exist in nature [113,114]. Triterpenoids are biosynthesized in plants by the cyclization of squalene, which is a big group of compounds that have thirty carbon atoms arranged in five rings with several oxygen atoms connected [115]. They can further be subclassified into diverse groups including cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes, hopanes, isomalabaricanes, lanostanes, limonoids, lupanes, oleananes, protostanes, squalenes, tirucallanes, ursanes and miscellaneous compounds [116–118].

They mostly are found in various plants including sea-weeds as well as in wax-like coatings of various fruits and medicinal herbs, including apples, cranberries, figs, olives, mistletoe, lavender, oregano, rosemary and thyme, and also in *Eucalyptus* genus [119–123]. Although triterpenoids were considered to be biologically inactive, accumulating evidence on their broad spectrum pharmacological activities coupled with a low toxicity profile has sparked renewed interest with regard to human health and disease [112].

Triterpenoids are used for traditional medicinal purposes in many Asian countries for anti-inflammatory, analgesic, antipyretic, hepatoprotective, cardiotonic, among other effects [72,116,121]. Recent studies have not only confirmed some of the aforementioned pharmacological properties of several triterpenoids, but also identified a variety of additional biological activities including antioxidant, antimicrobial, antiviral, and antiangiogenic activity [117,124]. An increasing number of triterpenoids have

been reported to exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells [116,117,120]. In a particular case, natural triterpenoids demonstrated the inhibitory effects against proliferation growth and invasion of breast cancer [45,125–127]. They also demonstrate antitumor efficacy in preclinical animal models of cancer, and many are being evaluated in clinical trials [117,127].

A large number of triterpenoids have been synthesized by structural modification of natural compounds for optimization of bioactivity, and some of these semi-synthetic analogs are considered to be the most potent anti-inflammatory and anticarcinogenic triterpenoids known to mankind [114].

1.4.1.1 Ursolic acid

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid, UA) [128] is a natural pentacyclic triterpene carboxylic acid of the ursane-type saponins **(Figure 3)**. It is widely present in several medicinal plants like sage, olive and rosemary as well as in some fruits, such as some berries and apples, in *Eucalyptus spp.,* and the percentage composition of UA varies between different species due to the presence of enzymes responsible for its biosynthesis [102,129,130].

The biosynthesis of UA is mainly achieved through the folding and cyclization of squalene into dammarenyl which further undergoes ring expansion and extra cyclization to form the fifth ring of UA [131]. It can behave as a bi- or tri-dentate ligand because of the presence of 3 oxygen atoms and can easily donate lone pairs of electrons to transition metal atoms [132].

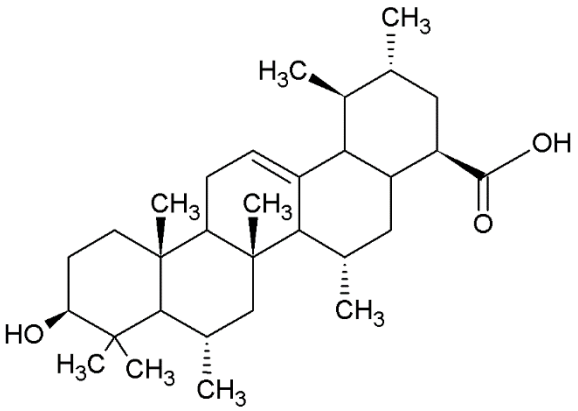


Figure 3 - Ursolic acid chemical structure. Designed in Chemsketch.

UA has a wide range of biological activities including anti-microbial, anti-inflammatory, anticancer, melanin biosynthesis, antidiabetic, anti-obesity, anti-atherosclerosis, anti-hepatic steatosis, analgesic,

anti-depression, neuroprotective effects and anti-osteoporosis, as well as it is able to provide protection against oxidative damage in DNA .

Interest in UA, as a promising therapeutic compound, has grown in recent years because of lower toxicity, potential antioxidant, anti-inflammatory and anticancer effects on normal and cancer cells, and it has been incorporated in many pharmaceutical applications claimed in different patents [133]. Furthermore, UA has been considerably known as chemopreventive agent against different types of breast cancer, *in vitro* and *in vivo* [131] **(Figure 4)**.

UA has been shown to inhibit EGFR, STAT3, and cyclin D1 expression in MCF-7 and MDA-MB-231 cells, which ultimately suppress epidermal growth factor (EGF) mediated cell proliferation [32]. Also, UA has been observed to bind with the ATP binding pocket of EGFR kinase domain, indicating UA could be an EGFR tyrosine kinase inhibitor [134].

Mitochondrial membrane permeability is mainly controlled by the Bcl-2 family of proteins, through regulation of the formation of apoptotic protein-conducting pores in the outer mitochondrial membrane [135]. UA studies showed that this triterpenoid acid downregulated Bcl-2 expression and upregulated Bax, caspase-3, -8, -9, and PARP expression, induced the release of cytochrome C to the cytosol from mitochondria and the appearance of Fas receptor, and reduced mitochondrial membrane potential in MDA-MB-231 cells, which indicate that UA may induce apoptosis through both mitochondrial death pathway and extrinsic death receptor dependent pathway [136].

UA reduced the expression of MMP-2 and urokinase-type plasminogen activator (u-PA), which are known to play an important role in cancer cell invasion and metastasis, by inhibiting Jun N-terminal kinase (JNK), AKT, and mTOR phosphorylation and reducing NF-kB protein level in the nucleus of MDA-MB-231 cells. Thus, treatment with UA leads to suppression of migration and invasion of MDA-MB-231 cells [17,137].

It has been observed that UA also induce apoptosis by promoting PARP cleavage and by reducing Bcl-2 expression in MCF-7 cells [136]. Furthermore, UA showed the ability to bind GR (glucocorticoid receptor) and translocate GR into nucleus, indicating UA could be GR modulator [137]. In the case of breast cancers that lack ER expression, GR activation appears to prevent death of cancer cells and therefore may promote tumor cell viability [138].

UA has low water solubility, which limits bioavailability in the human body, also its rapid metabolism restricts clinical applications for therapeutic effects [139,140]. To enhance the biopharmaceutical properties of this compound, modifications of chemical groups and new approaches,

as drug delivery technologies were developed. Several UA delivery systems have been used successfully, such as nanoemulsions and liposomes, for example [141].

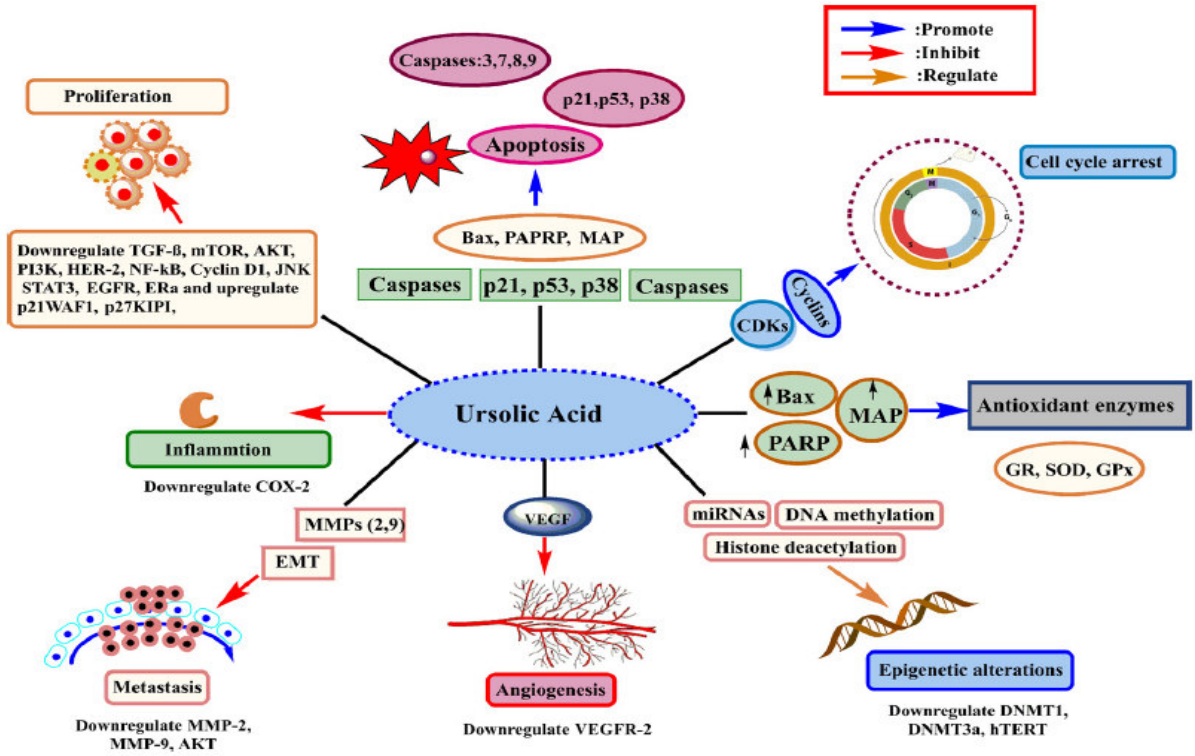


Figure 4 - Ursolic acid and their molecular mechanism of action in the prevention and treatment of breast cancer. Extracted from [17].

1.2.1.2 Betulinic acid

Betulinic acid (3β,hydroxy-lup-20(29)-en-28-oic acid, BA) is part of the lupane group, a pentacyclic triterpene, and it can be found in many fruits, plants and vegetables, but higher amounts can be obtained from the birch tree bark (*Betula sp.*), sycamore and *Eucalyptus* bark [142–144]. Betulinic acid **(Figure 5)** is the oxidized derivative of its precursor betulin, which was one of the first natural compounds to be isolated from plants more than two centuries ago [71,145].

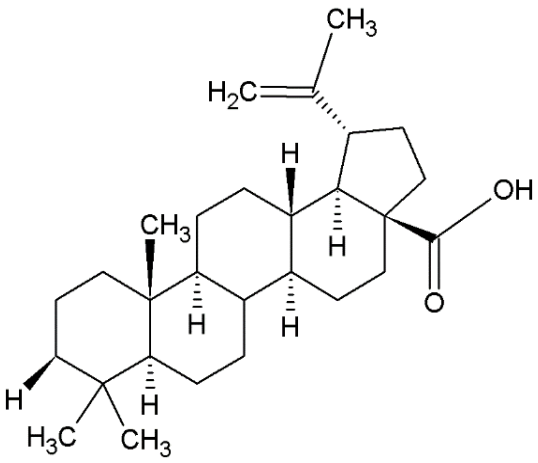


Figure 5 - Betulinic acid chemical structure. Designed in Chemsketch

BA is a bioactive compound that possess a wide range of pharmacological effects like anti-inflammatory, antimicrobial, cardiovascular, anti-proliferative, anti-angiogenic, cytotoxic, antimalarial, anti-viral, antioxidant, antiallergic, anti-tuberculosis effects, and cytotoxic towards various cancer cells, including, lung, prostate, skin, gastric and breast cancer [71,112,124,143,145,146].

Many drugs have carcinogenic potential in human cells. However, studies have demonstrated that BA selectively acts on cancer cells and not on normal ones, which means that BA is a very promising pipeline anticancer drug with pharmacological safety [147]. An interesting and clinically important fact is that BA has a higher effect in an environment with pH lower than 6.8, which is the pH at which almost every tumors develops [124,145].

BA can induce cell cycle arrest in breast cancer cell lines, triggered by decreased mitochondrial ATP production [125]. For example, MCF-7 cells arrest in G1 when treated with BA [148]. Alternatively, BA increases expression of p53 and p21, which are involved in cell cycle regulation, and can arrest MCF-7 in G1 phase [148]. MDA-MB-231 and T47D cell lines lacking p53 arrest in G1 phase, under BA treatment through activation of p21 [149].

BA decreases expression of Bcl-2 family anti-apoptotic proteins (Bcl-2, Bcl-XL), and increases expression of pro-apoptotic proteins Bax, Bak and Mcl-1, increasing the ratio of Bax/Bcl-2 [150]. These changes in gene expression also promote cell cycle arrest at G1 [151]. BA suppresses NF-kB mediated signaling by decreasing its expression and inhibiting its activation in breast cancer cells and appears to act upstream of IKK activation. Also suppresses TNF expression and inhibits interaction with its receptor (TNFR), thus preventing activation of IKK via IKKb, as well directly inhibits activation of IKK, thereby preventing all downstream events in the signaling pathway including expression of oncogenic response genes including COX-2 and MMP-9 **(Figure 6)** [152].

So, due to its low toxicity against normal tissues and cells, its remarkable anticancer activity and the fact that it can bypass drug resistance cancers, it can be expected that BA will eventually be accepted as an adjuvant therapeutic in the treatment of cancer cells. Though, BA is a highly lipophilic molecule with limited water solubility which can decrease its *in vivo* uptake [118,142,146]. To alleviate the problems associated with the poor aqueous solubility and bioavailability of BA, various nanostructured delivery systems have been used and hold great promise for future clinical applications [153].

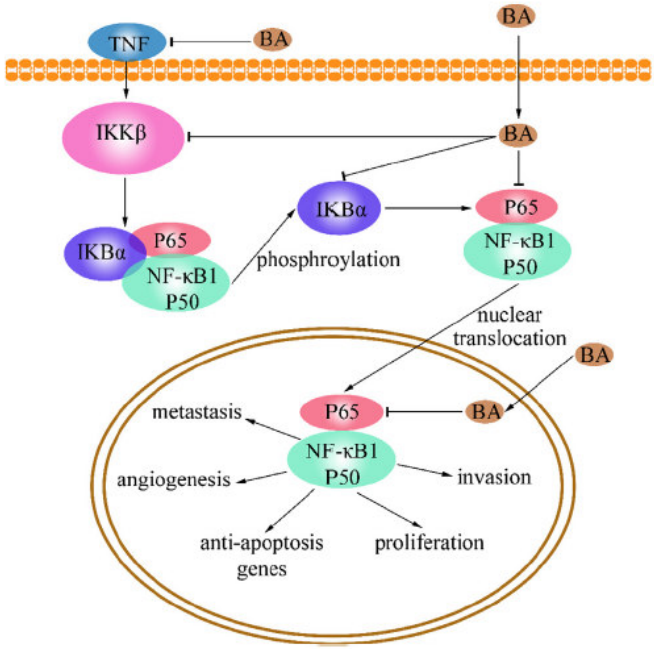


Figure 6 - Inhibition of NF-KB by betulinic acid to treat Breast Cancer. BA suppresses TNF expression, a membrane protein on the cell membrane, and inhibits interaction with its receptor, thus preventing activation of IKK via IKKB. BA also directly inhibits activation of IKK, phosphorylation of IKB-α, phosphorylation of p65, and nuclear transition. Also, NF-KB depend reporter gene transcription is inhibited by BA, so as to induce the inhibition of invasion, proliferation, angiogenesis and metastasis, to cause breast cancer cell death. Extracted from [152].

1.3 Cellular models

Cell cultures make it possible to understand cell biology, tissue morphology, mechanisms of diseases, drug action, protein production and the development of tissue engineering [154]. They are often used in the preclinical research of many drugs, in cancer research, and in studies on gene function [155]. The choice of the most appropriate cell culture methods in the area of cancer research may allow us to better understand tumor biology, and hence to optimize radio- and chemotherapy, or even to find new treatment strategies [156].

The most commonly used type of cell culture is the 2D model, but nowadays the 3D culture method has been gaining in popularity [157]. Depending on the type of culture chosen, cell behavior differs in many aspects [154].

In adherent 2D cultures, cells grow as a monolayer in a culture flask, attached to a plastic surface [158]. The advantages of 2D cultures are associated with simple and low-cost maintenance of the cell culture and with the performance of functional tests [154]. Unfortunately, 2D cultures also have numerous disadvantages. First, 2D cultured cells do not mimic the natural structures of tissues or tumors. In this culture method, cell-cell and cell-extracellular environment interactions are not represented as they would be in the tumor mass. These interactions are responsible for cell differentiation, proliferation, vitality, expression of genes and proteins, responsiveness to stimuli, drug metabolism and other cellular functions [154,155,157,158]. Another drawback of 2D culture is that the cells in the monolayer have unlimited access to the ingredients of the medium such as oxygen, nutrients, metabolites and signal molecules. For cancer cells *in vivo*, the availability of nutrients, oxygen, and so forth, is more variable because of the natural architecture of the tumor mass. Owing to the many disadvantages of 2D systems, there was a need to find alternative models, better able to mimic a natural tumor mass, such as 3D culture systems [157].

Due to the method of preparation, 3D models can be divided into suspension cultures, cultures in concentrated medium or in gel-like and cultures on a scaffold. Cells from the donor's tissues are cultured in multicellular, three-dimensional structures, imitating the architecture of the parental tissue more accurately than is possible in 2D models [159]. Cells can receive stimuli from the local environment, as happens *in vivo* [159,160].

The TNBC cell lines mirror the original tumors from which they were derived morphologically and molecularly. Thus, they are useful for the study of molecular aberrations in TNBC and the study of the pathways affected by those aberrations.

The interaction with ECM can affect the sensitivity of cells to therapy. Interestingly, work with cancer cell lines grown in 2D culture found that many TNBC cell lines are sensitive to MEK inhibition but the MDA-MB-231 cell line was relatively resistant. When grown in 3D cultures, the MDA-MB-231 cells were significantly more sensitive to MEK inhibition [161]. In contrast to the results with MEK inhibition, the transformed cells became more resistant to doxorubicin when cultured in 3D culture [161]. Other investigators have also found resistance to chemotherapeutic drugs and TNF family ligands when breast cancer cells are grown in 3D culture [157–159].

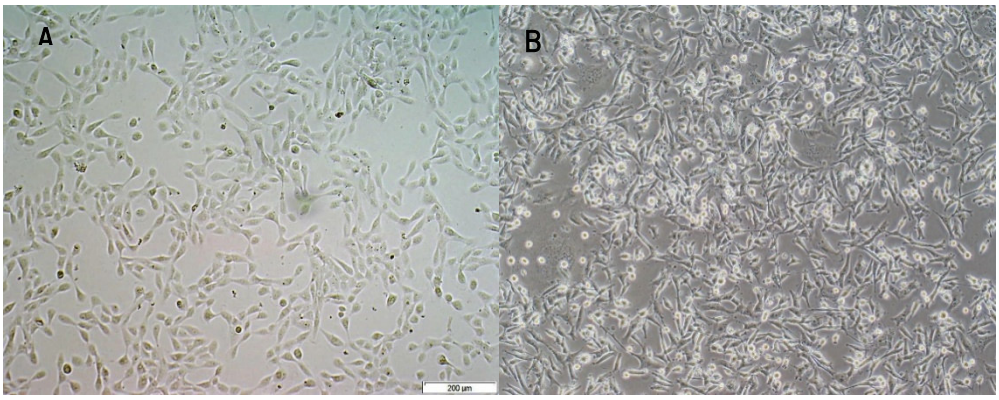


Figure 7 - A: MCF-10A is a human breast epithelial cell line and is the most commonly used normal breast cell model; **B**: MDA-MB-231, is an epithelial TNBC human cell line. Brightfield, 100X magnification. Scale bar: 200µm.

The MCF-10A human breast epithelial cell line **(Figure 7A)** is arguably the most commonly used normal breast cell model. These cells were derived from benign proliferative breast tissue and spontaneously immortalized without defined factors. They are not tumorigenic and do not express estrogen receptor [162]. When cultured on top of Matrigel, MCF10A cells are capable of forming acinus-like spheroids with a hollow lumen. This structure is covered by basement membrane and formed by polarized and organized cells [163].

MDA-MB-231 **(Figure 7B)** is an epithelial, highly aggressive, invasive and poorly differentiated TNBC human cell line as it lacks ER and PR expression, as well as HER-2 amplification [164]. It was established from a pleural effusion of a 51-year old caucasian female with a metastatic mammary adenocarcinoma [164].

Similar to other invasive cancer cell lines, the invasiveness of the MDA-MB-231 cells is mediated by proteolytic degradation of the extracellular matrix. This cell line has been widely used as cell model to study TNBC development and progression and to investigate new drugs against TNBC [164].

The 3D model using breast cell lines provides a useful tool for dissecting cell-cell interactions in mammary gland development, as well as for studying the molecular mechanisms on mammary tumor development, and screening of potential new drugs.

1.4 Aims

Previous studies on human breast cancer cell lines showed beneficial effects on the use of some natural compounds, due to their biological properties, as anticancer, antiproliferative, antiangiogenic and

antioxidant activities. The development of natural-based drugs gained the interest of investigators, in the last decades, as it can become valuable alternatives to the usual therapies, being less expensive, but mostly, being more efficient and with less side effects for the patients. Nevertheless, a better understanding of the chemical and biological characteristics is fundamental to the development of these new promising therapies.

The vast majority of natural bioactive compounds have been identified in plants and it is easy to extract them through optimized extraction methods. Portugal has a large production of *Eucalyptus* for the pulp and paper industry and this sector produces tons of bark residues that do not have a proper use. These industrial by-products can be an important source of bioactive compounds, such as ursolic (UA) and betulinic (BA) acids, which have recognized anticancer and antioxidant activities.

The overall aim of this work was the study of outer bark *Eucalyptus nitens* extract effects on two human breast cell lines, a tumoral and a non-tumoral model. Also, UA and BA were tested, individually and combined, as reference compounds.

The cellular response of triple negative breast cancer (TNBC) and normal epithelial breast cells to *E. nitens* compounds was evaluated. Therefore, cell viability was measured or evaluated by resazurin, MTT, trypan blue and acridine orange/propidium iodide double staining. The potential antioxidant effects of triterpenoids acids present in the extract and in its pure state, to balance redox cellular state were tested by DCFDA assay. The establishment of a 3D cell model was also tested. With this study we intend to give one more step ahead in the elucidation of the mechanisms of action of triterpenoids acids as promising anticancer compounds.

2. Materials and Methods

2.1 Chemicals and Equipments

Dubelcco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), antibiotic and antimycotic solution, horse serum (HS), recombinant human insulin, human epidermal growth factor (EGF), cholera toxin, hydrocortisone, dimethylsulfoxide (DMSO), tris acetate EDTA (TAE) buffer, resazurin, MTT, trypan blue, tBHP, acridine orange, ursolic and betulinic acids were supplied by Sigma-Aldrich. F12 and trypsin EDTA solution were from Biochrom, DCF and propidium iodide from Thermo Fisher, ethanol from Carlo Erba, agarose from Lonza and MTS from Promega.

The laminar flux chamber was a Telstar Bio II A cabinet; the centrifuge a 2-16k from Sigma and a plate centrifuge from Eppendorf; the fluorimeter was a Fluoroskan ascent Fl from Thermo Scientific; shaker was a Rotamax 120 from Heidolph; the CO2 incubator from Sanyo Eletric Co.; the inverted microscope was a Olympus IX71 with a DP72 Camera and the microplate reader was a SpectraMax Plus from Molecular Devices.

All the specific references are listed in Supplementary Data **(Appendix 1)**.

2.2 Preparation of the stock solution of *E. nitens* extract

The *Eucalyptus nitens* extract was kindly provided by Doctor Fátima Duarte (CEBAL). The extract was lyophilized, and a stock solution was prepared by dissolving the extract in ethanol, at 10 mg/ml concentration and filtered with a 0.22 µm PES filter. The stock solution was aliquoted, in order to avoid freeze-thaw cycles and stored at -20°C.

2.3 Preparation of pure acids

Ursolic and betulinic acids were both dissolved in ethanol. Stock solutions were prepared fresh by dissolving lyophilized acids in ethanol, at 1 mg/ml concentration. From this stock, several dilutions were performed to obtain the selected concentrations.

2.4 Mammalian cell culture

2.4.1 Cell lines

The MCF-10A and MDA-MB-231 cell lines were kindly provided by Doctor Fátima Duarte (CEBAL). MCF-10A is a non-tumorigenic epithelial cell line that was established from a mastectomy performed on

a 36-year old premenopausal caucasian woman with fibrocystic disease. The MDA-MB-231 is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year old causasian female with a metastatic mammary adenocarcinoma.

2.4.1.1 Subculture

MCF-10A cell line was cultured in growth medium consisting of DMEM:F12 (1:1) medium supplemented with 5 % ($^{v}/_{v}$) Horse Serum, 10 µM/ml recombinant human insulin, 20 ng/ml human epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.5 µg/ml hydrocortisone, and 1% antibiotic/antimycotic solution.

MDA-MB-231 was cultured in growth medium consisting of DMEM, supplemented with 10% ($^{v}/_{v}$) Fetal Bovine Serum (FBS) and 1% ($^{v}/_{v}$) antibiotic/antimycotic solution.

Cells were generally maintained in 25 cm^2 culture flasks in 5 ml of growth medium and kept in an incubator at 37°C with 5% CO_2. Cells were subcultured when 80-85% of confluency was reached, or about every 3 days. PBS 1x and 0.05% ($^{w}/_{v}$) or 0.25% ($^{w}/_{v}$) trypsin-EDTA was added to MCF-10A or MDA-MB-231 cell line, respectively. Cells were generally diluted 3 times and maintained for up to 25 passages.

All the assays were performed under sterile conditions in a laminar flux chamber, with sterile equipment, to avoid contaminations.

2.4.2 Viability assessment – Resazurin assay

Resazurin cell viability assay is widely used in drug discovery for the study of cytokines, growth factors, and cytotoxic agents. The resazurin cell viability assay provides high throughput screening tool, which can be used to assess mammalian cell toxicity, viability migration, and invasion in both early drug discovery compound screening and subsequent drug safety and toxicity studies [165]. It is reliable, sensitive, simple and cost-efficient. In addition, it keeps cells intact., which allow other parallel analyses.

Resazurin dye (7-hydroxy-3H-phenxazin-3-one-10-oxide) has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. Oxido-reductase reactions happen principally in living cells. The reduction of resazurin therefore, correlates with the number of living cells. The transference of electrons from NADPH to resazurin, will reduce the blue Resazurin to a pink fluorescent counterpart, resofurin.

The resazurin reduction test was carried out as follows. A stock solution was made by dissolving resazurin in phosphate-buffered saline (PBS 1x), at $2.5x10^{-3}$M concentration.

