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Janeiro de 2013

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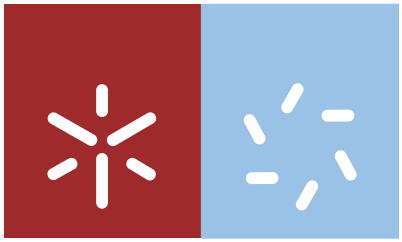

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Dissertação de Mestrado
Mestrado em Biotecnologia e Bioempreendedorismo em Plantas Aromáticas e Medicinais

Trabalho realizado sob a orientação do
**Doutor Cristóvão Lima**

e co-orientação da
**Doutora Cristina Pereira-Wilson**

Janeiro de 2013

## ACKNOWLEDGMENTS

*"Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning."*

Albert Einstein, *Relativity.*

Firstly, I would like to thank Doctor Cristóvão Lima, my main supervisor, for the opportunity of working in this project, as well as for all his support and time and the privilege to share his scientific knowledge with me.

Also a special thanks to Doctor Cristina Pereira-Wilson, my co-supervisor, for this opportunity and for her support throughout this work.

I would also like to thank Doctor Armando Silvestre and Doctor Fátima Duarte for providing extracts and compounds used in this work.

To the support of the project AFORE: Forest biorefineries: Added-value from chemicals and polymers by new integrated separation, fractionation and upgrading technologies", from the Seventh Framework Programme FP7/2007-2013, with the referece CP-IP 228589-2 AFORE.

To my laboratory colleagues, Dalila, Cristina, Cecília, Carla and Alice for all their help and conversations.

To Cláudia Silva and Joana Silvestre as well as Artur Oliveira and Filipe Pinto, for all their support, friendship and invaluable help.

To all my friends for just being there, for the good moments.

To my parents, sister, aunt and grandparents a very special thanks for always believing in me and for all the care, support and opportunities.

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## ABSTRACT

Cancer is one of the leading causes of death worldwide, being the colorectal cancer the third most occurring cancer in developed countries. The search for promising natural anticancer compounds is exponentially increasing, with the exploitation of new sources that were disregarded. *Eucalyptus nitens* crops are used in Portugal mainly by the pulp and paper industries, which produce substantial bark residues with no add-value use. They can, however, be an interesting source of triterpenic compounds. In this study, the potential anticancer effect of a lipophilic crude extract (CE) of *E. nitens*, a fraction of this more enriched in triterpenoids ($F_2$), as well as their main isolated compounds, betulinic acid (BiA) and betulonic acid (BoA), was studied in the colorectal cancer cells. All test extracts/compounds showed potent anticancer effects based on cell viability, colony forming and migration assays. The $F_2$ extract was shown to be