MCF-10A and MDA-MB-231 cell lines were plated in a 24-well plate at density of $1.2x10^5$ cells/ml. After 24h, the growth medium was then removed and cells were incubated with *E. nitens* extract at

different concentrations (0.1, 0.5, 1, 5, 10, 15, 25 and 50 µg/ml), dissolved in medium. In this assay, a positive control replacing the culture medium with fresh medium was performed, as well as negative control incubating cells with medium containing 30% (v/v) DMSO. Also, to ensure that the effect of the extract solvent (ethanol) had low impact toxicity in cells, three concentrations were tested, 0.5%, 0.25% and 0.15% ethanol, relatively to the amount of ethanol present in the highest extract concentrations.

At 24 and 48h, the solutions were removed and 500 µl resazurin diluted in cell medium was added to each well (1:10 dilution). Plates were incubated for 2h in the CO_2 incubator. The fluorescence intensity was measured on a scanning microplate spectrofluorometer, using an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

2.4.2.1 Resazurin assay optimization

In order to perform continuous experiments for assessment of cytotoxicity without toxicity caused by the contact with resazurin, which would create a bias in the results, the optimal concentration of resazurin was chosen from an optimization experiment with 4 different concentration, ranging from 2.5x10-4 to 5x10-5 M, with 9 replicates. Results are shown in **Table 6**, in the next section, and the selected resazurin concentration was $1.25x10^{-4}$ M. This optimization was done in MDA-MB-231 but the same concentration was applied in MCF-10A, which responded well.

2.4.3 Viability assessment – MTT assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This is a colorimetric assay based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells [166]. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting colored solution is quantified by measuring absorbance at 570 nm using a multi-well spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells.

The following protocol was used: cells were seeded in 96-well plate at a density of $1.5x10^5$ cells/ml for MCF-10A and $2x10^5$ cells/ml for MDA-MB-231, and then incubated at 37 °C in a humified CO_2 incubator. After 24h, the culture medium was replaced with fresh medium containing the *E. nitens* outer bark extract at increasing concentrations (5, 10 and 30 µg/ml), the equivalent concentration of UA present in the extract (0.495, 0.99, 2.97 µg/ml), the equivalent concentration of BA in the extract (1, 2

and 6 µg/ml) and the equivalent concentration of the mixture of UA and BA (1:1 - UB) in the extract. Three controls were considered: a positive control (untreated cells), a negative control (cells incubated with medium containing 10% DMSO (v/v), and ethanol control (relative to the amount of ethanol present in the highest *E. nitens* outer bark extract concentration (30 µg/ml)).

In the following 24 and 48 h, the medium was removed from the wells then, 100 µl of MTT reagent, prepared from 5 mg/ml stock solution and diluted with DMEM medium using a dilution factor of 1:10, were added to each well. The plates were incubated again at 37 °C for 1:30 h. The MTT reagent was then removed and replaced with 100 µl of DMSO/ethanol solution (1:1), to dissolve the purple formazan crystals. The absorbance of the samples was measured at 570 nm using the microplate reader. The results were expressed as percentage and normalized relatively to the positive control. The concentration of the extract that decreases the number of viable cells to half (IC_{50}) was calculated.

2.4.4 Cell membrane integrity – Trypan blue assay

The Trypan Blue exclusion assay distinguishes between live (unstained) and dead (stained) cells. Trypan Blue is a dye that permeates the compromised membranes of dead cells. Upon entry, the dye binds to intracellular proteins, resulting in a dark blue appearance [167].

To evaluate cell viability through cell membrane state, MCF-10A and MDA-MB-231 cells were grown in 24-well plate at a density of $1x10^6$/ml. Cells were treated with the same conditions of the MTT assay, for 8 and 24h. At these timepoints, the supernatant of each well was collected to a tube, the plates were washed with PBS 1x, which was transferred to the same tube. The cells were then harvested by trypsinization and centrifugated at 500 g for 10 min., and the supernatant discarded. The 20 µl cell suspension was then mixed with 20 µl of a 0.4% trypan blue solution and were subsequently resuspended. Dye-excluding viable cells were microscopically counted using a hemocytometer chamber.

The results were expressed as percentage and normalized relatively to the life control. The percentage of viable cell was then calculated through the following equation:

$$Viable\ cells\ (\%) = \frac{number\ of\ viable\ cells}{total\ number\ of\ cells} \times 100$$

2.4.5 Acridine Orange and propidium iodide double staining assay

Acridine orange (AO) is a nucleic acid-binding fluorophore that can permeate viable cell membranes to selectively stain all nucleated cells [168]. It is excited at wavelengths of approximately 502 nm when intercalated with dsDNA, emitting a green fluorescence with wavelengths of approximately 525nm. Propidium iodide (PI) is not suitable for total cell counting or nucleated cell identification due to the assay's inability to permeate intact cell membranes [168]. Instead, it is only able to cross the membranes of cells with compromised membranes. This makes it useful in differentiating viable from non-viable cells. It is excited at wavelengths of approximately 535nm and fluoresces red light in the region of 617nm. When cells in a sample are stained with both reagents, healthy nucleated cells fluoresce green exclusively while dead cells will only fluoresce red [168].

MCF-10A and MDA-MB-231 were seeded at $4x10^5$ cells/ml in 24-well plate. After 24 h of incubation, the medium in each well was removed and replaced with the same conditions of MTT assay, for 8 and 24 h, except for positive control. In this case, the positive control was cells incubated with tBHP and negative control was cells incubated with growth medium.

Two hours prior to the timepoints, 8 and 24 h, tBHP ($1x10^3$ µM) was incubated in the respective well. For following treatment for 8 and 24 h, media was removed, cell were washed with PBS 1x (250 µl) and were incubated with PI solution (50 µg/ml in PBS) for 15 min. and afterwards, incubated with AO solution (50 µg/ml in PBS) for another 15 min., always at 37 °C, 5% CO_2, in the dark.

The fluorescence was analyzed qualitatively by an inverted fluorescent microscope, using FTIC and TRITC filters.

2.6.1 Oxidant activity – DCF-DA assay

The DCFDA assay quantitatively measures ROS in cells using a microplate reader. It uses a permeant reagent 2',7'-dicholorofluorescin diacetate (DCFDA), which is a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell [169]. After diffusion into the cell, DCFDA is cleaved by intracellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy.

MCF-10A and MDA-MB-231 were seeded in 24-well plates at a density of $1.2x10^5$/500 µl/well. Cells were treated with same conditions of MTT assay for 3, 8 and 24 h, but in this experiment the positive control was tert-Butyl-hydroperoxide (tBHP) at 1 mM and negative control contained only growth medium.

DCF-DA stock solution was prepared by diluting in DMSO at $2x10^{-3}$ µM concentration. Cell were washed with warmed PBS 1x, DCF-DA solution ($1x10^{-2}$ µM in PBS) was added to all the wells and cells incubated for 30 min., at 37 °C and 5% CO_2, in the dark. Then, for positive control, tBHP ($1x10^{-3}$ µM in PBS) was incubated for 15 min., in the same conditions. DCF-DA solution was removed, and cells washed with PBS 1x. After this, cells were lysated with a DMS0/PBS solution (9:1 solution), at room temperature, for 10 min. in a shaker.

Relative changes in the intracellular ROS levels were monitored by fluorometric detection using a fluorescent microplate reader at excitation and emission wavelengths of 485 nm and 538 nm, respectively. The fluorescence intensity of DCF is proportional to the level of ROS generated intracellularly. The results were expressed as percentage and normalized relatively to the life control. Fluorescence was also monitored qualitatively by an inverted fluorescent microscope, using FTIC filter.

2.4.6.1 DCFDA assay optimization

For MCF-10A, 1 and 10 µM of DCFDA were used to detect the intracellular ROS [170], and 10 and 20 µM of DCFDA, in MDA-MB-231 cells [95,162]. All these concentrations were tested for each cell line, and the final concentration selected was 10 µM for both cell lines.

For positive control, H_20_2 was also tested but it showed no significant difference compared to tBHP, in all timepoints, so tBHP continued to be the selected oxidant reagent.

2.4.7 3D cell culture

After literature research, some methods were selected to attempt the formation of multicellular breast cancer spheroids [171,172]. The first approach to the protocols was developed with MCF-10A cell line and two protocols were tested, suspension culture on non-treated plates and the hanging drop technique.

2.4.7.1 Suspension culture

Cell suspensions were seeded with two different densities, $5x10^{3}$ 000 cells/drop and $7.5x10^{3}$ cells/drop, and two different volumes, 30 and 100 µl, in 24-well suspension plates, and allowed to aggregate for 3 days. MTS assay was performed, which is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays [173]. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in

metabolically active cells [173]. Assays are performed by adding MTS reagent diluted in cell medium (0.2%/ml ($^{v}/_{v}$)) directly to culture wells, which were incubated for 2 h, at 37 °C. The medium was transferred to a 96-well plate and absorbance was measured at 490 nm.

2.4.7.2 Hanging Drop

Cell suspensions were seeded in 30 µl growth media, containing two different densities, $5x10^3$ cells/drop and $7.5x10^3$ cells/drop, in a 10 cm Petri dishes for 3 days to allow aggregation.

This process was repeated with MDA-MB-231 cell line. This time, cell suspensions were seeded with 40 µl growth media, containing two cell densities, $5x10^3$ cells and $1x10^4$ cells/drop, in a 10 cm Petri dish and in 24-well suspension plate, for 3 days to allow aggregation.

2.4.7.3 Multi-well agarose-coated plates

An 1.5% agarose hydrogel coating was prepared by dissolving 1.5g agarose in 48 ml of ultra-pure H_2O and 2 ml of 50x TAE buffer. The solution was heated in microwave at maximum potency for 30 s., until it boiled and then, 10 s. of heating-cooling cycles, three times. Agarose coating (100 µl) was added to each well of a 96-well suspension plate. After the coating cooled, cells were seeded in 50 µl at a concentration of $5x10^3$ cells/well and $1x10^4$ cells/well. Plates were incubated for 7 days to allow formation of aggregates.

2.4.7.4 Spheroids optimization

From the evaluation of the above-mentioned methods and conditions, $1x10^4$ cells density was selected for both cell lines and only Hanging Drop and multi-well agarose-coating plates methods continued to be carried out, as it produced better results. Several modifications have been attempted to the methods specified above.

To the hanging drop method cells suspensions were seeded with 40 µl growth medium, and to prevent dehydration of the drops, 5 ml of PBS 1x were added to the bottom of the plate. Cells were incubated at 37 °C and 5% CO_2, for 4 days to allow aggregation. Once cells created aggregates in the drops, spheroids were transferred to a 10 cm petri dish coated with 0.75% agarose in 10 ml growth medium for a further 3 days.

To the multi-well agarose coated plates method, the agarose percentage was reduced to 0.6%, and instead of using ultra-pure H_2O to dissolve agarose and dilute 50x TAE buffer, PBS 1x was selected

as it produced more homogeneous aggregate along the plate. Also, PBS 1x was incubated in the outer wells of the plate to prevent dehydration.

Plates were centrifuged for 10 min. at 1000 g to ensure equal cell numbers for initiation of the formation of a single spheroid in each well.

2.4.7.5 Bioactivity analysis of *E. nitens* outer bark extract in spheroids.

E. nitens outer bark extract bioactivity was evaluated in MCF-10A aggregates, 3 days after its transference from Hanging Drop petri dish to petri dish coated with 0.75% agarose. Growth medium of one petri dish was carefully renewed (life control) and in a second petri dish 30 μg/ml of extract was diluted in growth medium (treatment). Same extract concentration was incubated in 0.6% agarose-coated wells, for both cell lines. Morphological changes were evaluated, over 24 h, trough microscopy.

2.2 Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA), and all experiments were carried out in triplicate, at least in three independent tests. Continuous variables were presented as the mean ± standard error of the mean (SEM), and categorical variables were presented as the number (percentage).

Two-way analysis of variance (ANOVA) and *post hoc* Tukey's multiple comparison tests were used to determine the differences among the means. Value of $p<0.05$ was considered to be statistically significant.

3. Results and Discussion

Approximately 15% of breast cancers are compromise in the category of triple-negative phenotype owing their lack of ER, PR and HER-2 receptor. ER negative hormone independent breast cancers are more aggressive, presenting high potential to metastasize and are unresponsive to hormonal therapy, so its prognostic is poorer than hormone-dependent subtypes. Current cancer therapies have associated clinical problems such as normal tissue toxicity and multidrug resistance [44,47,174].

As a cell model, MDA-MB-231 cell line (triple negative) is used to investigate the mechanism underlying migration and invasion, and it is important to find preventive, adjuvant and therapeutic compounds which possess multi-targeted and multi-functional potential with antimetastasis activity. The search for alternative drugs that are effective in the treatment of cancers, as well as non-toxic to normal tissues is also an important research line [175].

In the present work, the potential anticancer effect of a lipophilic extract obtained from the outer bark of *E. nitens* was studied in non-tumor and tumor breast cancer cells. Although *E. nitens* is not the main species of *Eucalyptus* in Portugal, the outer bark fraction possesses higher content of triterpenic acids than *E. globulus* species, mainly BA, UA, betulonic and oleanolic acids, as showed in **Table 4** [176].

Table 4 - Chemical composition of *E. nitens* and *E. globulus* outer bark extract in triterpenic acids. Adapted from [159].

Compound	E. *nitens* outer bark % of compound (mg of compound/g of total extract)	E. *globulus* outer bark % of compound (mg of compound/g of total extract)
Betulonic acid (BOA)	14.72 ± 0.69	1.01 ± 0.12
Oleanolic acid (OA)	10.71 ± 0.56	0.78 ± 0.08
Betulinic acid (BA)	20.02 ± 0.90	1.48 ± 0.16
Ursolic acid (UA)	9.90 ± 0.63	2.80 ± 0.33
3-Acetyloleanolic acid	3.16 ± 0.36	2.72 ± 0.38
3-Acetylursolic acid	2.54 ± 0.28	11.88 ± 1.47
3-Acetylbetulinic acid	-	0.30 ± 0.01

Additionally, after an initial screening with *E. nitens* outer bark extract, pure UA and BA were also tested individually, according to their concentrations within the extract, 9.9% and 20%, respectively.

Combination of the two pure acids, in the same proportion as presented in the extract, was also tested for comparison.

The results were normalized relatively to the life control, which was considered as 100% of viability. The line Y=50 represents the half percentage.

3.1 Cell viability in exposure to *E. nitens* outer bark extract – Resazurin assay

MCF-10A and MDA-MB-231 cells were incubated for 24 and 48h with a range of different concentrations (0.1, 0.5, 1, 5, 10, 15, 25 and 50 µg/ml) of extract. Cell viability was evaluated by the resazurin assay.

To assess whether resazurin can cause cell toxicity, the optimal concentration of resazurin was chosen from an optimization experiment with 4 different concentrations, ranging from $2.5x10^{-4}$ to $5x10^{-5}$ M, with 9 replicates. Results are shown in **Table 5**.

Table 5 - Resazurin assay optimization in MDA-MB-231 cells at 24 and 48 h, using 4 different resazurin concentrations (C1-C4). Results are expressed in fluorescence intensity ± standard error of means.

	Fluorescence intensity (%)		
	24 h	48 h	Difference (48-24 h)
C1 = $2.5x10^{-4}$ M	18.58 ± 0.38	21.71 ± 0.81	3.13
C2 = $1.25x10^{-4}$ M	15.72 ± 0.49	22.41 ± 1.28	6.69
C3 = $8.3 x10^{-5}$ M	13.16 ± 0.33	18.49 ± 0.76	5.53
C4 = $5.0 x10^{-5}$ M	13.11 ± 0.39	17.1 ± 0.27	3.99

Cell morphology was tracked with microscopy, which allowed the detection of some signs of possible cytotoxicity with higher concentrations, particularly at 24 h **(Figure 8).** Based on these results and the highest difference in detected fluorescence between the 48 and the 24 h timepoint assays, the resazurin concentration selected for all subsequent experiments was $1.25x10^{-4}$ M.

The effects on cell viability of continuous exposure to increasing concentrations of *E. nitens* outer bark extract was then evaluated with the optimized resazurin assay, in MCF-10A and MDA-MB-231 cells **(Figure 9).** After the incubation period, fluorescence intensity was measured on a spectrofluorometer,

using an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The results were expressed as percentage of cell viability for each timepoint.

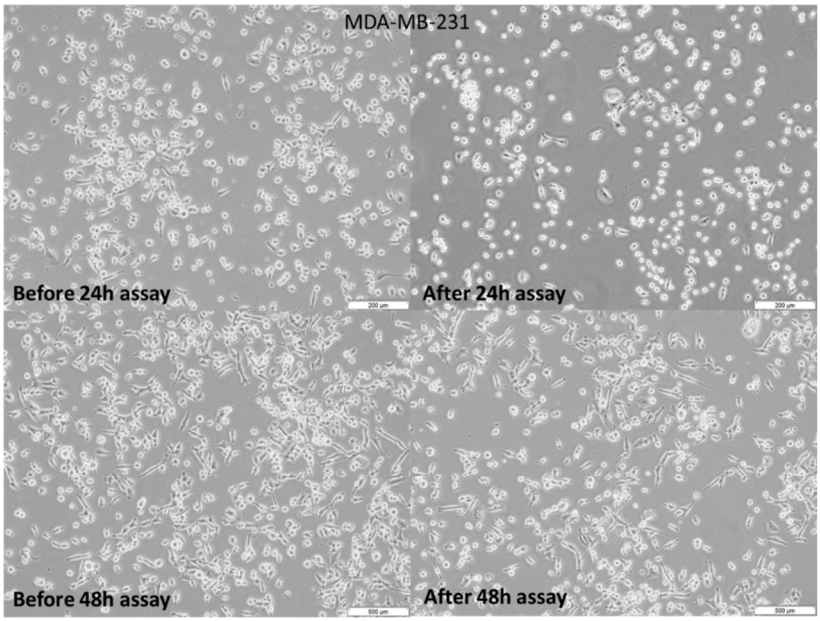


Figure 8 - Resazurin optimization showing the cell morphology of MDA-MB-231 cells after resazurin incubation, at $5x10^{-5}$ M, for 24 and 48 h. Brightfield, amplification of 40X, scale bar: 500µm.

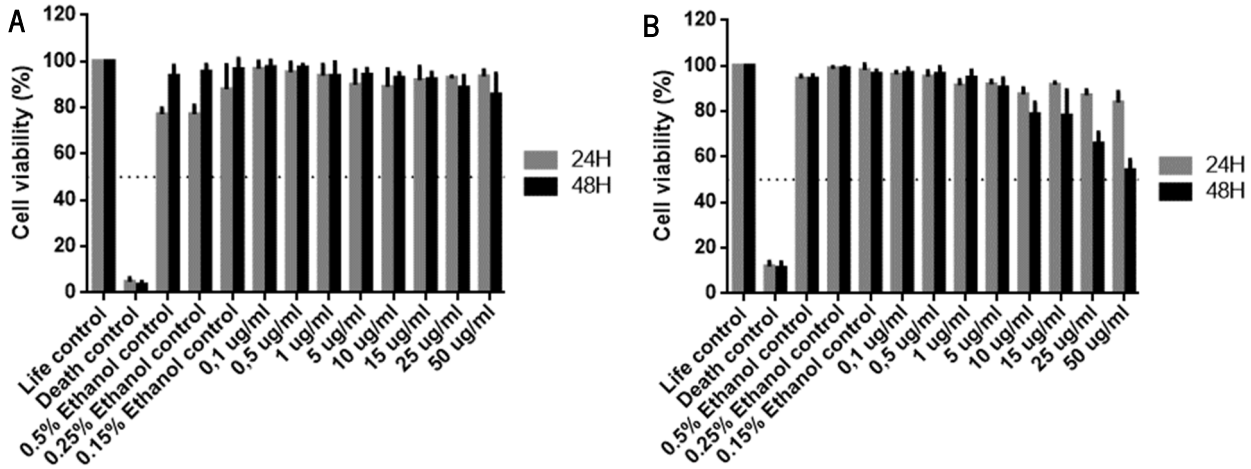


Figure 9 - Resazurin assay showing the cell viability percentage after continuous exposure to *E. nitens* outer bark extract. Cells were treated with increasing concentrations of the extract (0.1, 0.5, 1, 5, 10, 15, 25, 50 µg/ml), at 24 and 48 h. **A-** MCF-10A; **B -** MDA-MB-231 cell line.

The results indicated higher sensitivity of MDA-MB-231 cells to the extract when compared to MCF-10A cells, consistently from 10 µg/ml upwards and particularly detectable at 48h of exposure. Also, assessing the three solvent controls (0.15, 0.25 and 0.5% of ethanol), non-tumor cells seems to be more sensitive to ethanol rather than tumoral ones. However, this sensitiveness does not suggest to be

correlated with extract cytotoxicity in MCF-10A, specially at 48h. Therefore, we can speculate the solvent is not significantly causing cellular cytotoxicity.

Tumoral cell line, MDA-MB-231, showed higher toxicity than the non-tumor cell line, but cytotoxicity was expected to be higher in the tumor cell line. The extract effect upon MDA-MB-231 cells led to almost 50% cell survival for the 50 µg/ml concentration, at 48 h of treatment, and for MCF-10A more than 80% of cells survived, increasing the possibilities of treatment with compounds present in *E. nitens* outer bark extract.

In general, the results were representative, considering the expected effects of the triterpenoid acids present in the extract, but presented low cytotoxicity to tumoral cells. Accordingly, to the literature, triterpenoid acids selectively produce cytotoxic effects on tumor breast cell lines and are non-toxic to normal epithelial breast cells. Namely, 25-Hydroxy-3-oxoolean-12-en-28-oic acid (Amooranin-AMR) is a triterpene acid isolated from the stem bark of a tropical tree (*Amoora rohituka*) and displayed a strong inhibitory effect on survival of MCF-7 and MDA-MB-468 breast carcinoma cells compared to MCF-10A normal breast epithelial cells [177].

Combining all the results and limitations from this first screening, we limited the range of extract concentrations to further confirm the results obtained with resazurin assay and to determine IC_{50} values.

3.2 Cell viability in exposure to *E. nitens* outer bark extract, UA, BA and UB – MTT assay

To confirm resazurin assay data and to gain mechanistic insight into *E. nitens* outer bark extract cellular effects, we evaluated cell viability by MTT assay. For that purpose, MCF-10A and MDA-MB-231 were treated with 3 selected concentrations of the extract (5, 10 and 30 µg/ml), for 24h and 48h. In order to determine whether the *E. nitens* outer bark extract cytotoxic effects are due to UA and BA, cells were treated with pure acids, in the equivalent concentrations as their percentage composition within the extract (individually and combined), as determined by Parreira *et al.* [176] **(Table 5).**

Thus, UA were tested for 0.495, 0.99 and 2.97 µg/ml and BA for 1, 2 and 6 µg/ml. The concentrations of pure acids combination were designated as 5, 10 and 30 µg/ml UB (**U**rsolic-**B**etulinic acid). The effect of *E. nitens* outer bark extract on non-tumor (MCF-10A) and tumor breast cancer (MDA-MB-231) cells viability is shown in **Figure 10 and 11**, respectively and IC_{50} of *E. nitens* outer bark extract, UA and BA, is represented in **Table 6**.

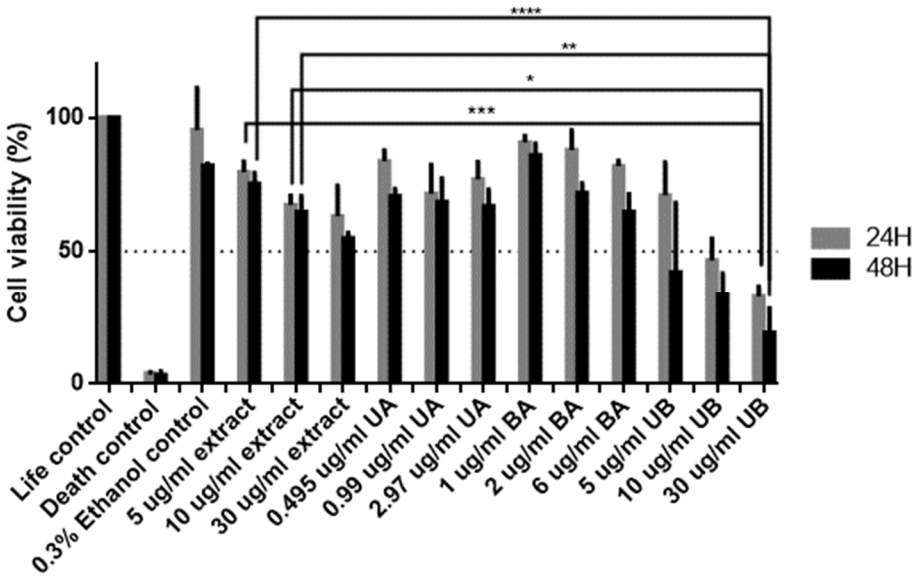


Figure 10 - Cell viability of MCF-10A after continuous exposure to *E. nitens* outer bark extract, ursolic (UA) and betulinic acid (BA), and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, was evaluated by MTT, at 24 and 48 h. ANOVA analysis by Tukey's test ($p<0.05$).

In MCF-10A cell line it was observed that, the extract did not suppress cell viability in a dose and time-dependent manner. In the three concentrations of extract tested, 30 µg/ml presented higher difference between 24 and 48 h, and reduced cell viability to 63% and 55%, respectively. These results indicate that this concentration presents significant toxicity to normal cells. With 10 µg/ml, the difference between the two timepoints was the lowest, with cell viability being reduced in the order of 65%, and 5 µg/ml inhibited cell viability around 80%.

UA effect on these normal cells had no significant differences between dose and time response. Cell viability was reduced to 77% and decreased 10% at 48 h. BA effect in cell viability was lower than UA at 24h, with a cell viability around 90%, but at 48 h BA toxicity increased, reducing cell viability to around 65%.

The combination of the two pure acids showed more cytotoxicity at 24 and 48 h, in all three concentrations, than the extract and the pure acids individualized.

These results suggest that the compounds present in *E. nitens* extract outer bark exerts a synergistic effect, decreasing the cytotoxic effect in this normal epithelial mammary cell line, as the cytotoxicity is higher when the pure acids are combined. No evidences were found in the literature proving this hypothesis.

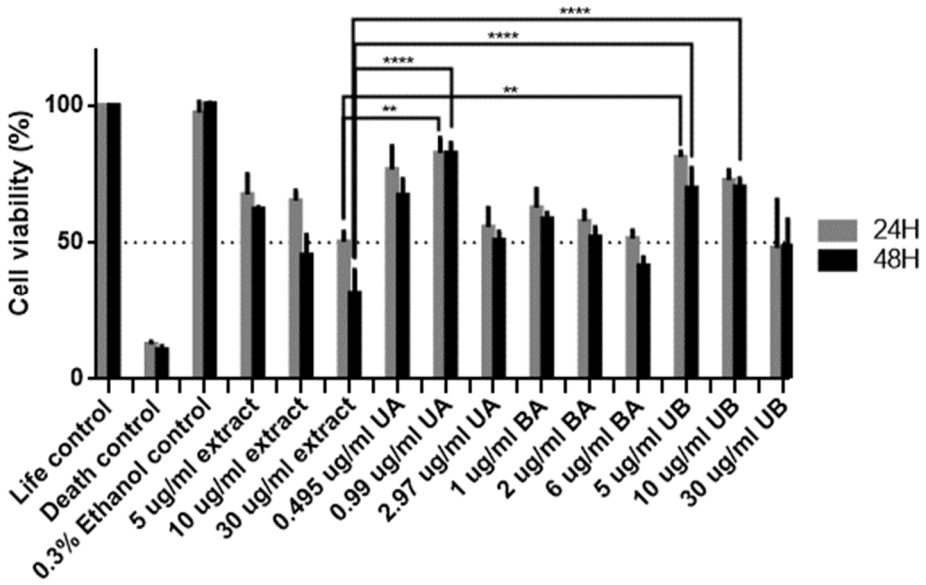


Figure 11 - MTT assay showing the cell viability of MDA-MB-231 cells after continuous exposure to *E. nitens* outer bark extract, ursolic (UA) and betulinic acid (BA), and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, at 24 and 48 h. ANOVA analysis by Tukey's test ($p<0.05$).

In MDA-MB-231 cell line the results showed that the extract did not inhibited cell viability in a dose and time-dependent manner. At 24h, 5, 10 and 30 µg/ml of extract suppressed cell viability to 67, 65 and 50%, respectively. At 48 h, the viability was reduced to 62, 25 and 32%, respectively. Dose-response was more significant with higher concentrations (10 and 30 µg/ml).

UA effect on these TNBC cells had no significant differences in time response manner. A dose with 2.97 µg/ml was found to markedly reduce cell viability to 50% at 24 and 48 h. Increasing concentrations of BA treatment showed increased cell sensitivity. At 24h, 1, 2 and 6 µg/ml of BA suppressed cell viability to 63, 58 and 52%, and at 48 h, was reduced to 59, 52 and 39%, respectively.

UB combination was less cytotoxic to tumoral cells with 5 and 10 µg/ml than extract, with 30 µg/ml presenting equivalent effect than the extract (50% cell viability), at 24 h. At 48 h, the combination showed lower toxicity for all concentrations, than the extract. Comparatively to UA individually, the combination was less cytotoxic also with 5 µg/ml, at 24 h. UB were less cytotoxic to MDA-MB-231 cells with 5 and 10 µg/ml than BA, with 30 µg/ml presenting equivalent effect than the extract (50% cell viability), at 24 h. At 48 h, the combination continues to be less cytotoxic, compared to 6 µg/ml BA.

It is observed that the pure acids individually produce cytotoxic effects to these tumoral cells, but, for some reason, when the two are combined the cytotoxic effect is decreased. From that we can hypothesize that the extract has in its composition certain compounds that enhance the cytotoxicity produced in MDA-MB-231. Further tests are needed to answer this hypothesis.

It is clear that *E. nitens* extract is able to produce a dramatic effect on breast cancer cell viability, while in non-tumor breast cell line displays lower cytotoxicity, mainly with higher concentrations. Due to the observed differences in both cell lines, different mechanisms may be at play and it is expected to see varying results from the treatments in further testing.

Table 6 - Half inhibitory concentration (IC_{50}) of *E. nitens* outer bark extract, ursolic acid (UA) and betulinic acid (BA), for non-tumoral and tumoral breast cell lines, at 24 and 48 h. IC_{50} values are theoretical and were calculated using GraphPad 6.0 version software.

	IC50 (µg/ml)					
	E. nitens outer bark extract		Ursolic acid		Betulinic acid	
	24 h	48 h	24 h	48 h	24 h	48 h
MCF-10A	122.2 ± 12.15	42.43 ± 6.270	5850 ± 350	42910 ± 271	201.1 ± 27	23.05 ± 2.1
MDA-MB-231	33.840 ± 2.11	9.133 ± 0.4400	4.733 ± 1.51	4.350 ± 0.83	7.3 ± 1.9	2.5 ± 0.9

The analyses of IC_{50} values confirms the selectively effect of *E. nitens* outer bark extract to MCF-10A and MDA-MB-231 cell lines. IC_{50} is around 4-fold higher in the normal cell line, at 24 h, and 5-fold higher, at 48 h than in the tumoral cells.

Considering the percentage of UA present in the extract (9.9%) and the extract IC_{50} obtained value, the theoretically IC_{50} expected was 3.35 µg/ml and 0.90 µg/ml, at 24 and 48 h, in MDA-MB-231. This is confirmed by our results at 24 h (4.733 ± 1.51 µg/ml), but not at 48 h, with a higher value (4.350 ± 0.83 µg/ml). In the case of BA, its percentage in *E. nitens* outer bark extract is 20%, therefore considering the obtained extract IC_{50} value, BA should have an IC_{50} of 6.76 µg/ml and 1.82 µg/ml, at 24 and 48 h, respectively, for MDA-MB-231. These values were also confirmed by our results, with an IC_{50} at 24 h (7.3 ± 1.9 µg/ml) and 48 h (2.5 ± 0.9 µg/ml).

Comparing with the literature, a study presented a lower UA IC_{50} value of 1.83 µg/ml, in MDA-MB-231, at 24 and 48h [136]. Some other studies exhibited UA IC_{50} values, but at 72 h. BA studies in the same cell line, indicated an IC_{50} of 10 µg/ml [178] and 9.3 µg/ml [179], for 24 h, confirming our results.

If the same extrapolation is done for MCF-10A values, considering extract IC_{50}, UA IC_{50} should be 12 µg/ml (24 h) and 4.2 µg/ml (48 h), and BA IC_{50} would be 24 µg/ml (24 h) and 10 µg/ml (48 h). These values present a huge difference to our results obtained by MTT assay. UA present an IC_{50} of 5850 ± 350 µg/ml (24 h) and 42910 ± 271 µg/ml, and for BA the IC_{50} was 201.1 ± 27 µg/ml (24 h) and 23.05 ± 2.1 µg/ml (48 h).

The pure acids (UA and BA) are indeed more cytotoxic to tumor cells than non-tumoral ones. However, extract cytotoxicity does not seem to be correlated with the two pure extracts, in MCF-10A. These data indicate that other compounds present in the extract could have cytotoxic effects in this normal epithelial breast cell line. **Table 5** indicates that betulonic and oleanolic (OA) acids are present in similar percentages to ursolic and betulinic acids.

A study showed that a concentration of 10μg/ml OA increased cell viability of MCF-10A after 6 h (110.75%) and 20μg/ml increased cell viability after 72 h (110.35%) but significantly decreased after 24 h (90.31%) and 48 h (81.47%) compared to control [180]. Another study demonstrated that, for MCF-10A cells, OA at 10 and 100 μM promoted cell death (cell survival was 83% and 13% for OA, respectively) [181]. No studies of betulonic acid in MCF-10A were found.

3.3 Cell viability in exposure to *E. nitens* outer bark extract, UA, BA and UB – Trypan blue assay

This assay was performed to study the cellular membrane integrity, when the cells where exposed to the extract, to UA and BA, and to the combination of both of them (UB). This study was performed at 8 and 24 h, in order to observe, in a shorter period of time, the possible effects in the cellular membrane. Within the previous assays (resazurin and MTT), cells easily detached from the plate with increasing concentrations of each treatment.

Since this dye is unable to penetrate the cellular membrane, viable cells will not internalize it. However, cells with a compromised plasma membrane will be stained blue since the dye can easily cross their damaged membrane. Results are showed in **Figure 12 and 13**.

In the normal cell line **(Figure 12)**, all treatments had no significant differences in a dose and time manner. For 8 and 24h, cell viability was not lower than 81%. In BA treatment, no toxicity was observed, at cell membrane level.

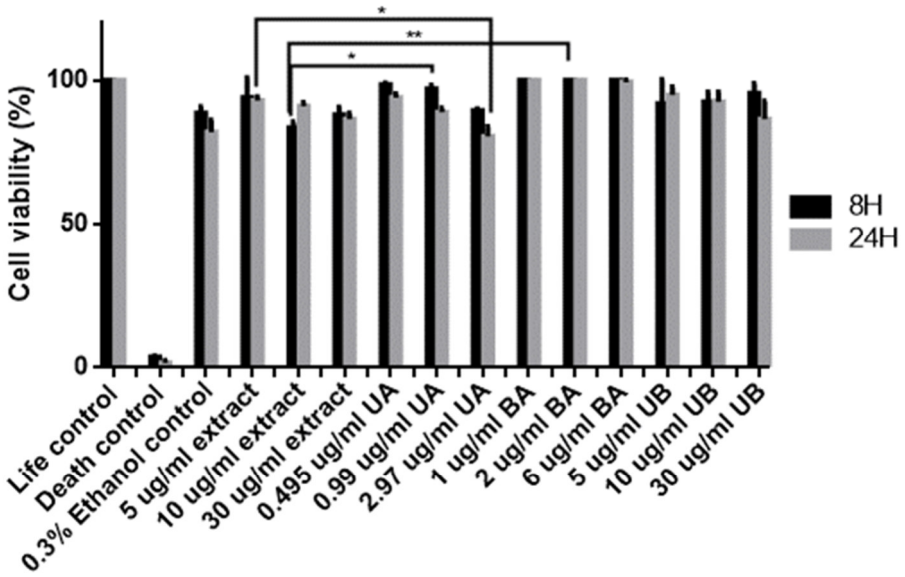


Figure 12 – Cell viability of MCF-10A cells was evaluated by trypan blue after continuous exposure to *E. nitens* extract, ursolic (UA) and betulinic acid (BA), and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, at 8 and 24 h. ANOVA analysis by Tukey's test ($p<0.05$).

In MDA-MB-231 **(Figure 13)**, treatment with BA showed significant differences in a dose and time manner. The most significant reduction of cell viability was observed with 6 µg/ml BA treatment, to 57% and 51%, at 8 and 24 h, respectively. This condition present higher toxicity when compared to the equivalent concentration in the extract and combination (30 µg/ml extract: 24 h – 69% and at 48 h – 82%; UB combination equivalent to 30 µg/ml extract: 24 h – 88% and at 48 h 79%, of cell viability).

When comparing both cell lines, the results reveal a more consistent and significant impact of all treatments on the metastic MDA-MB-231 cell line.

These results are not similar to those observed in MTT for both cell lines. Data suggests that the pure acids, when individualized, affect the cell's metabolism and cell's membrane in different ways, as their percentages of cell viability does not have the same reduction proportion.

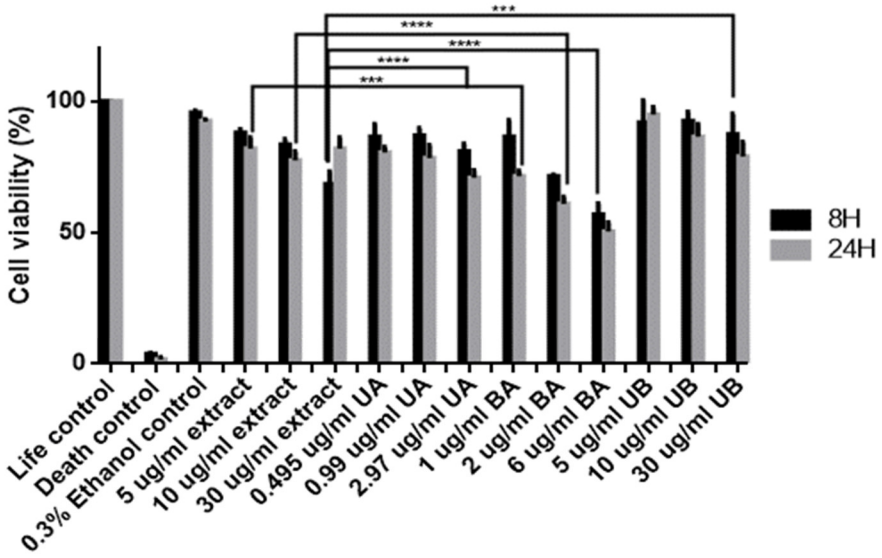


Figure 13 - Trypan blue assay showing the cell viability of MDA-MB-231 cells after continuous exposure to *E. nitens* outer bark extract, ursolic (UA) and betulinic (BA) acid, and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, at 8 and 24 h. ANOVA analysis by Tukey's test ($p<0.05$).

3.4 Cell viability in exposure to *E. nitens* outer bark extract, UA, BA and UB – AO/PI assay

A fluorescent microscopy study was undertaken to understand cell death mechanisms. The microscopic examination showed that the live cells had a normal morphology, took up AO, and stained uniformly in green. In contrast, cells incubated with treatments showed different characteristics of cell death and altered cell morphology such as condensed and fragmented nucleus, cellular shrinkage, and membrane blebbing. Early apoptotic cells with bright green nuclei and late apoptotic cells with condensed orange-red nuclei were observed in incubation with extract, UA, BA and combination(UB)-treated cells **(Supplementary Data – Appendix 2 and 3).** There was a significant increase in apoptotic cells and apoptotic bodies after treatment with both pure acids, neither with its combination, being these characteristics more expressive in MDA-MB-231 cell line.

3.5 Oxidant activity of *E. nitens* outer bark extract, UA, BA and UB – DCFDA assay

ROS, cellular metabolites that regulate the various signaling transduction pathways of the cell, are important cell fate regulators. Mitochondria are regarded as an important target of ROS damage, and an increase in ROS levels can lead to apoptosis by oxidizing mitochondrial pores. Therefore, the production of ROS in MCF-10A and MDA-MB-231 cells was evaluated. *E. nitens* outer bark extract, UA and BA, and their combination (UB), were used as treatment and DCF-DA, at 3, 8 and 24 h, was used. Micrographs were taken to evaluate fluorescence emission qualitatively (showed at **Supplementary Data – Appendix 4 and 5**). Quantitatively, the intracellular ROS levels were evaluated by fluorimetric detection.

Under the above-mentioned conditions, cells detached too easily from the well plates, making it impossible to obtain reliable results, so multiple experiments were carried to optimize the concentration of DCFDA and the concentration of the oxidant reagent in positive control, in both cell lines, as explained in *2.4.6.1 DCFDA assay optimization.*

The quantitatively results did not confirm that the production of ROS was increased by those treatments. The controls worked in the optimization but in the presence of treatment conditions, the controls did not give consistent results, as it should present higher fluorescence than negative control. Other limitation is the fact that MDA-MB-231 cells detached more easily than normal cells, during the experimental procedure, consequently reducing the number of cells in the assay. The results for both cell lines are presented in **Figure 14** and **15**.

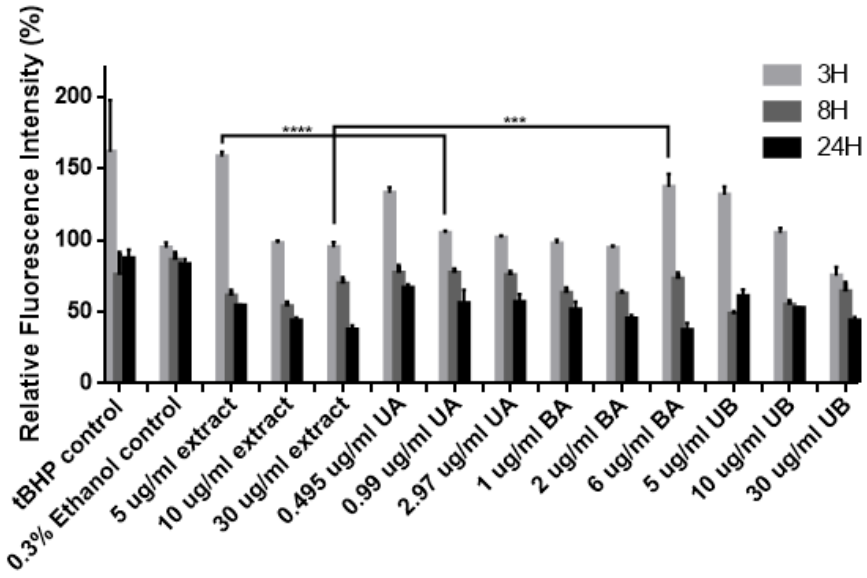


Figure 14 - DCFDA assay showing the fluorescence intensity of MCF-10A cells after continuous exposure to *E. nitens* outer bark extract**,** ursolic (UA) and betulinic (BA) acid, and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, at 3 ,8 and 24 h. ANOVA analysis by Tukey's test ($p<0.05$).

For the normal cell line, the positive control worked, and the more significant results were at 3 h timepoint. It was exhibited that the DCF fluorescence intensity levels are significantly increased, in all conditions, when compared to 8 and 24 h timepoints. It is also observed that the lower concentrations of each treatment, exhibit higher fluorescence intensity, except for BA treatment. From these data, although the lower concentrations of each treatment are those causing higher ROS production, these results must be confirmed further because cells detached more with increasing extract concentrations.

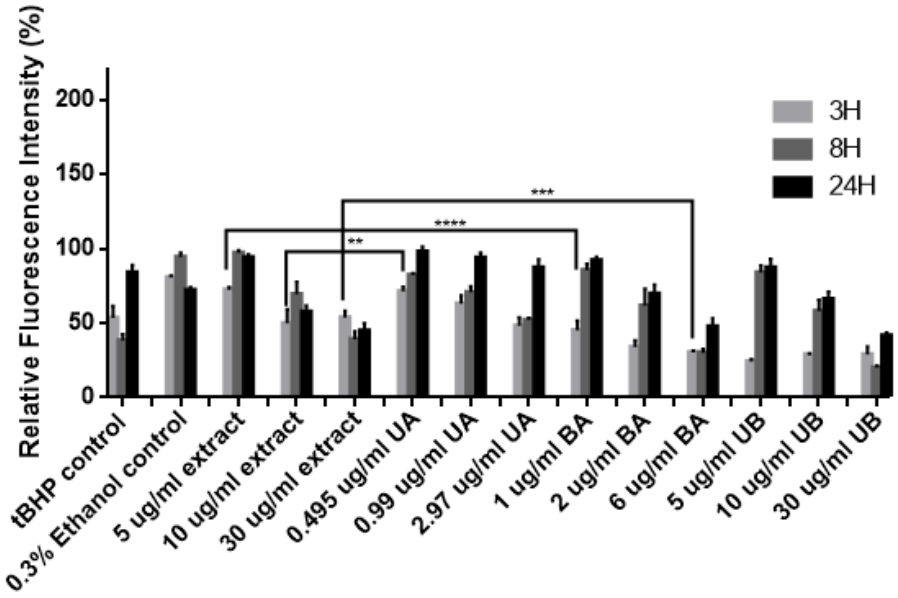


Figure 15 - DCFDA assay showing the fluorescence intensity of MDA-MB-231 cells after continuous exposure to *E. nitens* outer bark extract**,** ursolic (UA) and betulinic (BA) acid, and their combination (UB), with the equivalent concentrations relatively to their percentage composition in the extract, for 3 ,8 and 24 h. ANOVA analysis by Tukey's test ($p<0.05$).

For TNBC cell line, the positive control did not work appropriately, and higher fluorescence intensity increased up to 8h (exceptionally at 30 µg/ml and 30 µg/ml UB treatment) and 24 h timepoints.

Overall, from these data we cannot conclude that exposure to extracts and pure acids compounds interfere with ROS cellular production. Further studies are needed.

3.6 3D cell culture models

Monolayer cell culture is traditionally used as *in vitro* model to investigate tumor behavior and identify effective antitumor therapies [157]. Promising activities observed in 2D cell culture could not always be satisfactorily confirmed in animal studies or in clinical trials, because of the inability to replicate the extracellular microenvironment where cells reside in tumor tissues [182]. Therefore, 3D multicellular spheroid models provide valuable tools for *in vitro* identification of potential anticancer drug targets [158,172]. Particularly due to more realistic microenvironment, extracellular matrix and adhesion features.

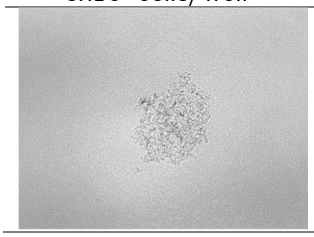
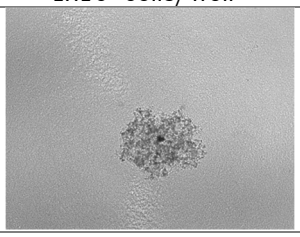
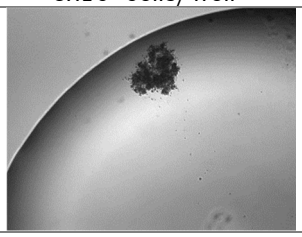
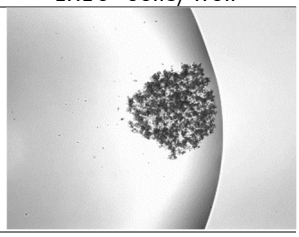
Therefore, in this study the normal epithelial breast cell line, MCF-10A, and breast cancer cell line, MDA-MB-231, were both used to generate different spheroid models by testing different techniques such as, suspension culture techniques, hanging drop and agarose-coated plates and different parameters based on Shoval *et al.*, and Froelich *et al.* [171,172].

3.6.1 Suspension culture

In a first step, MCF-10A was seeded with two cell densities ($5x10^3$ and $7.5x10^3$ cells) and two different volumes (30 and 100 µl) in 24-well suspension plate, for 3 days, in order to define optimal conditions. From this screening, it was observed that cells concentrated in the well center, forming an aggregate with circular shape, but with low cellular density **(Supplementary Data – Appendix 6).**

This process was then repeated with $5x10^3$ and $1x10^4$ cell seeding densities, in a total of 40 µl cell suspension, for both cell lines. MCF-10A produced more compact aggregates, in higher density. With TNBC cell line, in initial seeding density, the aggregate obtained is more compact, but with irregular shape, whereas a more uniform shape in displayed when cell density was increased. Furthermore, this method led formation of heterogeneous aggregates, which makes standardization difficult for subsequent experiments **(Table 8)**.

Table 4 - MCF-10A and MDA-MB-231 aggregates formation by suspension culture method, after 3 days. Different seeding densities were used in a 40 µl suspension.

24-well suspension plate (40µl volume)			
MCF-10A		MDA-MB-231	
$5x10^3$ cells/well	$1x10^4$ cells/well	$5x10^3$ cells/well	$1x10^4$ cells/well
			

MTS assay was used to support the selection of the optimal seeding density, but it did not show significant differences among the two densities in both cell lines. Results are presented in **Table 9**.

Table 5 - Absorbance reading values in MCF-10A and MDA-MB-231 aggregates, in two different seeding densities, at day 3.

	Absorbance at 490 nm (Reading values)					
	$5x10^3$/well			$1x10^4$/well		
MCF-10A	0.291	0.307	0.238	0.238	0.241	0.244
MDA-MB-231	0.393	0.379	0.392	0.432	0.463	0.387

This method was thus discarded for further testing, in this optimization.

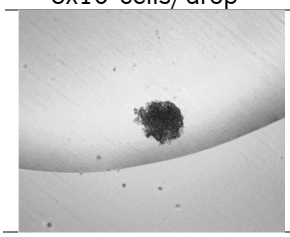
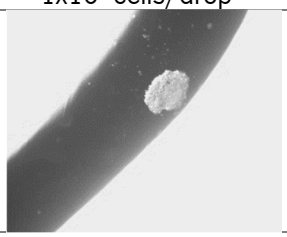
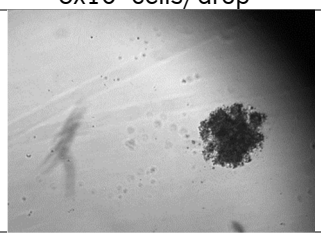
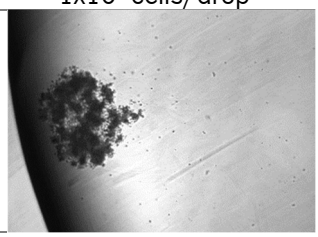
3.6.2 Hanging Drop

For comparison of cell number influence on generation of spheroids, different numbers of MCF-10A cells ($5x10^3$ and $7.5x10^3$ cells) were seeded in hanging drops (30 µl) under the lids of 10 cm Petri dish, for 3 days to allow aggregation.

In this method, formed aggregates were very circular in shape, primarily with initial seeding density (**Supplementary Data – Appendix 7**).

This process was repeated with $5x10^3$ and $1x10^4$ cells in a 40 µl cell suspension drop, for both cell lines. MCF-10A aggregates were easily reproduced and were more compact in the higher density, while in breast cancer cell line, the aggregates were very heterogeneous and disaggregated easily. In general, hanging drop worked better than suspension culture method, and continued to be optimized.

Table 6 – Hanging Drop method in MCF-10A and MDA-MB-231, at day 3.

10 cm Petri dish (40µl volume)			
MCF-10A		MDA-MB-231	
$5x10^3$ cells/drop	$1x10^4$ cells/drop	$5x10^3$ cells/drop	$1x10^4$ cells/drop
			

3.6.3 Multi-well agarose-coated plates

Polymer or natural hydrogels, such as agarose are the most widely used non-adherent substrates. Agarose forms a macroporous matrix which allows rapid diffusion of molecules including macromolecules unrestricted by the gel [183]. Agarose, being nontoxic to living cells, is used for cellular-models research , including cell migration study, bio mimetics of vasculature, creation of synthetic analogs of basement membrane [183,184], and as a half-liquid medium for cellular 3D structure formation [185]. Finally, optical properties of agarose gel are excellent for live cell visualization due to its refractive index being comparable to water [186].

For our study, 1.5 %agarose hydrogel was added to each well of a 96-well culture plate. After the agarose cooled, MCF-10A cells were seeded in 50 or 100 µl at a concentration of $5x10^3$ cells/well and $1x10^4$ cells/well. Plates were incubated for 5 days, to allow aggregate formation. Micrographs were taken to evaluate aggregation. Multiple-small aggregates were formed, in all conditions, so the method was

repeated with 0.6% of agarose, and micrographs were taken to evaluate aggregation, as shown in **Supplementary Data – Appendix 8**.

A spheroid per well was obtained in MCF-10A cell line, with $1x10^4$ cells, in a suspension volume of 100 µl. No spheroids were obtained with MDA-MB-231, up to 14 days. This process continued to be optimized for better results.

3.6.4 Spheroids optimization

As mentioned in *2.4.7.4 Spheroids optimization,* several modifications were set. Based on the obtained results, $1x10^4$ cell density was selected for both cell lines, to allow comparable parameters. With hanging drop method, cells suspensions were seeded with 40 µl growth medium and incubated 4 days. Once spheroids were formed, they were transferred to a 10 cm petri dish coated with 0.75% agarose, for 3 days. Under these conditions, spheroids were formed in both cell lines.

MCF-10A spheroids were formed and, after transfer to the coated petri dish, it was possible to maintain them for 5 more days, until them began to disaggregate. A concentration of 30 µg/ml of *E. nitens* outer bark extract was added to the coated petri dish upon spheroid transfer, and spheroids were morphologically evaluated, by microscopy, compared to a life control **(Table 11)**. It is observed that spheroid morphology is maintained in life control but, with *E. nitens* outer bark extract incubation, the spheroid is disaggregated, over 24 h.

With breast cancer cell line, many techniques were tried to transfer aggregates from drops to the coated petri dish, but none enabled the transfer without disaggregating them **(Table 12)**. For this reason, it was not possible to test the extract. These results may indicate differences of expression patterns of adhesion molecules, as well as tight junction proteins between MCF-10A and MDA-MB-231.

The breast gland is a mechanically active tissue, where the basement membrane plays a key role for force homeostasis and mechanical stress shielding [159]. This balance is assumed to be deregulated in breast cancer development and to contribute to aggressive phenotypes. Expansive loads of a hyperproliferating tumor mass could cause a progressive basement membrane thinning and breakdown [187]. MCF-10A parent cell lines were reported to form acinus-like structures devoid of apical polarity as their acini usually lack apical tight junctions [188].

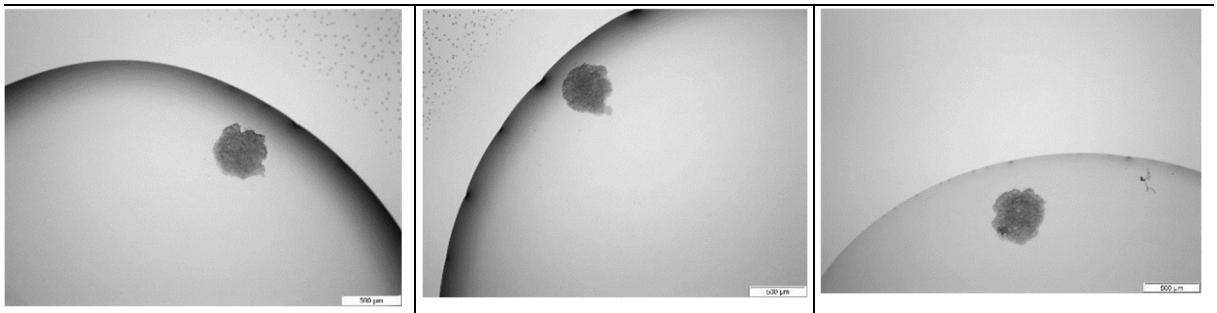
It is also identified that, selectins and integrins are two major cell adhesion molecule families, that are crucial factors in metastization process [189,190]. Integrins are glycoproteins receptors responsible for cells attachment to ECM proteins, as fibronectin, vitronectin, laminins, collagens and others. Integrins dependent cell attachment to ECM proteins initiates activation of signaling pathways that

regulate behavior and motility of cells [190]. Integrin α2β1, has higher expression in MDA-MB-231 as compared to non-invasive breast cancer line, MCF-7. It has been suggested that the difference in this integrin expression in one of numerous features responsible for the MDA-MB-231 highly-metastic behavior [191].

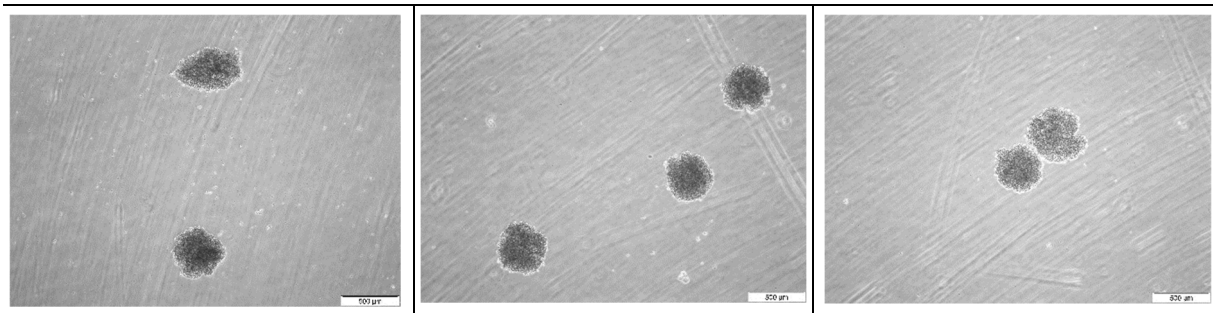
Furthermore, some cell lines require specific components mimicking the extracellular matrix (ECM) such as laminin or collagen IV, in their culture medium [163]. These components are responsible for the cell/extracellular matrix connections and are able to generate an environment leading to the formation of intercellular connections [192,193]. Moreover, it is known that MDA-MB-231 cell line express low protein intercellular junctions such as E-cadherin, which limit the ability of cells to organize themselves spontaneously into compact spheroids [194]. In another study, the similarity of cell-cell adhesions of spheroids generated rapidly with the centrifugation technique compared to spheroids grown from a few cells over extended periods of time, was evaluated. Authors investigated the expression patterns of adhesion and tight junction proteins of 24 h spheroids derived from 10,000 cells with 10-day-old spheroids derived from 1000 cells: integrin β1 and claudin 4, and Western blot revealed that there was no difference in the expression patterns of these proteins in the different culture setups [195].

Table 7 - MCF-10A aggregates in Hanging Drop method. Representative micrographs of 4 day spheroids. 7 days, spheroids before treatment (A) and after 24 h treatment (B) with either complete medium (Life control) or *E. nitens* outer bark

A - MCF-10A aggregates in 0.6% agarose-coated plate (1x10⁴ seeding cells in 40 µl drop) at day 4



MCF-10A cells transferred to 0.75% agarose coated petri dish, at day 7.



B - MCF-10A aggregates in 0.75% agarose-coated plate - after 24 h treatment

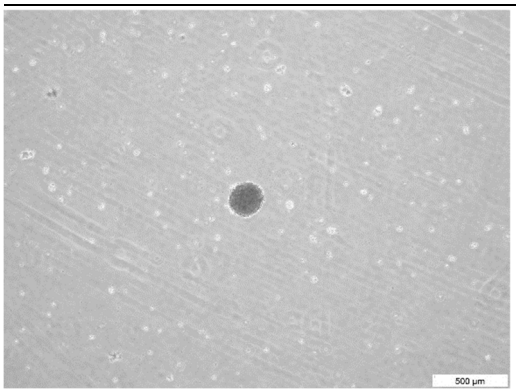
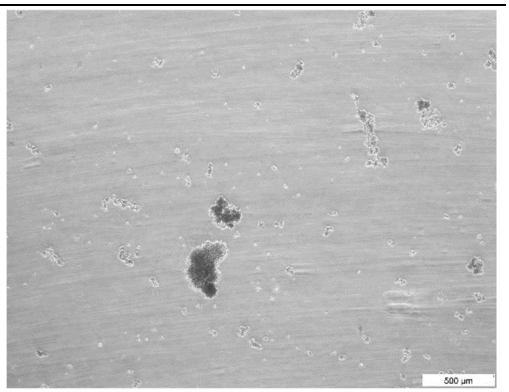
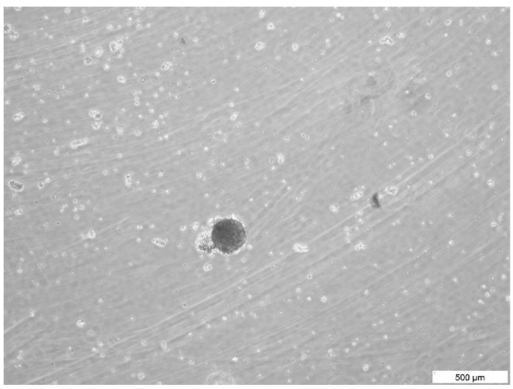
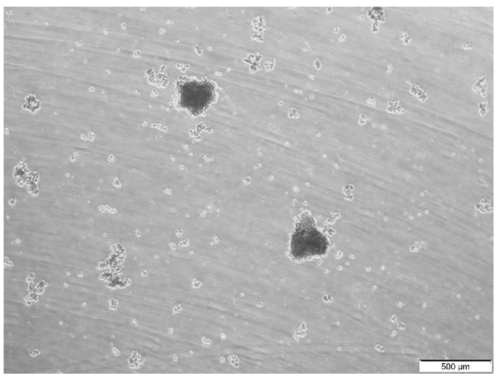
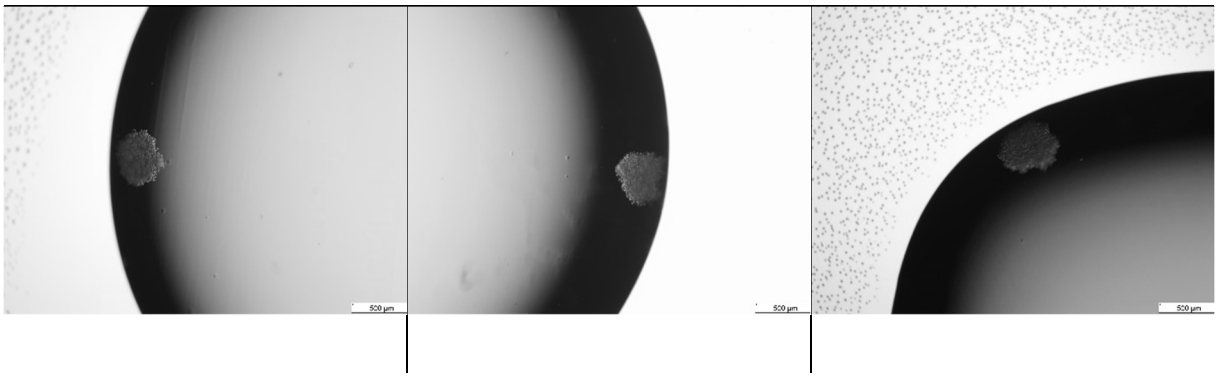
Life control	30 µg/ml *E. nitens* extract
	
	

Table 8 - MDA-MB-231 spheroid formation by Hanging Drop method, at day 4.

MDA-MB-231 spheroids in 10 cm petri dish



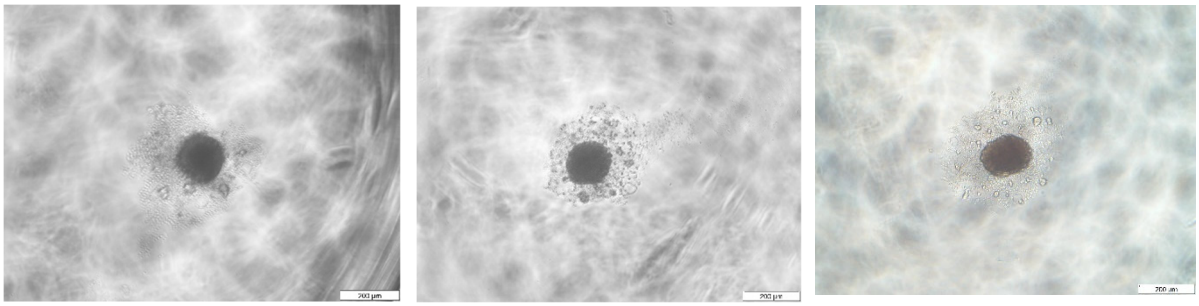
In agarose-coated plate method, cells were seeded in a 100 µl suspension cell, and growth medium was refreshed every 2 days. MCF-10A also formed uniform spheroids, within 7 days. These spheroids were also incubated with 30 µg/ml *E. nitens* outer bark extract, and spheroid morphology was evaluated, through microscopy, over 24 h. It was observed that after 8 days spheroids start to disaggregate, even with growth medium only **(Table 13)**. When compared with treated spheroids, the core is maintained, whereas the presence of *E. nitens* outer bark extract promotes total disintegration.

MDA-MB-231, formed only loose aggregates of cells after 3 days. The cell-cell contacts established by these cultures were again weak, and the aggregates were easily dispersed mechanically by pipetting, and difficult to reproduce homogeneously. This initially observed morphology did not change within 14 days of cultivation. These aggregates were also incubated, at day 7, with 30 µg/ml of extract, for 24 h. In this cell line, aggregates fragmented in pieces, differently from MCF-10A **(Table 13 and 14)**.

To understand the effect of distinct morphological features of both cell lines, in the same spheroid generation method, and the impact of *E. nitens* outer bark extract on them, more studies are needed. Also, the establishment of standard spheroid generation protocols will allow a more complete study of the application of natural compounds in novel anticancer drugs, as multicellular tumor spheroids are a valuable tumor research model.

Table 9 - MCF-10A aggregates in agarose-coated plates method. Representative micrographs of 7 day spheroids, before treatment (A) and after 24 h treatment (B) with either complete medium (Life control) or *E. nitens* outer bark extract.

A - MCF-10A aggregates in 0.6% agarose-coated plate (1x10⁴ seeding cells in 100 µl cell suspension), at day 7 – before treatment



B - MCF-10A aggregates in 0.6% agarose-coated plate (1x10⁴seeding cells in 100 µl cell suspension) - after 24 h treatment

Life control	30 µg/ml *E. nitens* extract

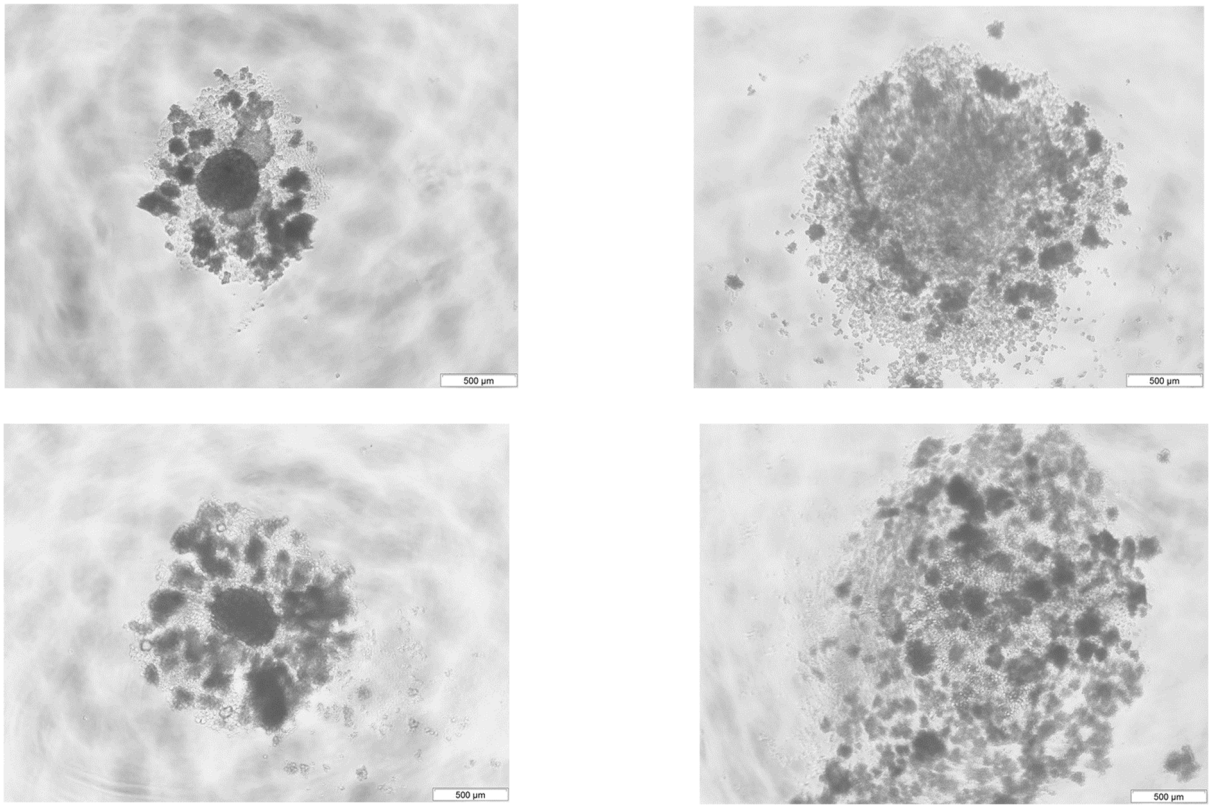
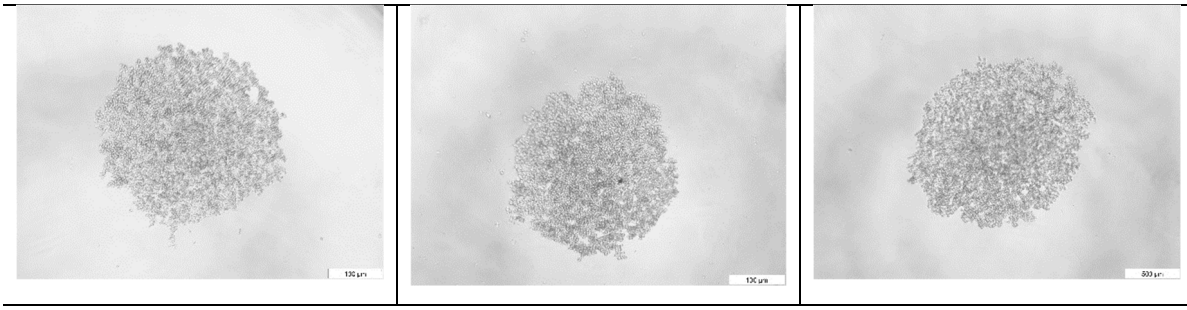


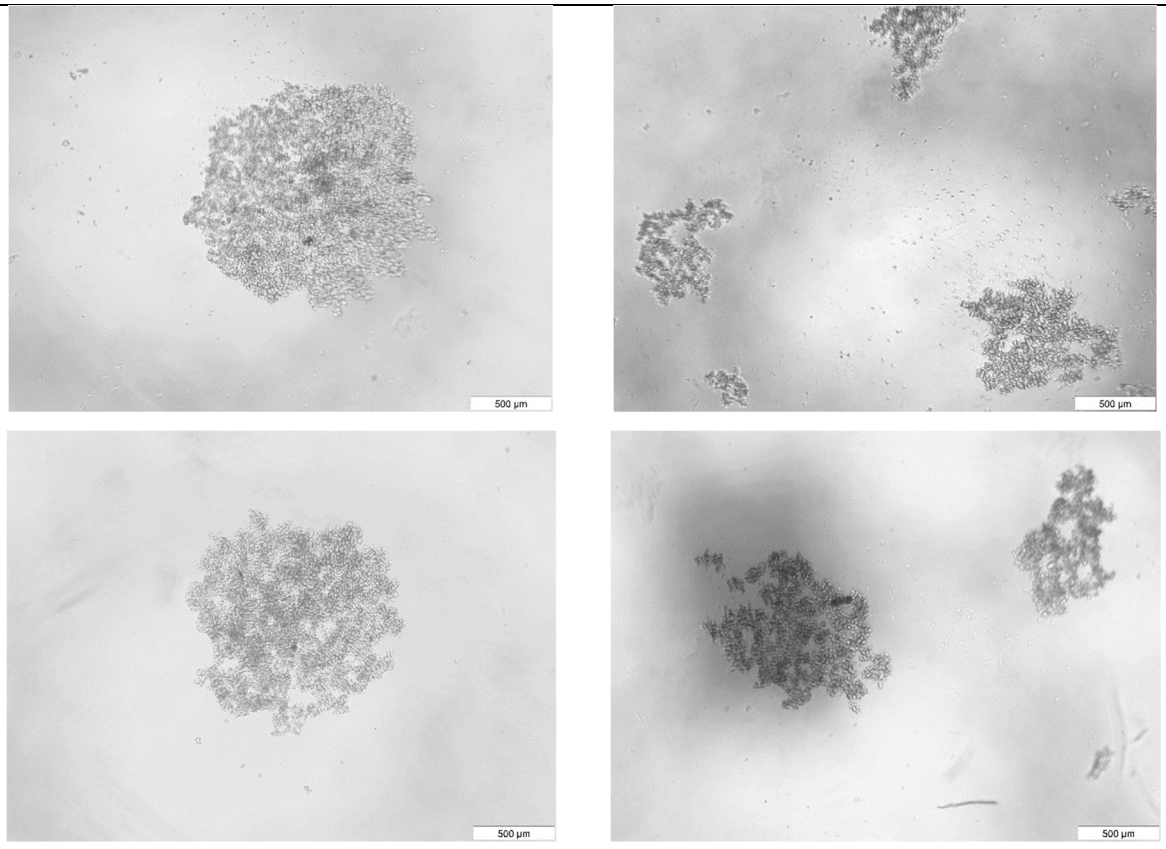
Table 10 – MDA-MB-231 aggregates in agarose-coated plates method. Representative micrographs of 7 day spheroids, before treatment **(A)** and after 24 h treatment **(B)** with either complete medium (Life control) or *E. nitens* outer bark extract.

A – MDA-MB-231 aggregates in 0.6% agarose-coated plate (1x10⁴ seeding cells in 100 µl cell suspension), at day 7 – before treatment



B – MDA-MB-231 aggregates in 0.6% agarose-coated plate (1x10⁴ seeding cells in 100 µl cell suspension) - after 24 h treatment

Life control	30 µg/ml *E. nitens* extract



4. Conclusion and future perspectives

Cancer and, in particularly, breast cancer mortality rates, remain high, despite clinical improvements. This emphasizes an urgent need to better understand the disease and to search novel and effective breast cancer treatments, with the particular concern to reduce side effects, thereby improving patient quality of life. *Eucalyptus nitens* outer bark extract has in its composition two triterpenoids, UA and BA, with proven anticancer and antioxidant properties. In this study, we investigated the effect of *E. nitens* outer bark, UA, BA, and synthetic mixture of UA plus BA, named UB, in tumor (MDA-MB-231) and non-tumor (MCF-10A) breast cells. An initial screening with the extract showed a higher sensitivity of tumor cells, demonstrating the potential of the extract as a source of natural compounds able to modulate tumor cells viability. This potentiality was further confirmed in the following experiments for extract, as well for both and both pure acids, which act at metabolic and cell membrane level, indicating higher interference in cellular processes, that contribute to cell death, more strongly in MDA-MB-231 cells. However, the same cytotoxic effect does not occur with the synthetic combination. This needs to be in further studied, in order to fully understand the biological impact of the pure acids, regarding the activity of *E. nitens* outer bark extract. It is also known that oxidative stress has a role in breast carcinogenesis. Even after an exhaustive DCF-DA protocol optimization, it was not possible to elucidate about the effect of our treatments in intracellular ROS production for technical reasons. Thus, further investigation is needed.

Multicellular aggregates of cells, or spheroids, are of interest for studying tumor behavior and for evaluating the response of pharmacologically active agents. Spheroids more faithfully reproduce the tumor macrostructure found *in vivo* compared to classical 2D monolayers. In the present work, we tested several experimental approaches to form spheroids with MDA-MB-231 and MCF-10A cells. While the normal cell line easily formed spheroids under different conditions, the tumoral cell line only generated loose aggregates. These differences may be due to distinct adhesion molecules and tight junction proteins. For that, MDA-MB-231 cells may require the presence of an extracellular matrix hydrogel, to enhance cell-cell interaction, Cultrex basement membrane matrix could be applied to generate spheroids, with this particular cell line. More studies will be needed to further explore the link between extracellular environment and invasive capacity. Also, the different spheroid formation capabilities of different cell lines stress the need for standardization of spheroid generation protocols for reliable comparison of therapeutic agents.

In further studies, optimized protocols could be applied to evaluate the effect of UA, BA and UB in the spheroid integrity and to compare the results with 2D cell models.

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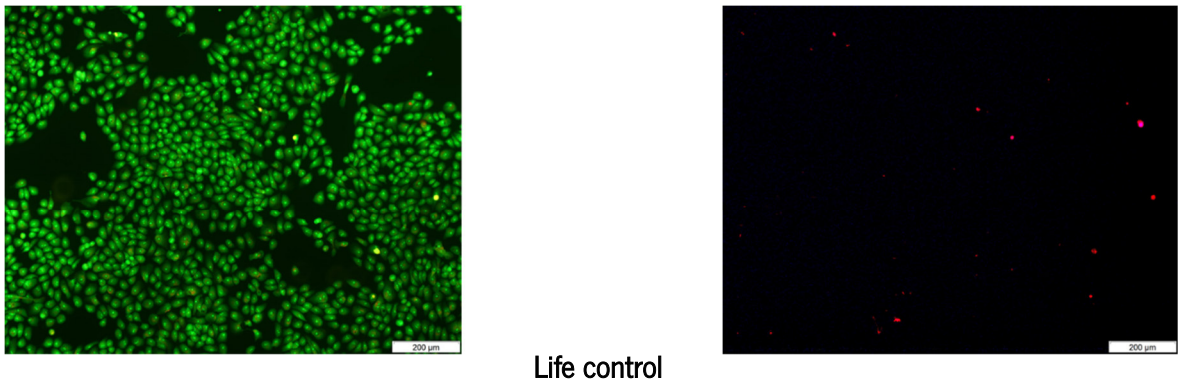
6. Supplementary Data

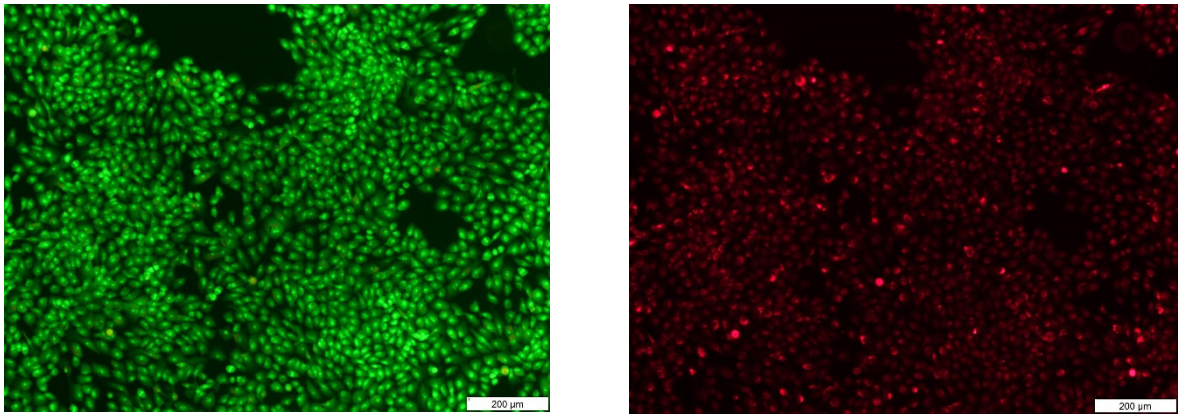
Appendix 1

Reagents and Equipments	Reference	Company
Acridine orange (AO)	A6014	Sigma Aldrich
Agarose	50004	Lonza
DCF	D399	Thermo Fisher
Propidium iodide (PI)	2060562	Thermo Fisher
F12	FG0815	Biochrom
tBHP	458139	Sigma Aldrich
Ursolic acid	U6753	Sigma Aldrich
Resazurin	R7017	Sigma Aldrich
DMEM	1001417958	Sigma Aldrich
FBS	A7030	Sigma Aldrich
Ethanol	4146052	Carlo Erba
DMSO	101719820	Sigma Aldrich
Trypsin EDTA solution	L2103	Biochrom
MTT	M2128	Sigma Aldrich
Trypan blue	T8154	Sigma Aldrich
Antibiotic and antimycotic solution	A5955	Sigma Aldrich
MTS	G3582	Promega
Tris Acetate EDTA (TAE) buffer	SRE0033	Sigma
Betulinic acid	B8936	Sigma Aldrich
Horse Serum (HS)	H1270	Sigma Aldrich
EGF	E5160	Sigma Aldrich
Hydrocortisone	H0888	Sigma Aldrich
Cholera Toxin	C8052	Sigma Aldrich
Insulin	I2643	Sigma Aldrich
Culture Chamber	Bio II A	Telstar
Inverted Microscope	IX71	Olympus
Inverted Microscope Camera	DP72	Olympus
Fluorimeter	Fluoroskan ascent FL	Thermo Scientific
Centrifuge	2-16 K	Sigma Aldrich
Shaker	Rotamax 120	Heidolph
Plate centrifuge	5804 R	Eppendorf
CO_2 incubator	-	Sanyo Electric Co.
Microplate reader	SpectraMax Plus	Molecular Devices

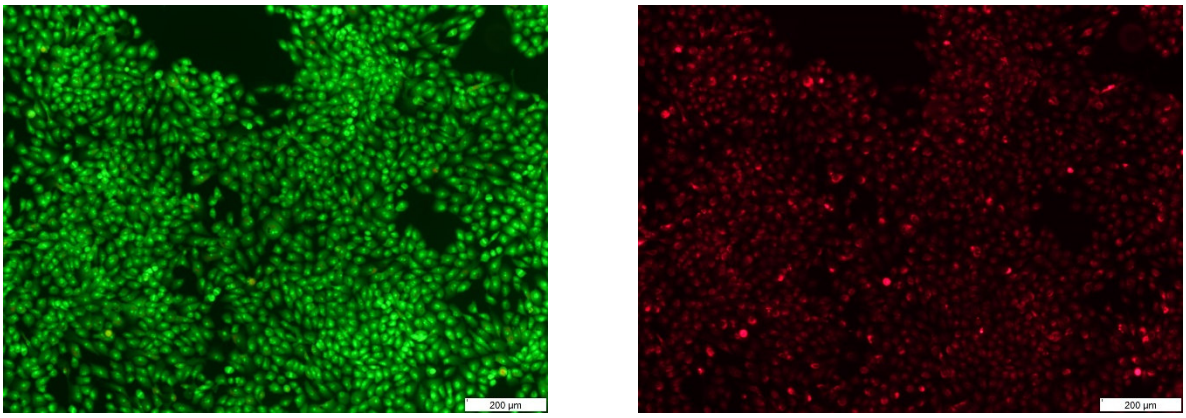
Appendix 2 – AO/PI assay in MCF-10A (8 H)



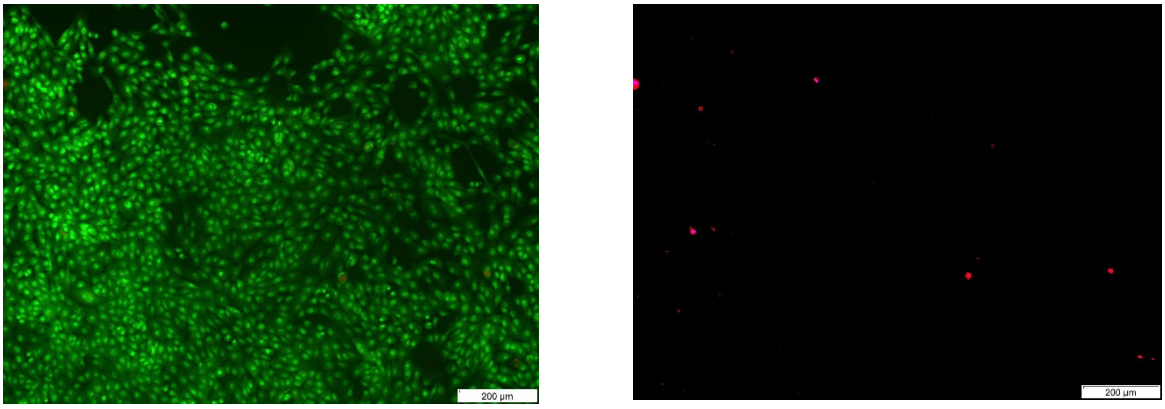
Life control



5 µg/ml extract

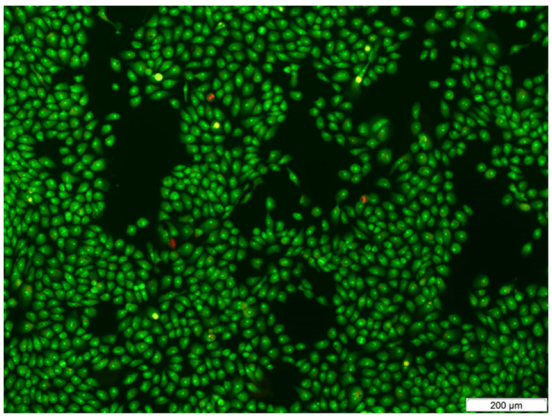


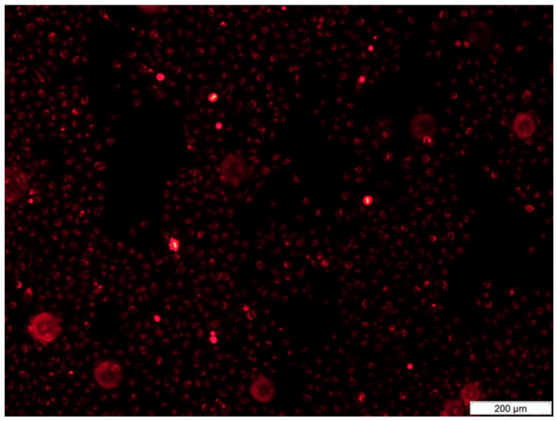
10 µg/ml extract



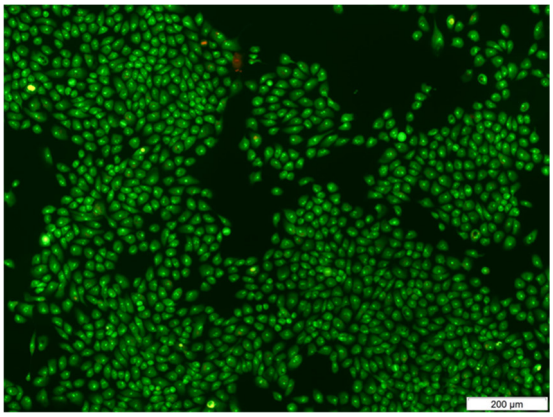
30 µg/ml extract

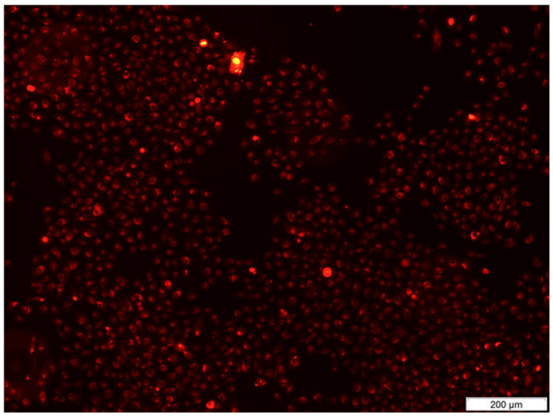
Appendix 2 – AO/PI assay in MCF-10A (8 H)



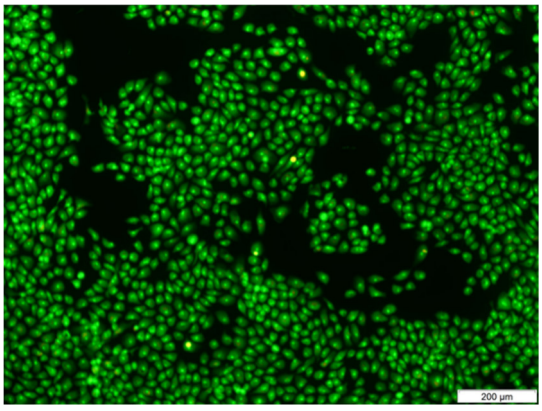


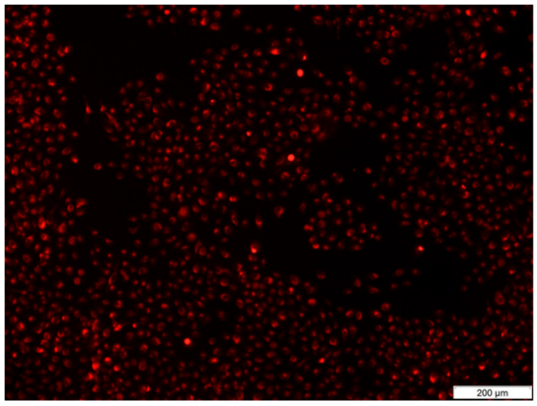
0.495 µg/ml UA





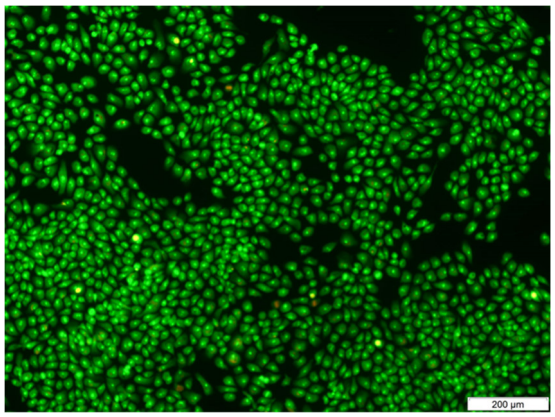
0.99 µg/ml UA

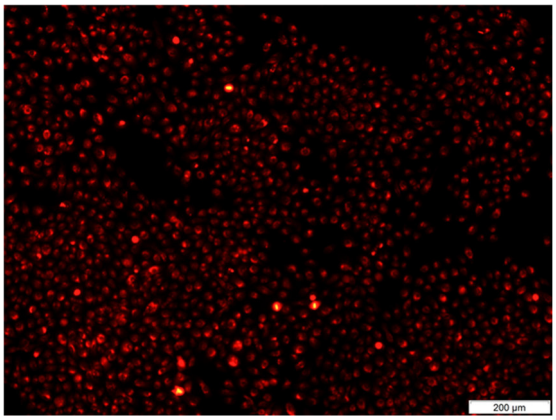




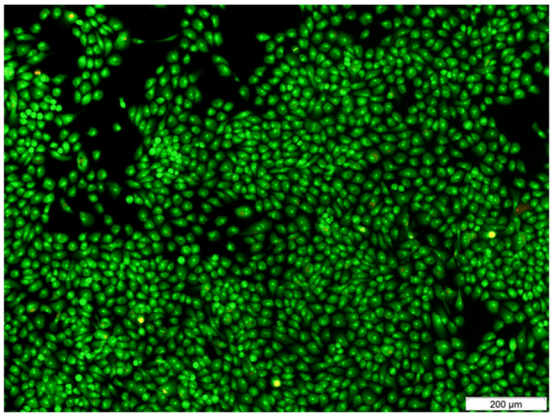
2.97 µg/ml UA

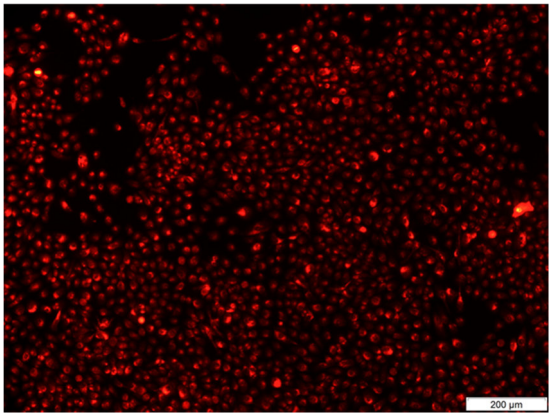
Appendix 2 – AO/PI assay in MCF-10A (8 H)



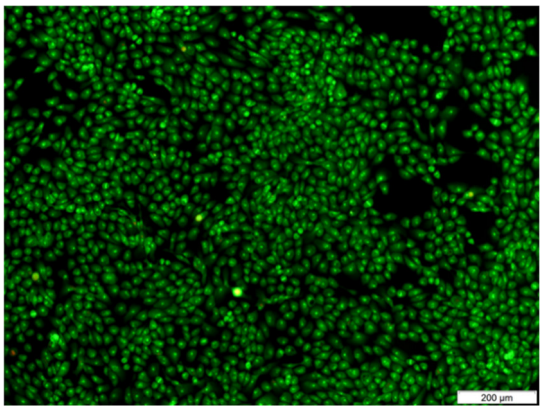


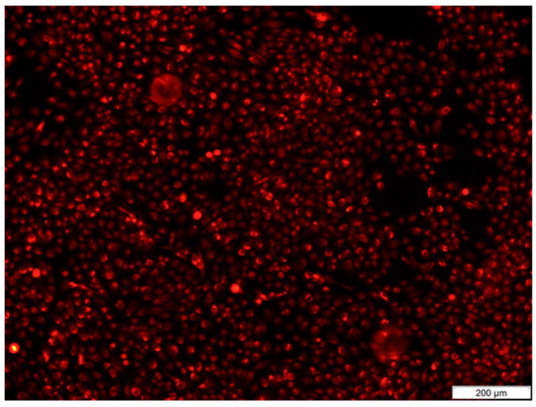
1 µg/ml BA





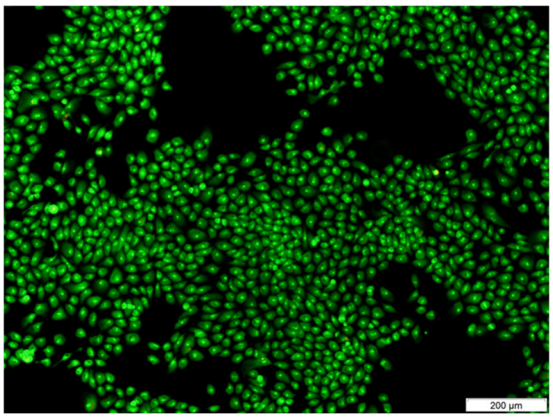
2µg/ml BA

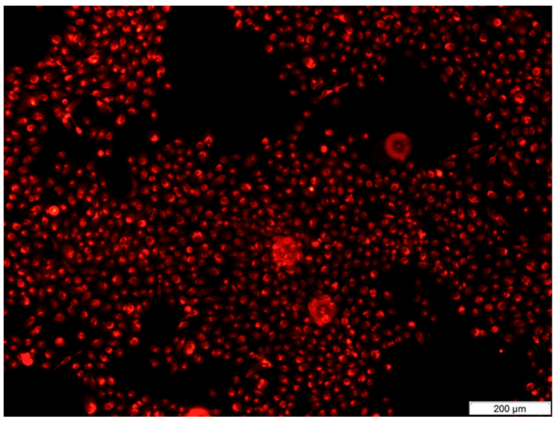




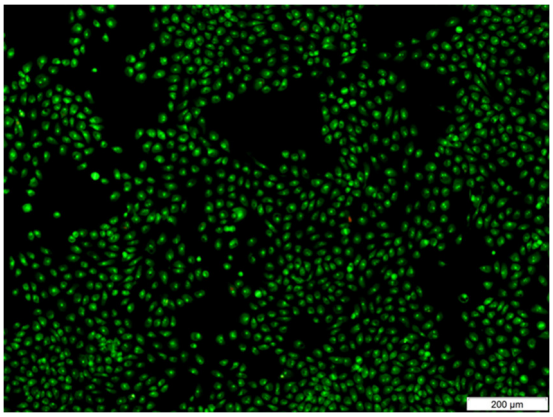
6 µg/ml BA

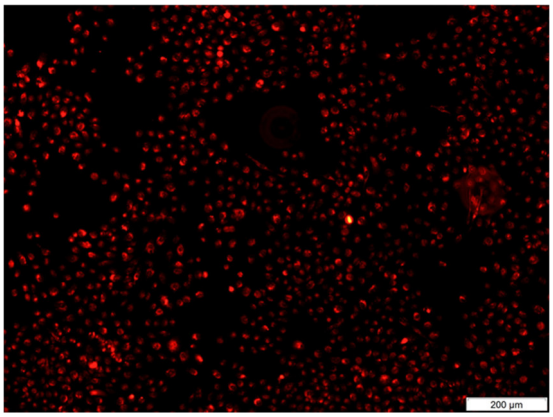
Appendix 2 – AO/PI assay in MCF-10A (8 H)



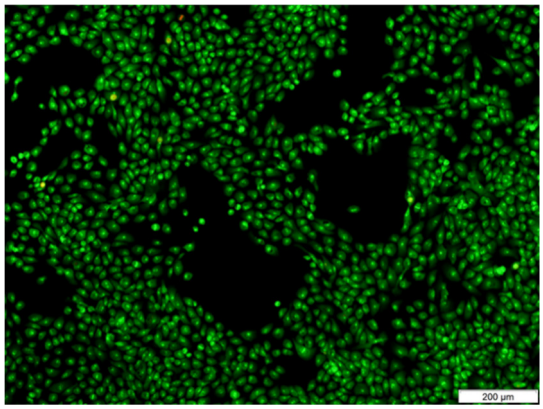


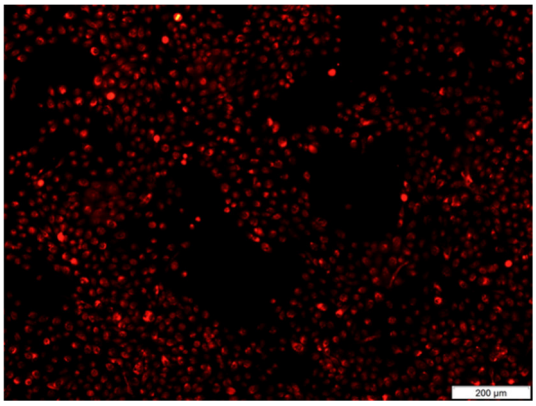
5 µg/ml UB





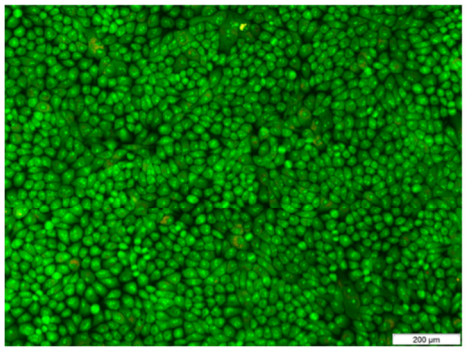
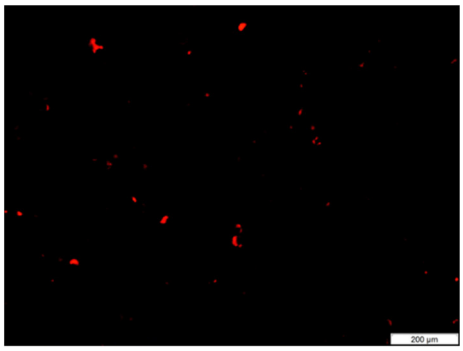
10 µg/ml UB



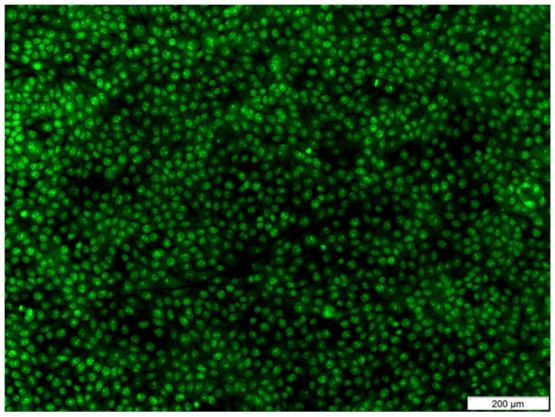
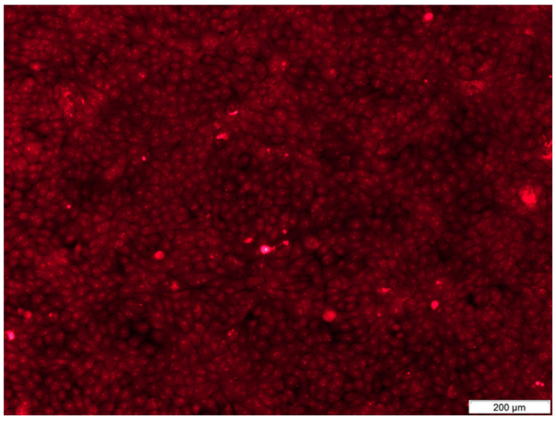


30 µg/ml UB

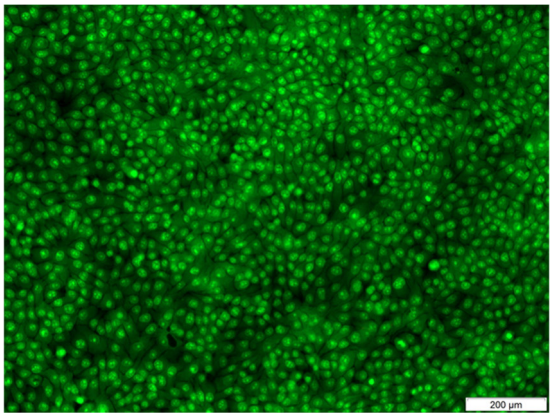
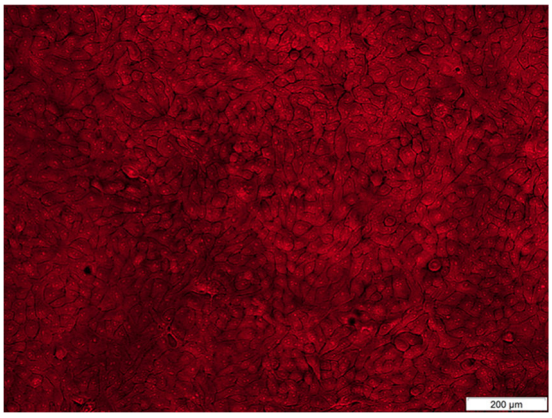
Appendix 2 – AO/PI assay in MCF-10A (24 H)

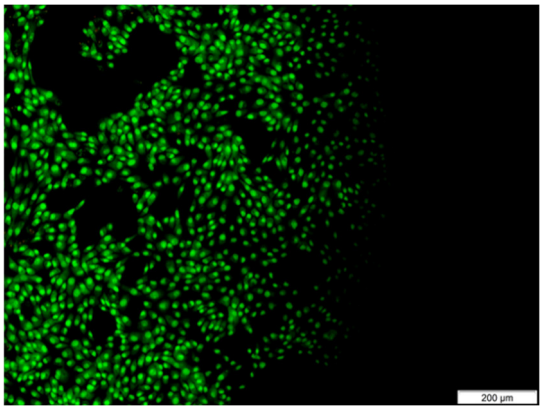
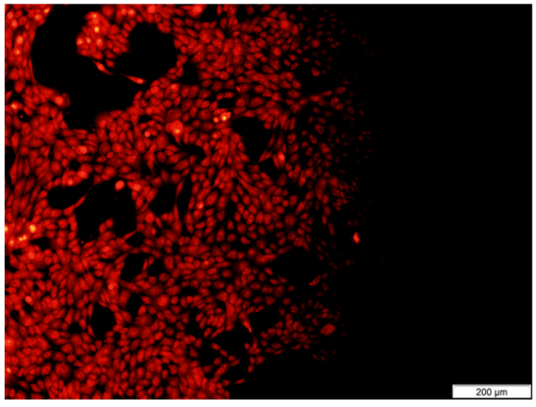
Life control

5 µg/ml extract

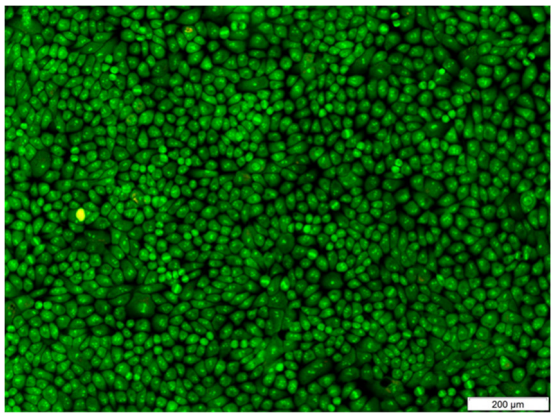



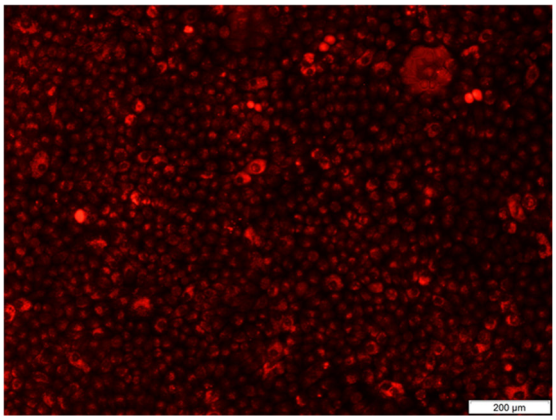
10 µg/ml extract

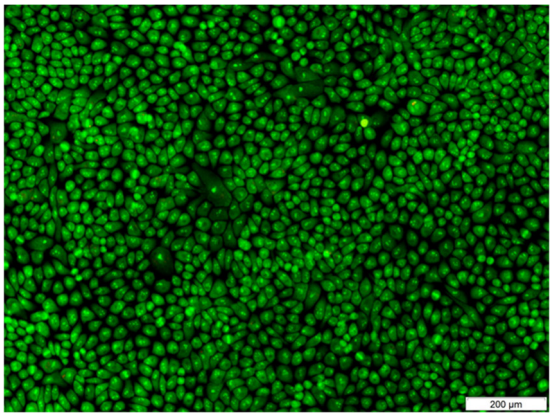
30 µg/ml extract

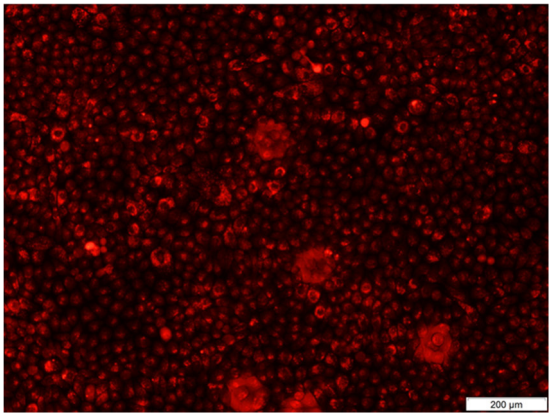
Appendix 2 – AO/PI assay in MCF-10A (24 H)



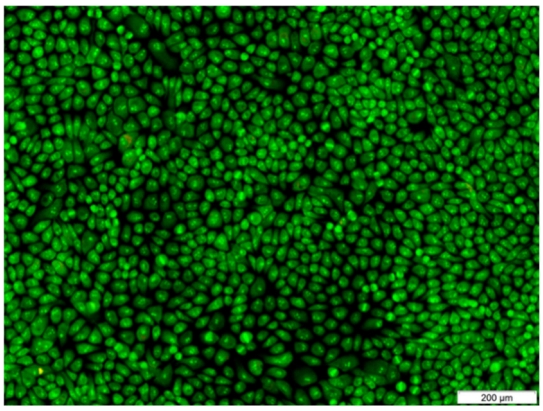


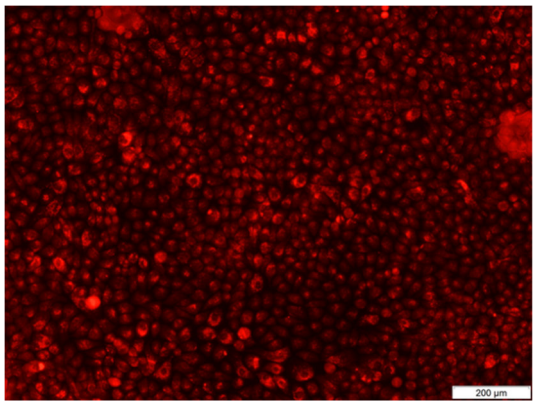
0.495 µg/ml UA





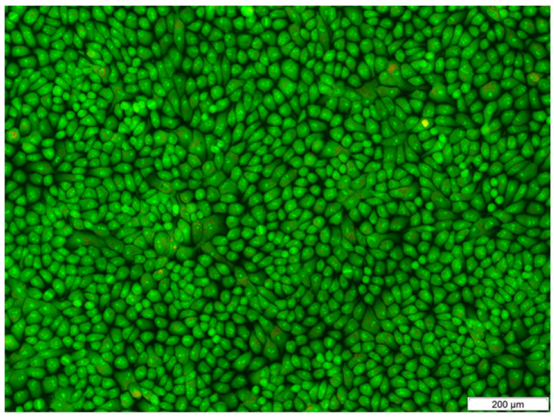
0.99 µg/ml UA

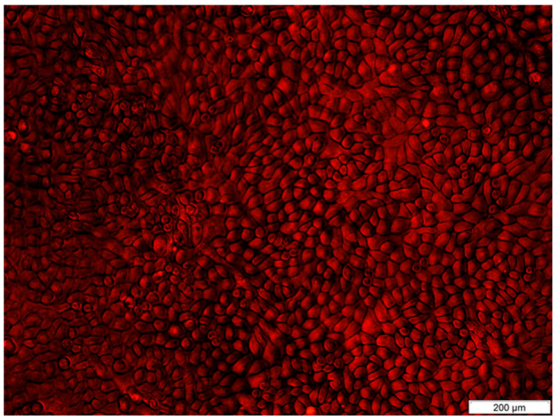




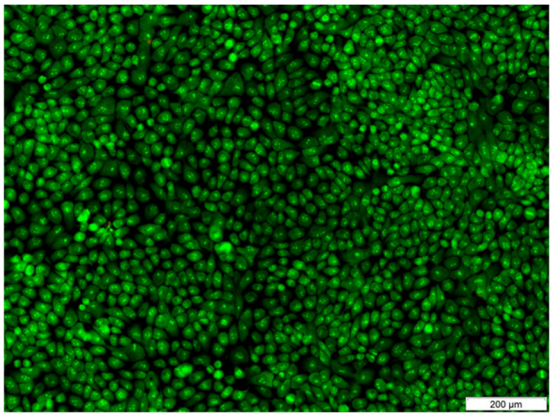
2.97 µg/ml UA

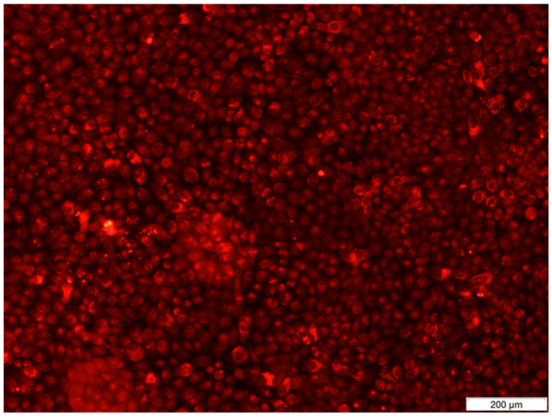
Appendix 2 – AO/PI assay in MCF-10A (24 H)



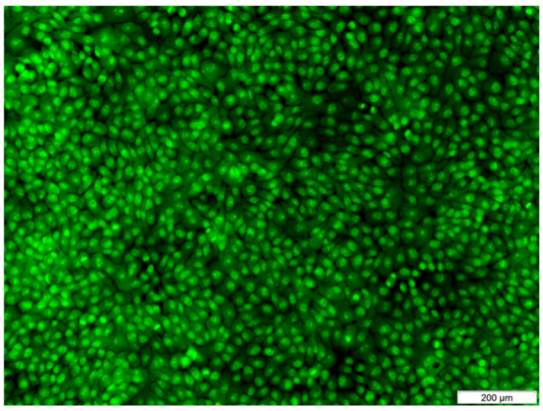


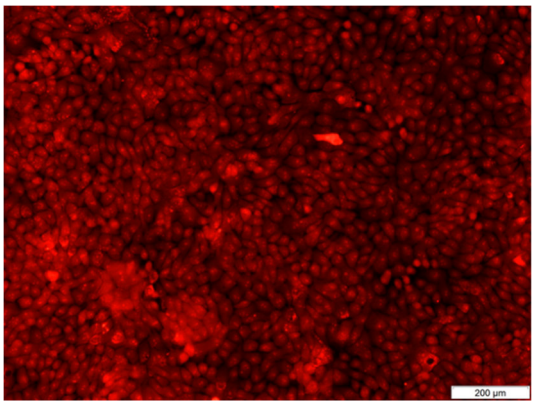
1 µg/ml BA





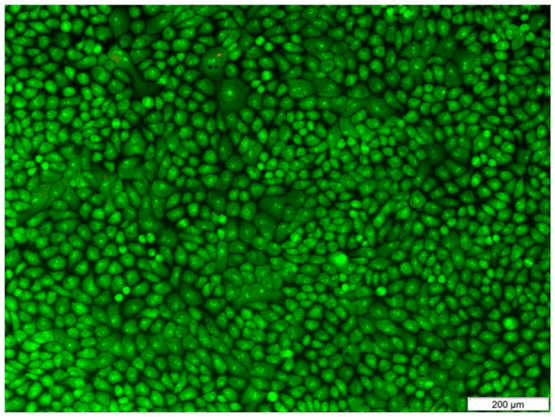
2µg/ml BA

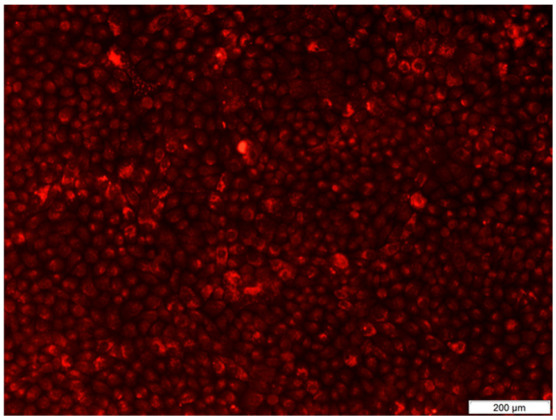




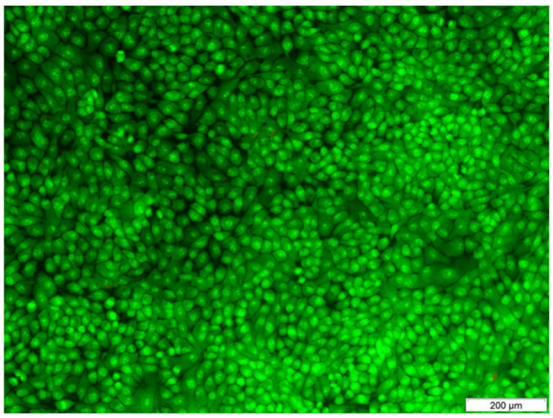
6 µg/ml BA

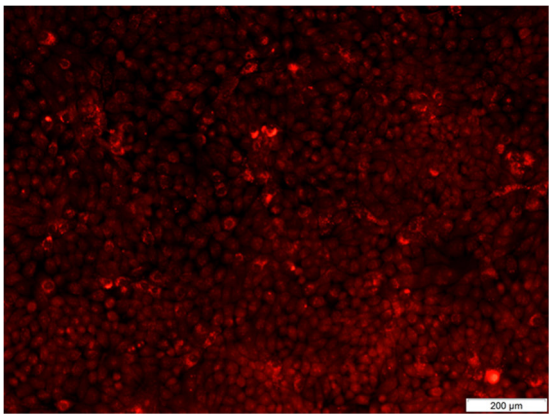
Appendix 2 – AO/PI assay in MCF-10A (24 H)



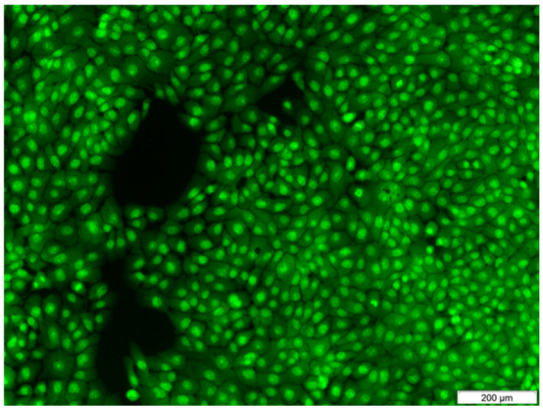


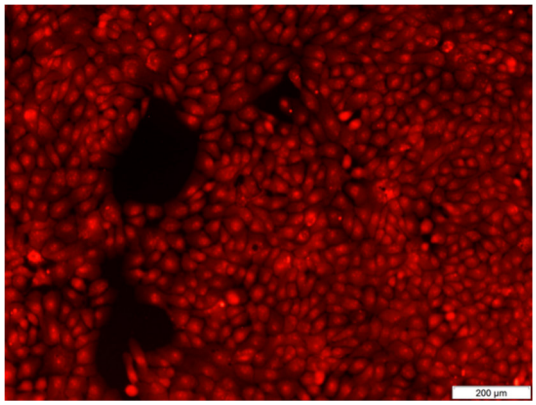
5 µg/ml UB





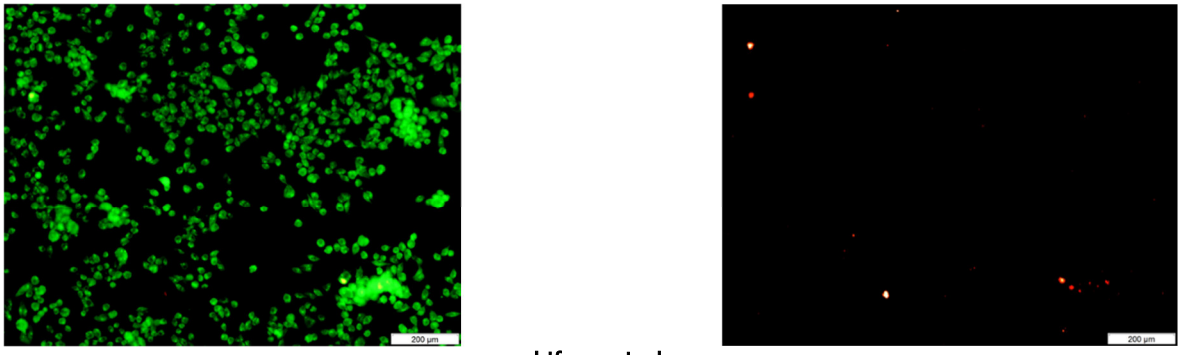
10 µg/ml UB



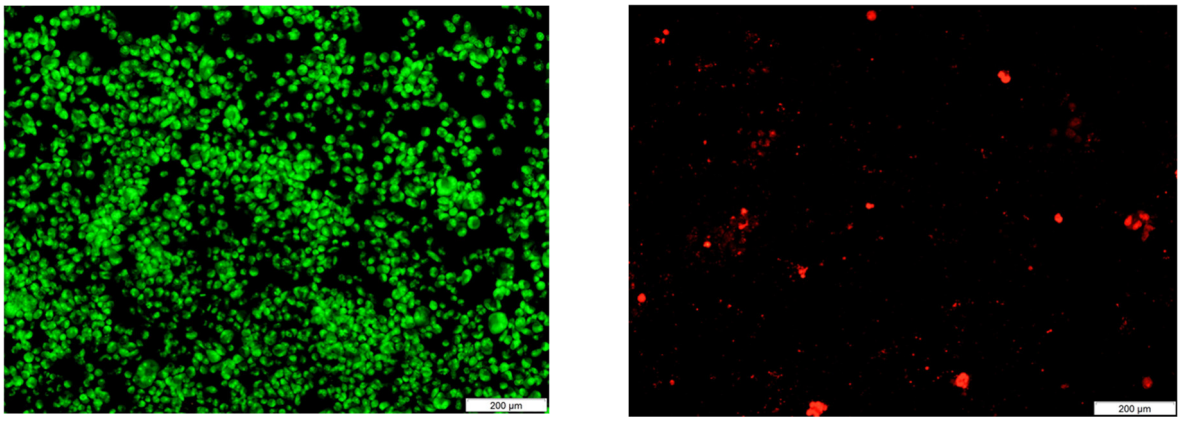


30 µg/ml UB

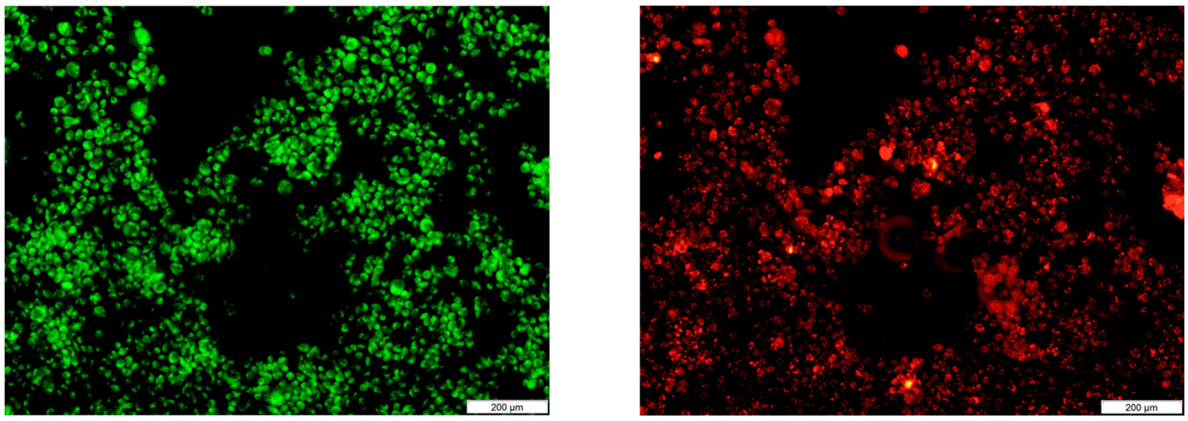
Appendix 3 – AO/PI assay in MDA-MB-231 (8 H)



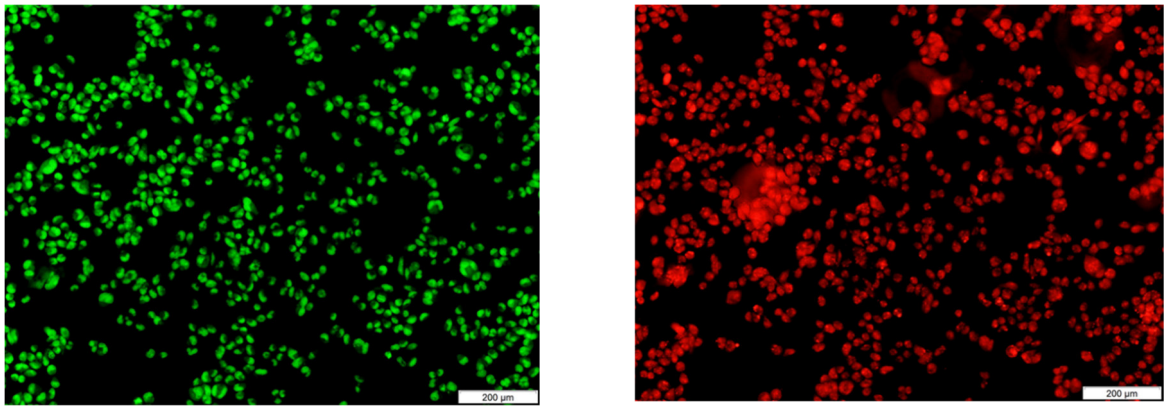
Life control



5 µg/ml extract

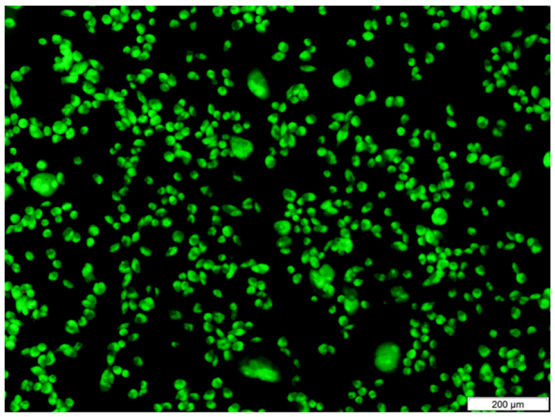


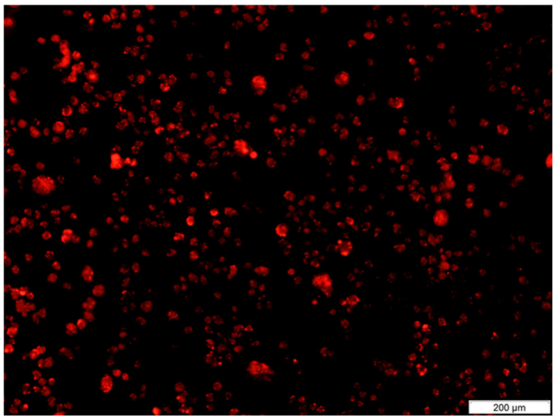
10 µg/ml extract



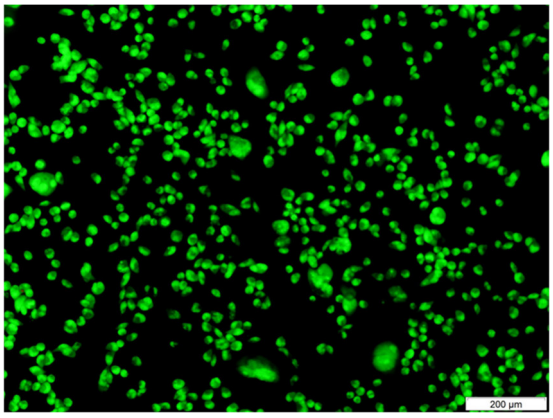
30 µg/ml extract

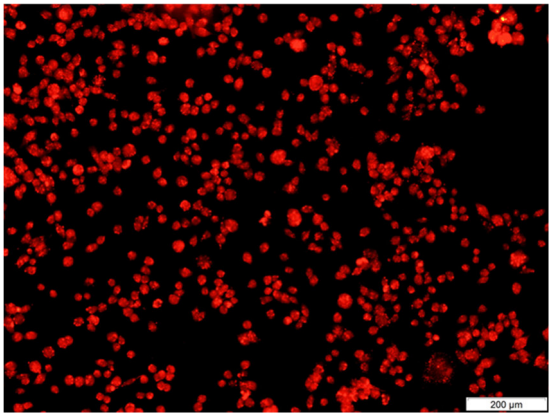
Appendix 3 – AO/PI assay in MDA-MB-231 (8 H)



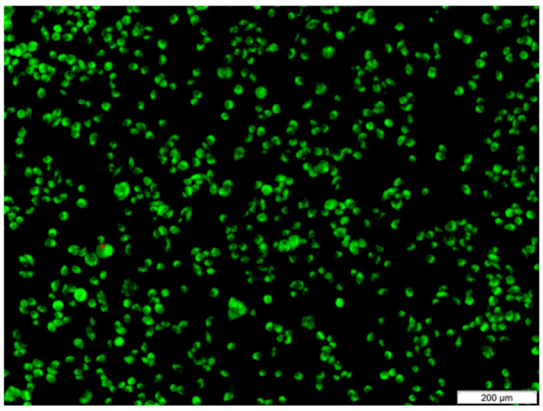


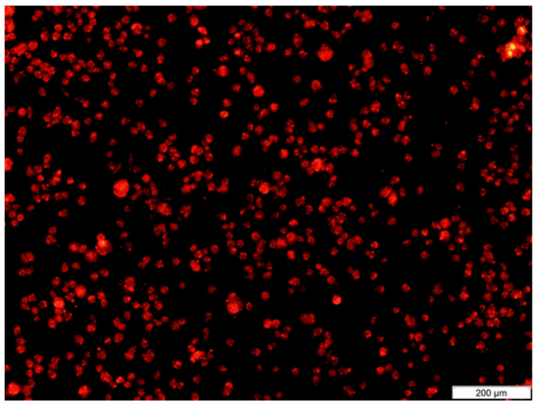
0.495 µg/ml UA





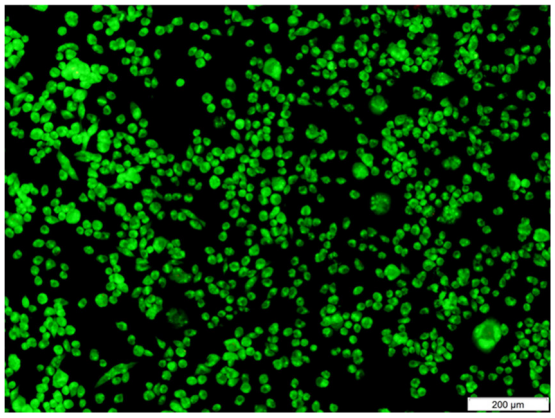
0.99 µg/ml UA

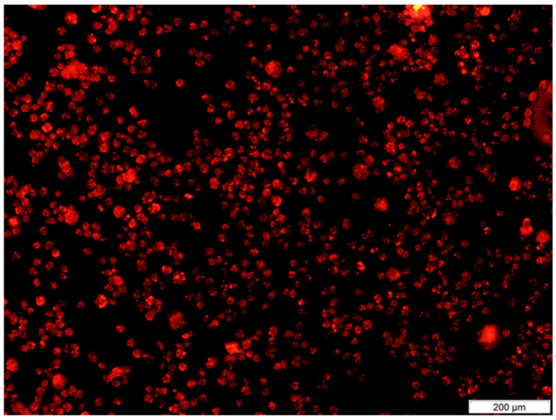




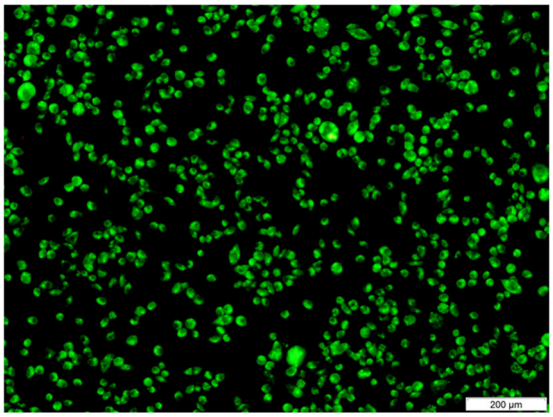
2.97 µg/ml UA

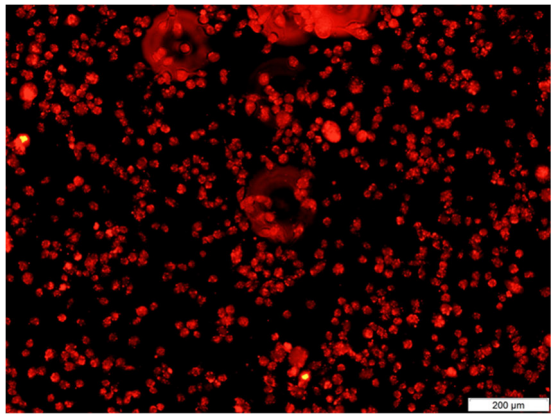
Appendix 3 – AO/PI assay in MDA-MB-231 (8 H)



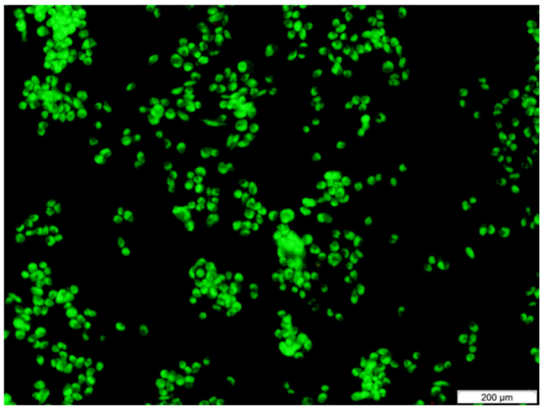


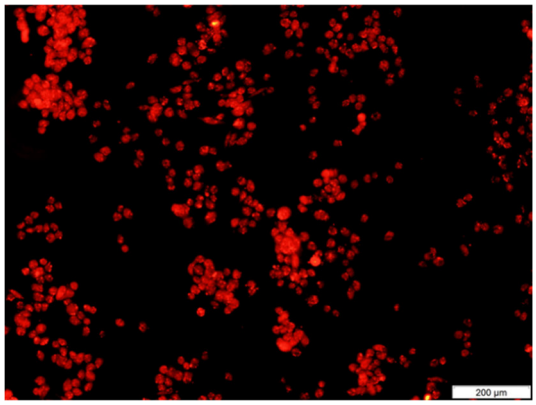
1 µg/ml BA





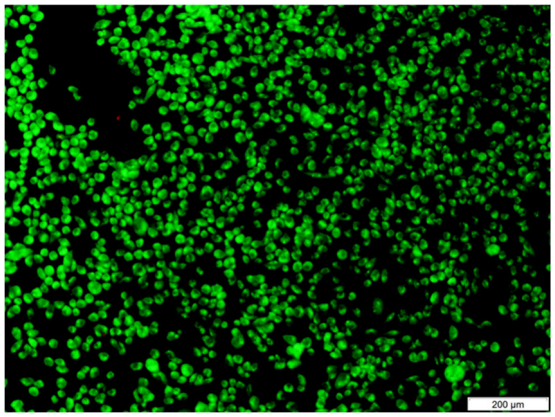
2µg/ml BA

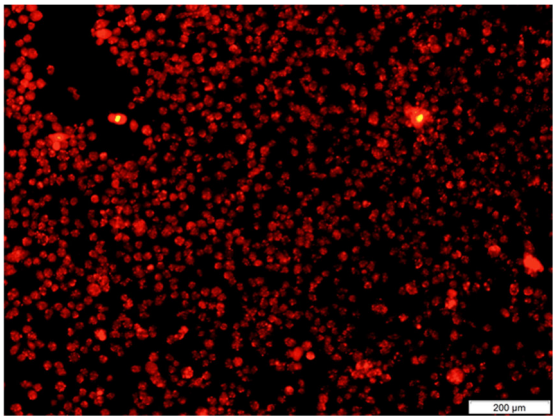




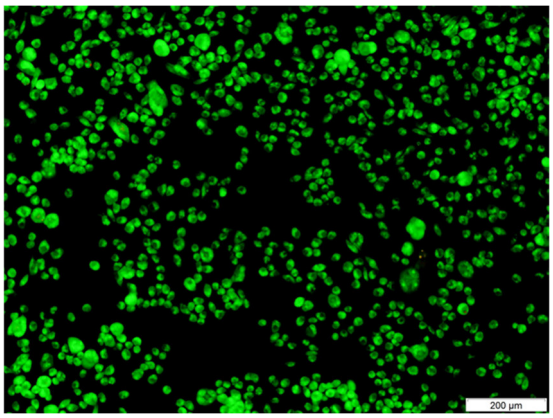
6 µg/ml BA

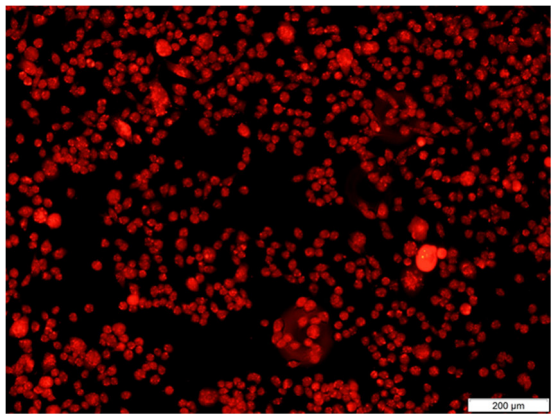
Appendix 3 – AO/PI assay in MDA-MB-231 (8 H)



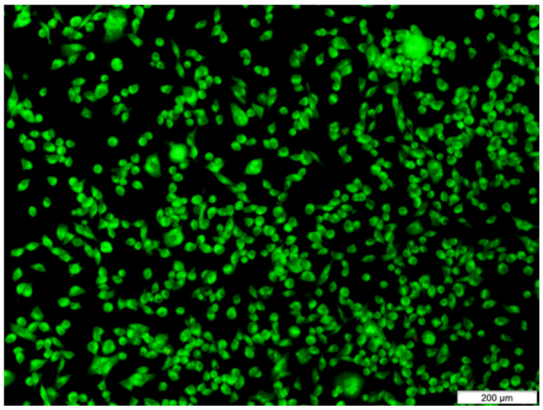


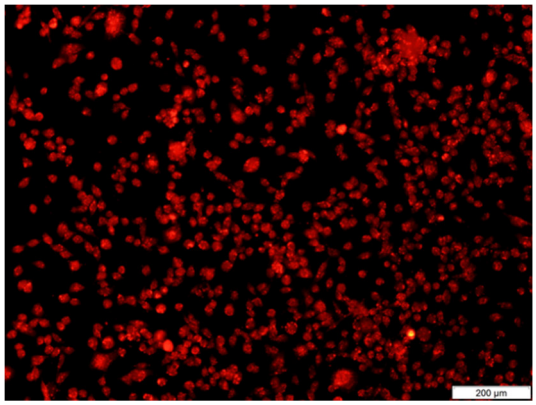
5 µg/ml UB





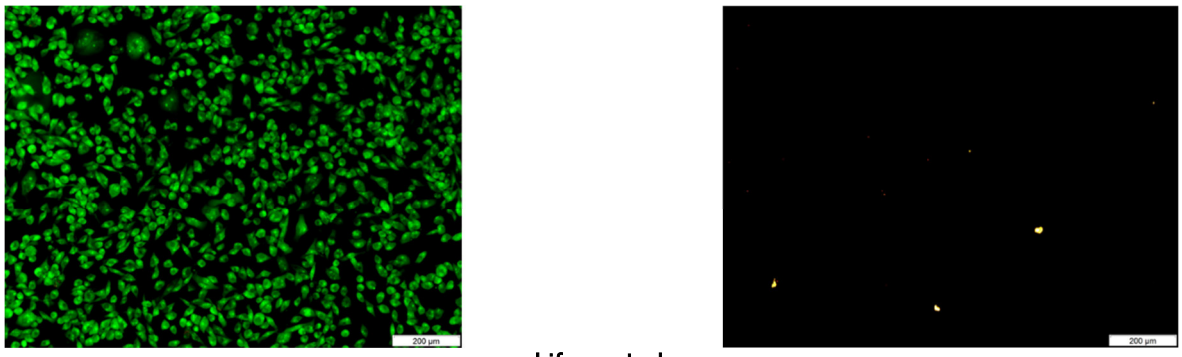
10 µg/ml UB



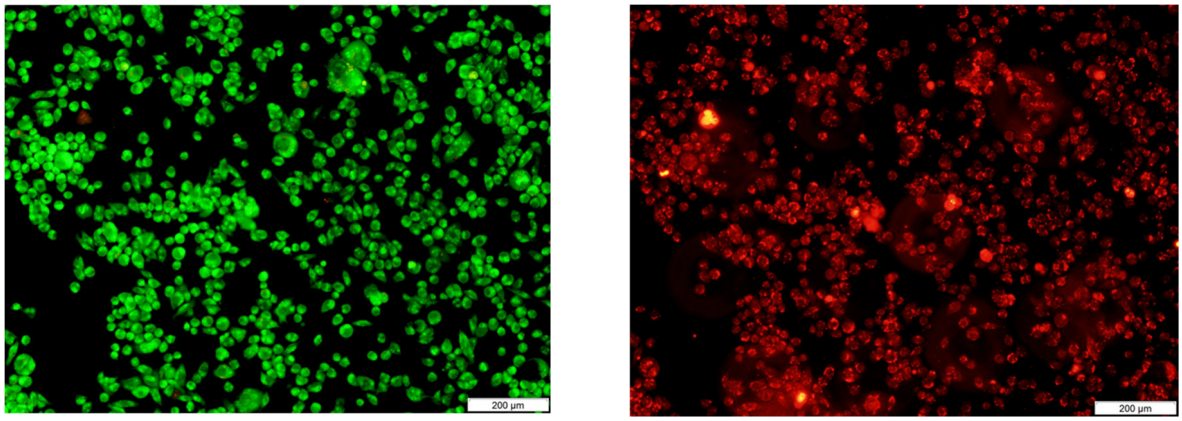


30 µg/ml UB

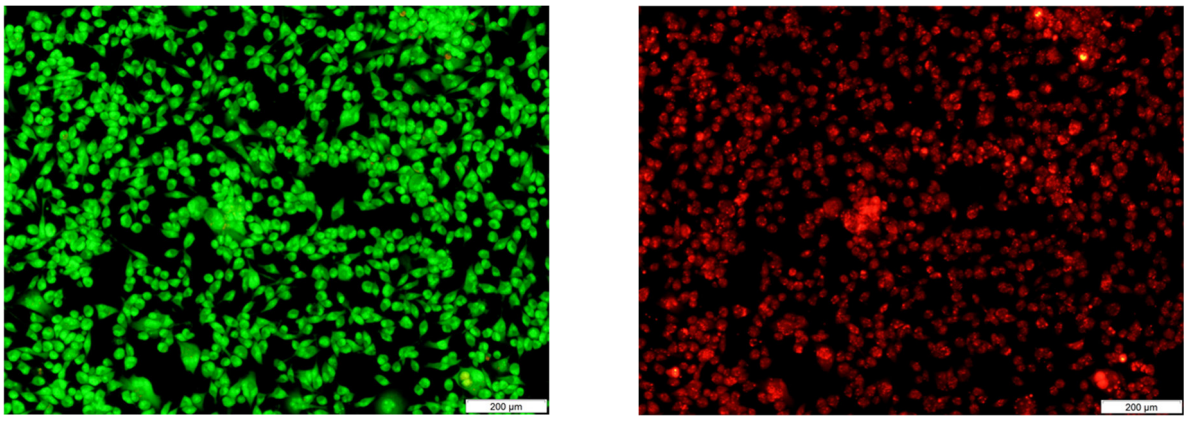
Appendix 3 – AO/PI assay in MDA-MB-231 (24 H)



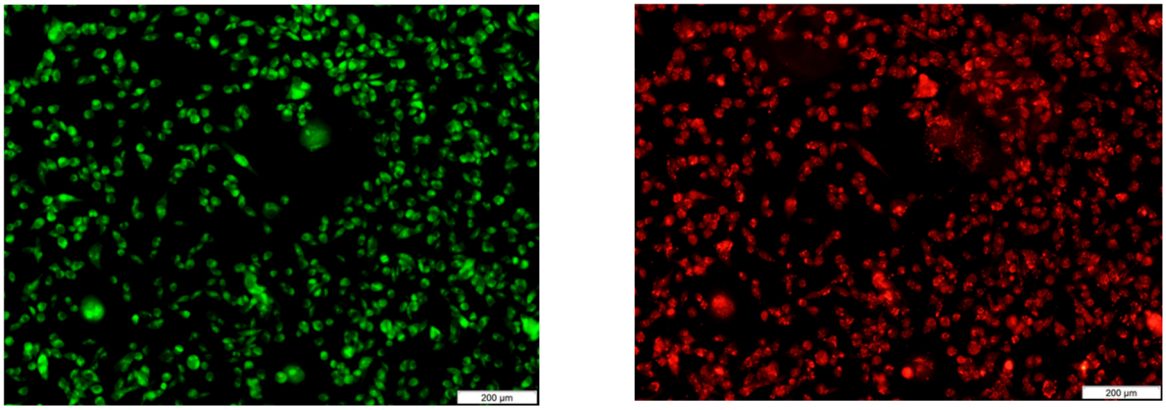
Life control



5 µg/ml extract

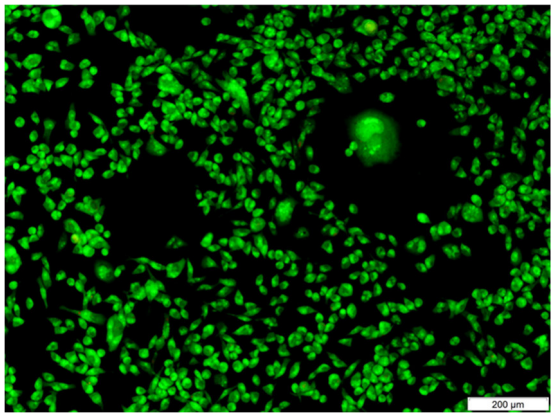


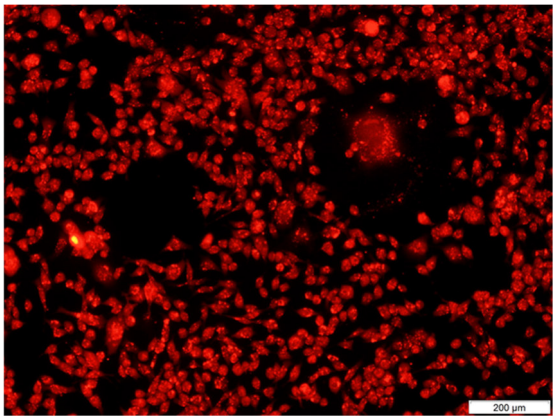
10 µg/ml extract



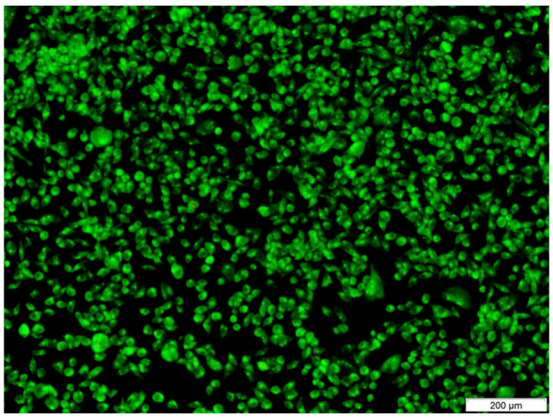
30 µg/ml extract

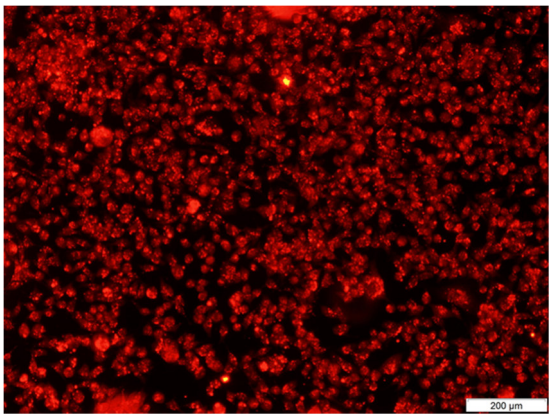
Appendix 3 – AO/PI assay in MDA-MB-231 (24 H)



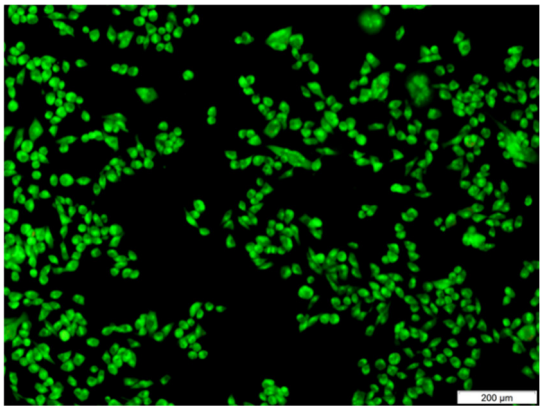


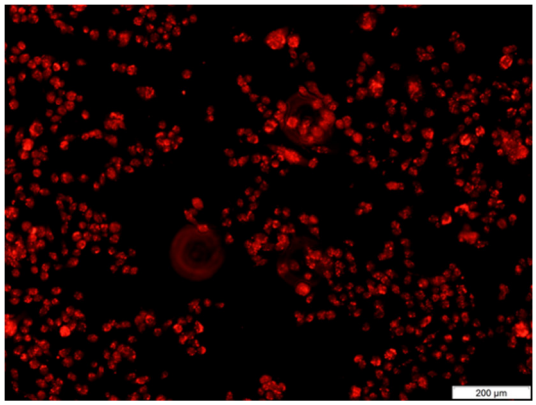
0.495 µg/ml UA





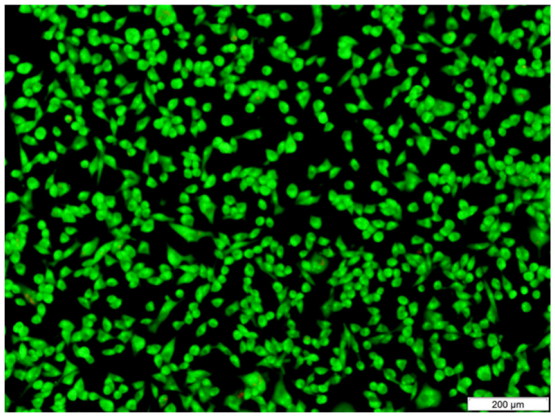
0.99 µg/ml UA

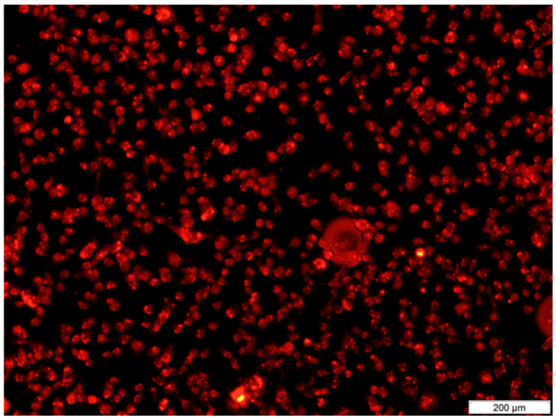




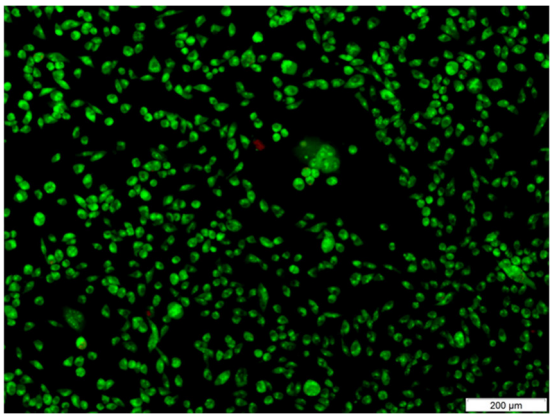
2.97 µg/ml UA

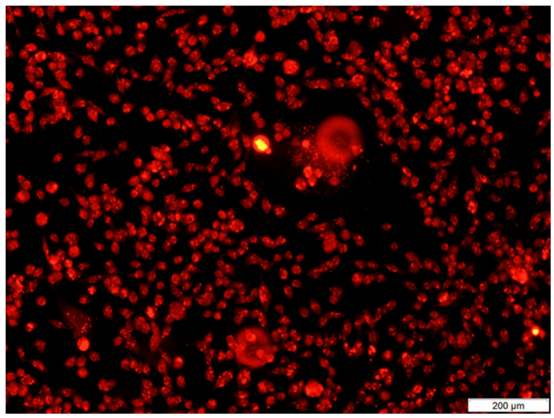
Appendix 3 – AO/PI assay in MDA-MB-231 (24 H)



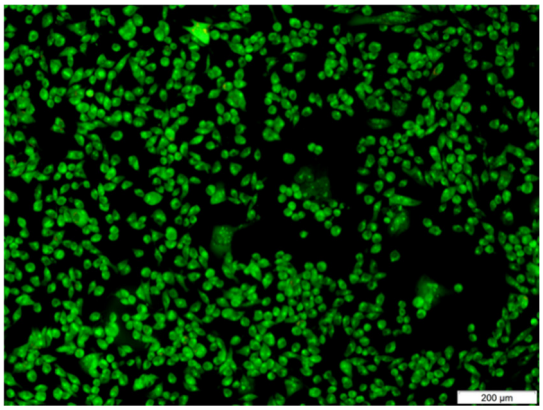


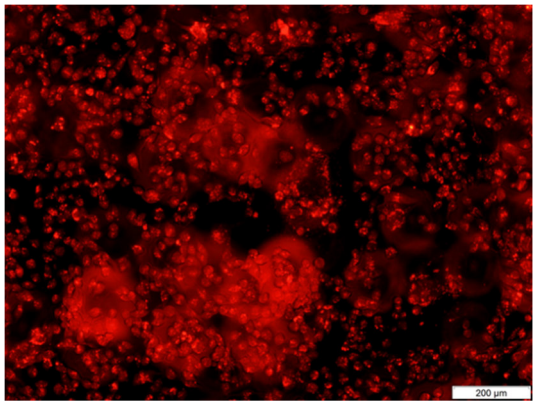
1 µg/ml BA





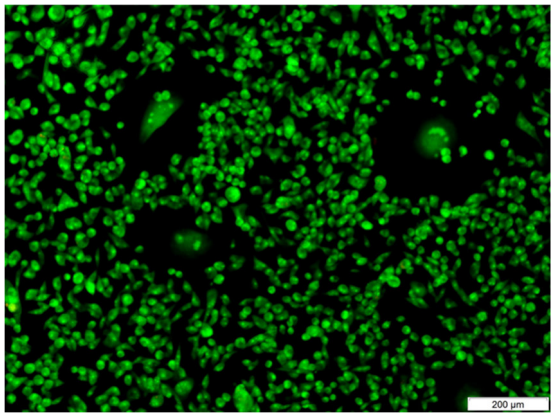
2µg/ml BA

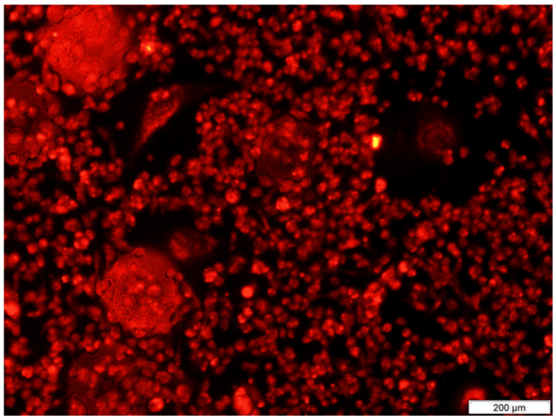




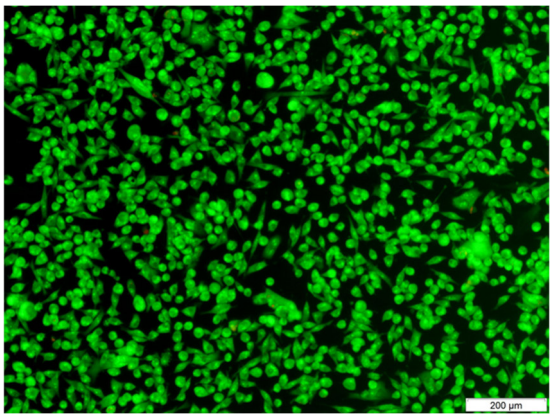
6 µg/ml BA

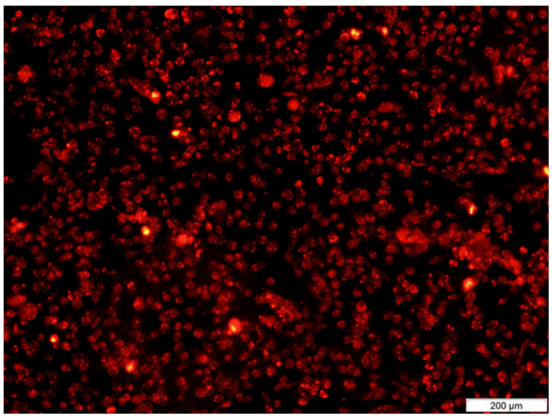
Appendix 3 – AO/PI assay in MDA-MB-231 (24 H)



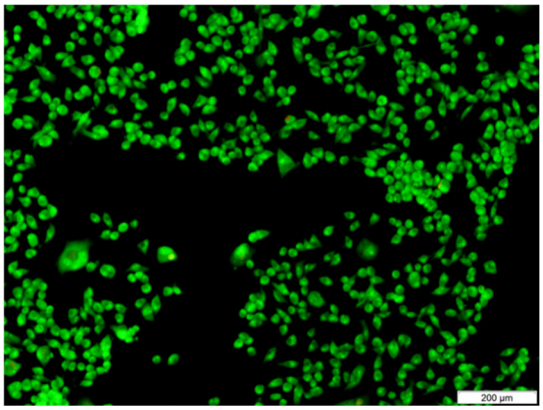


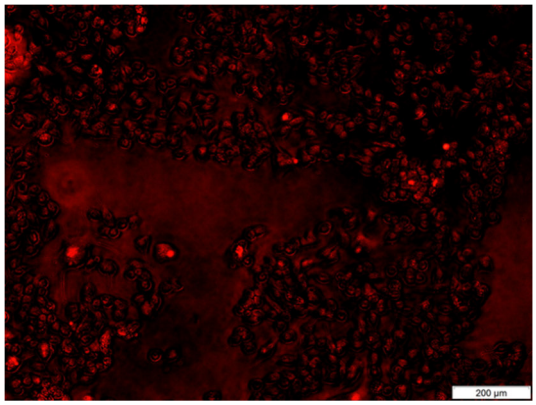
5 µg/ml UB





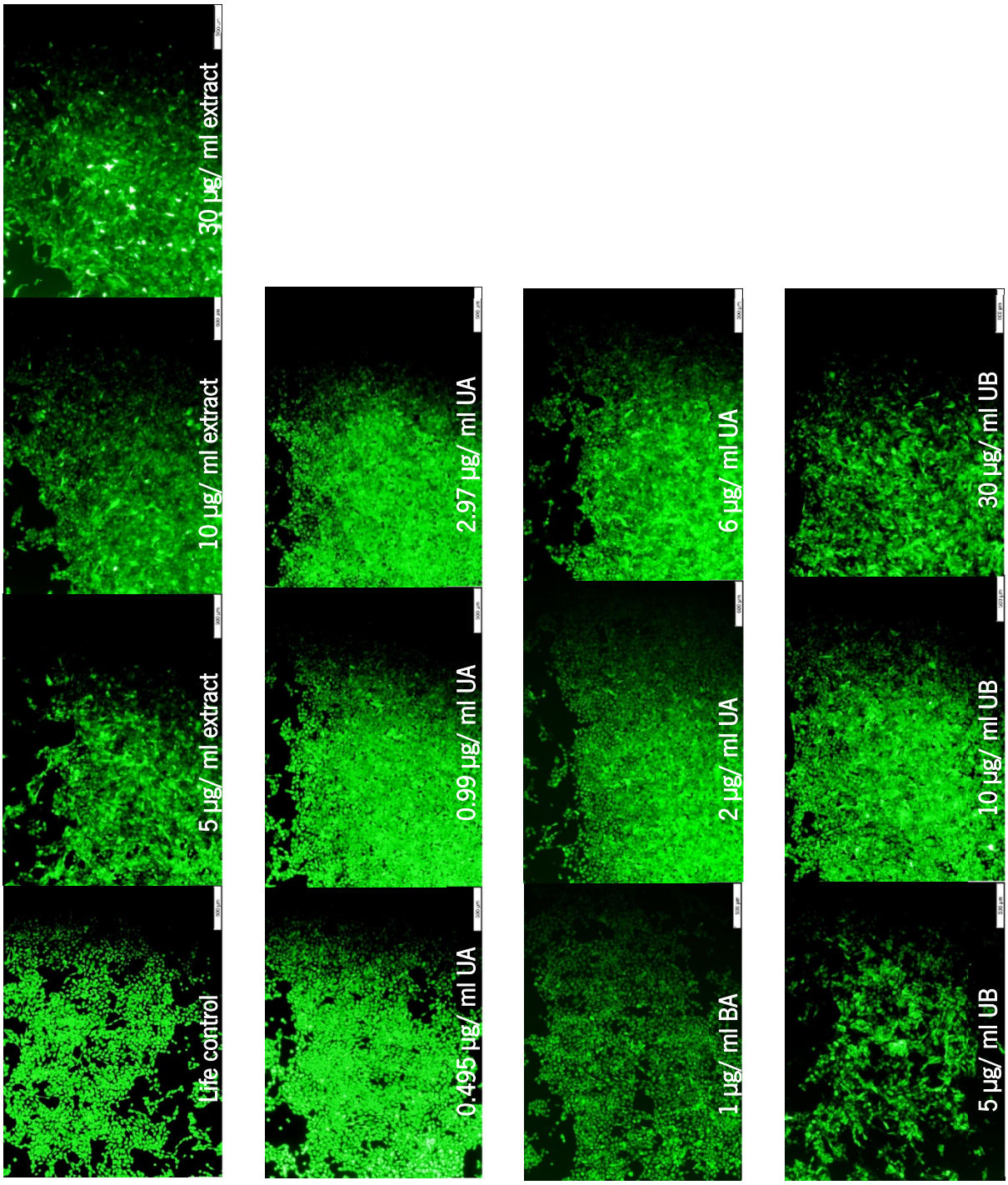
10 µg/ml UB



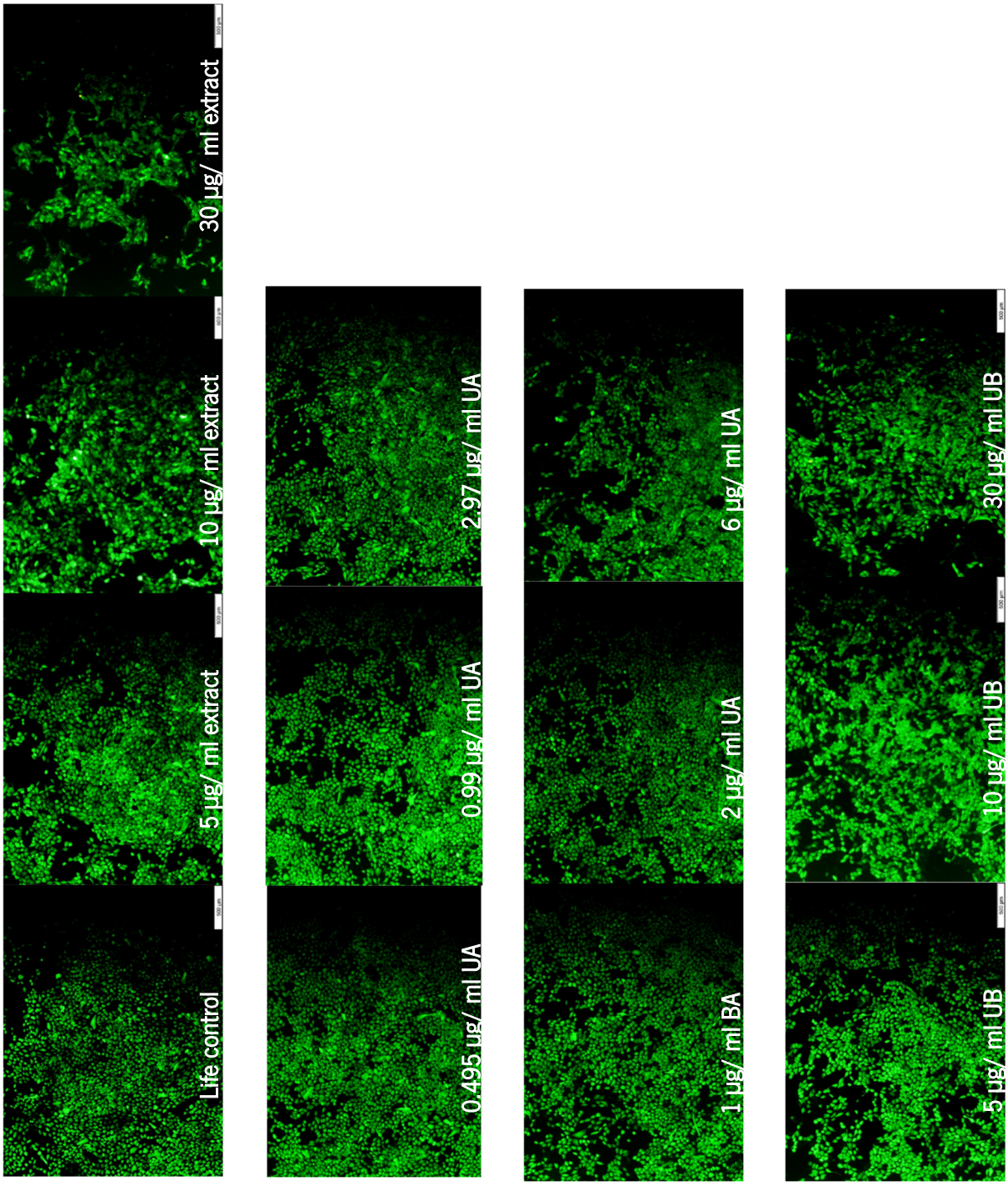


30 µg/ml UB

Appendix 4 – DCF-DA assay in MCF-10A (3H)

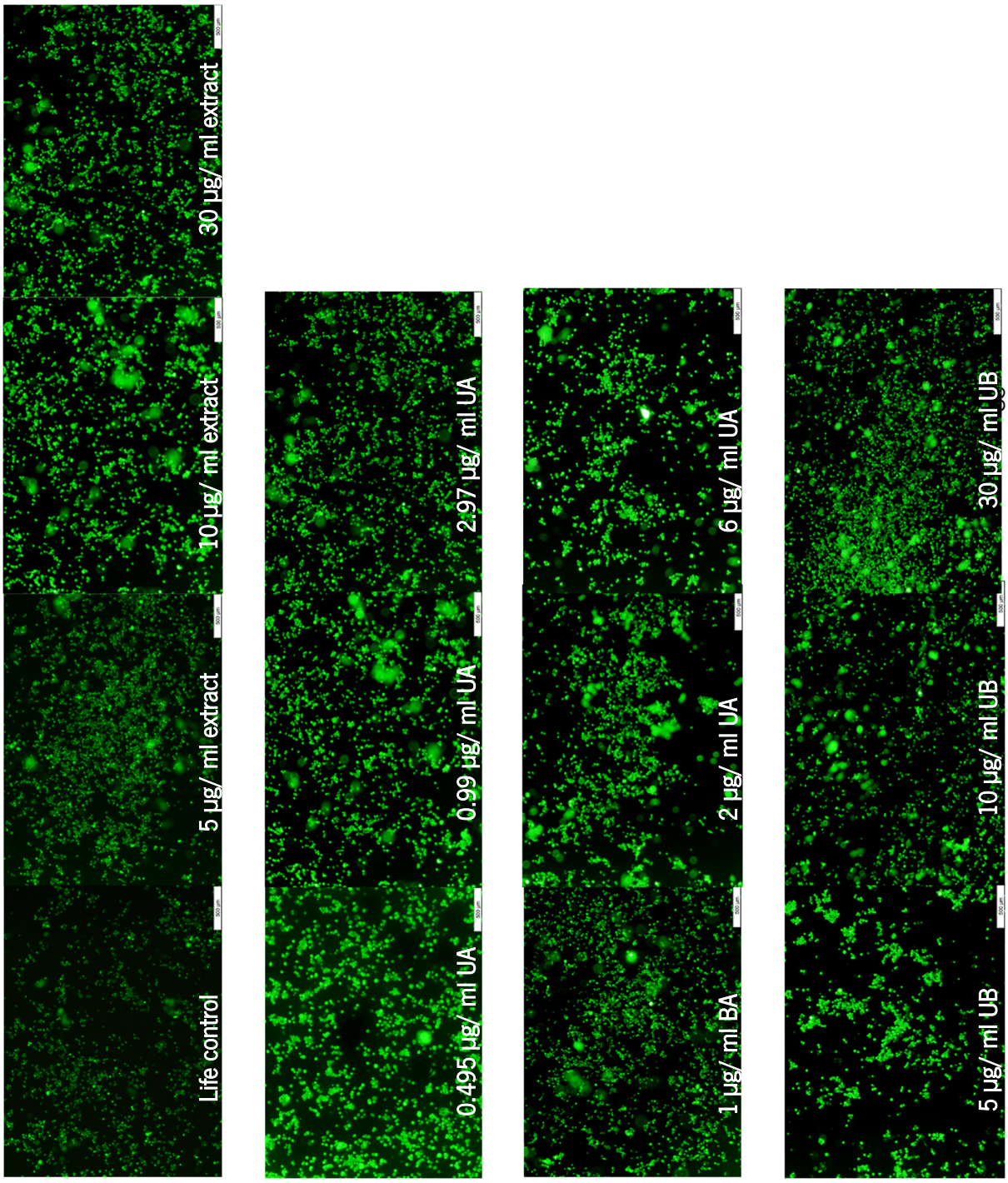


Appendix 4 – DCF-DA assay in MCF-10A (8 H)

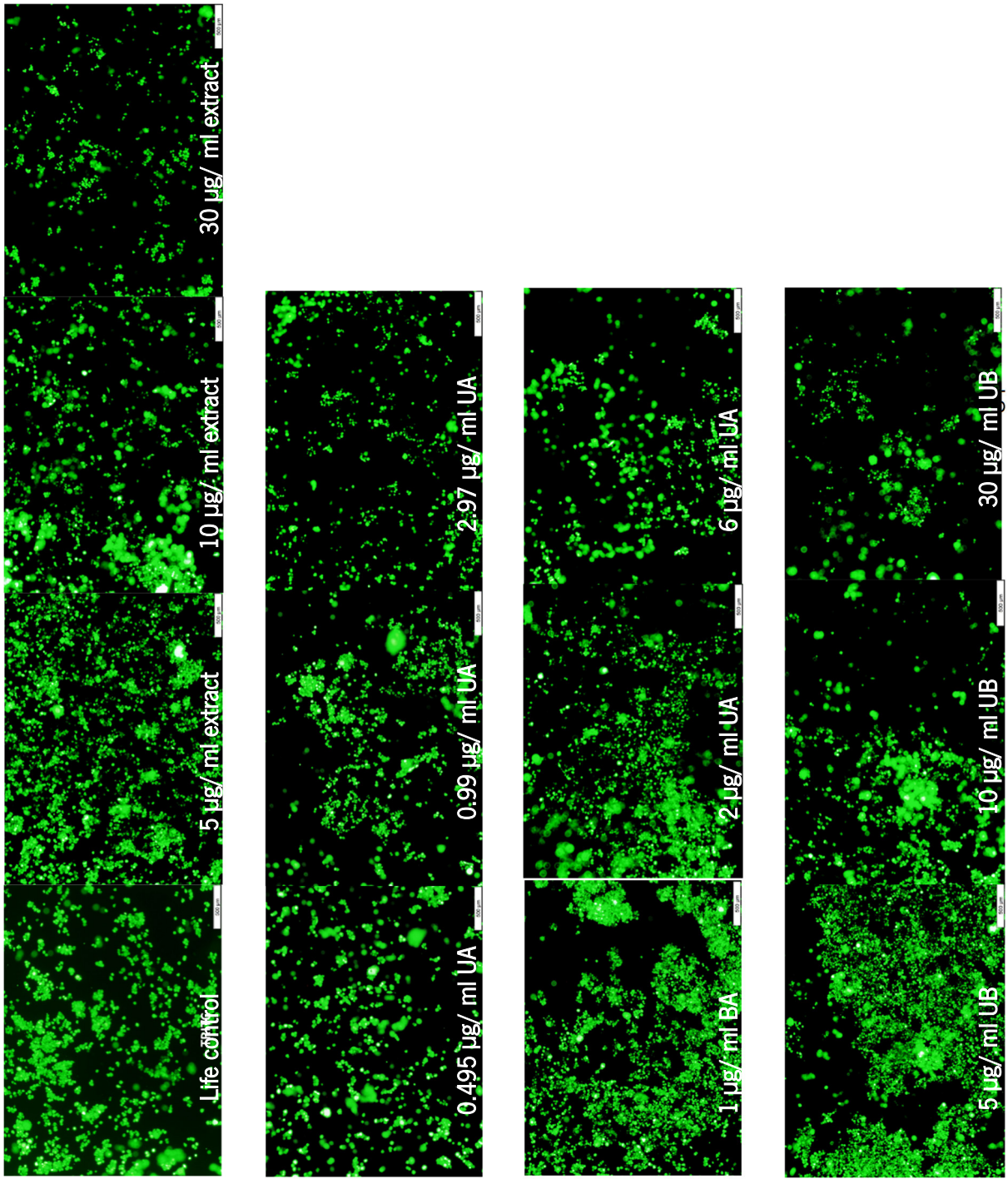


Appendix 4 – DCF-DA assay in MCF-10A (24 H)

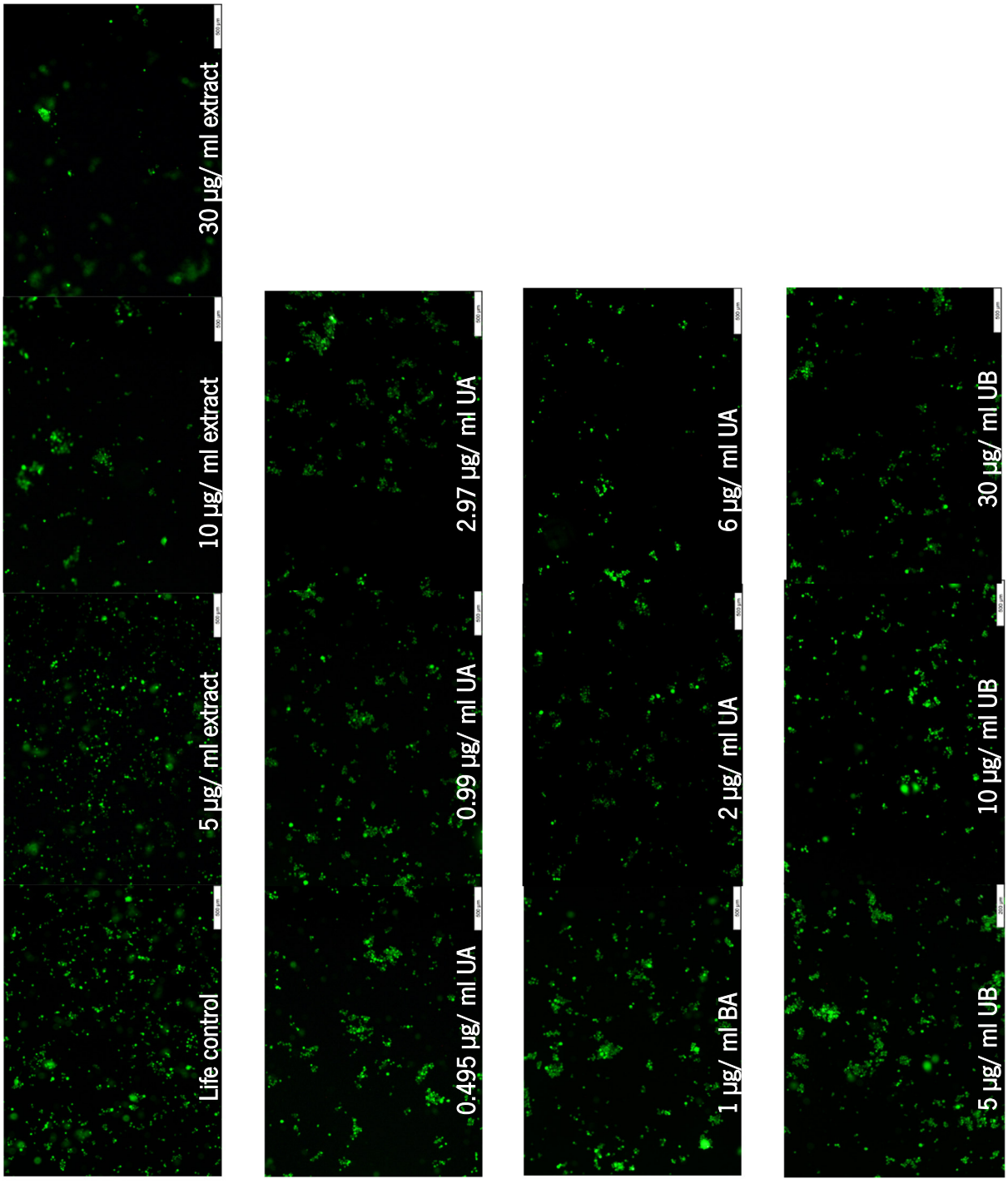
Appendix 5 – DCF-DA assay in MDA-MB-231 (3 H)



Appendix 5 – DCF-DA assay in MDA-MB-231 (8 H)

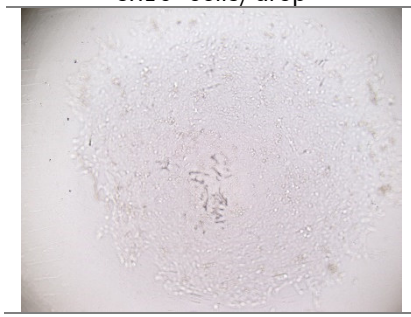
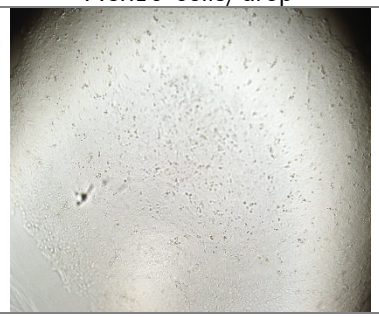
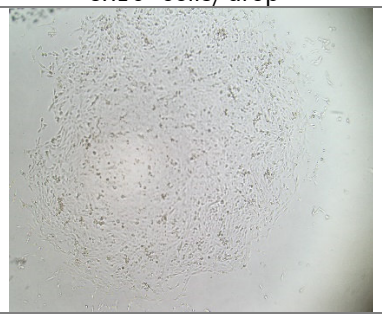
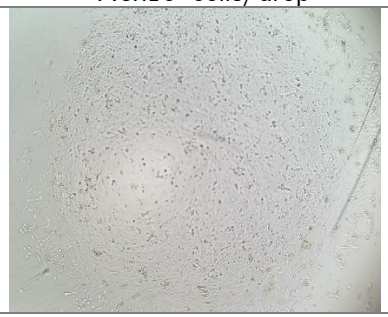


Appendix 5 – DCF-DA assay in MDA-MB-231 (24 H)

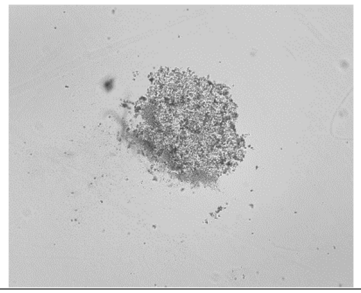
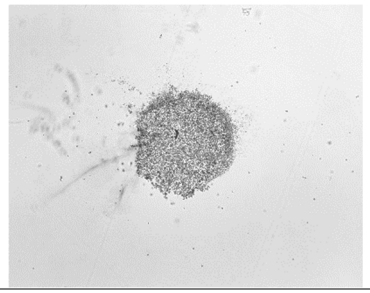


Appendix 6

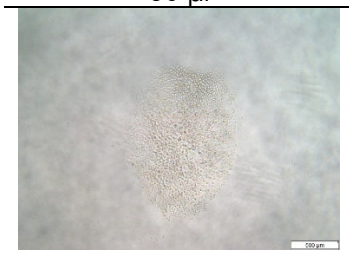
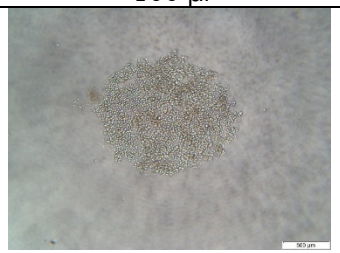
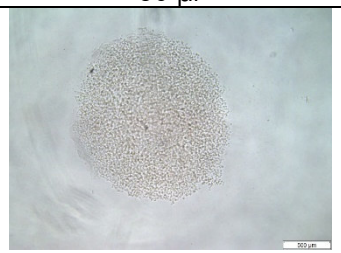
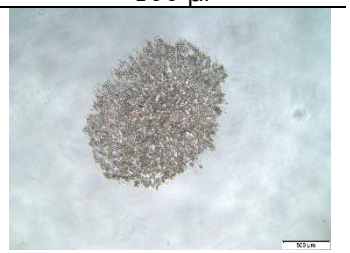
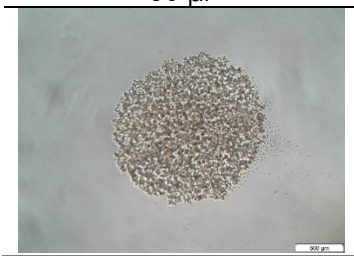
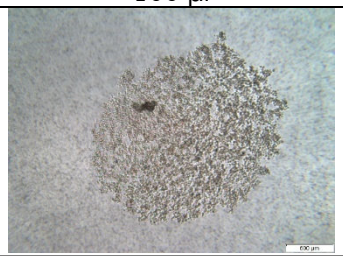
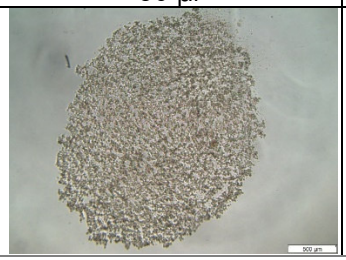
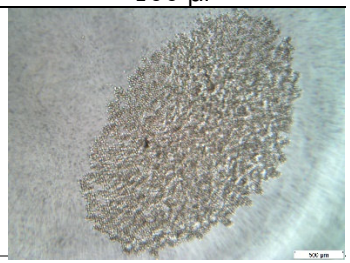
Suspension culture method in MCF-10A

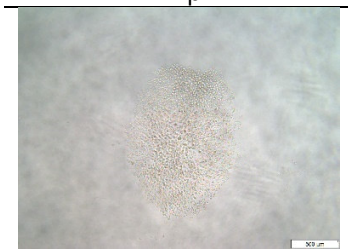
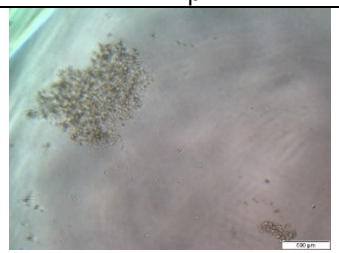
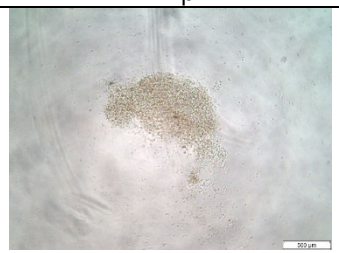
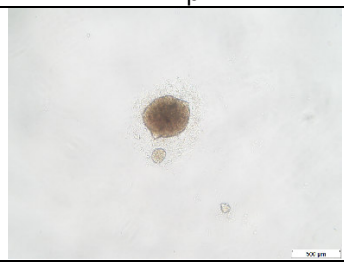
Suspension culture method in MCF-10A			
30µl		100 µl	
$5x10^3$ cells/drop	$7.5x10^3$ cells/drop	$5x10^3$ cells/drop	$7.5x10^3$ cells/drop
			

Appendix 7

Hanging drop method in MCF-10A	
$5x10^3$ cells/drop (30µl volume)	$7.5x10^3$ cells/drop (30µl)
	

Appendix 8

0.6% agarose coating			
MCF-10A (72H)			
$5x10^3$ cells		$1x10^4$ cells	
50 µl	100 µl	50 µl	100 µl
			
0.6% agarose coating			
MDA-MB-231 (72H)			
$5x10^3$ cells		$1x10^4$ cells	
50 µl	100 µl	50 µl	100 µl
			

0.6% agarose coating			
MCF-10A (120H)			
$5x10^3$ cells		$1x10^4$ cells	
50 µl	100 µl	50 µl	100 µl
			
0.6% agarose coating			
MDA-MB-231 (120H)			
$5x10^3$ cells		$1x10^4$ cells	
50 µl	100 µl	50 µl	100 µl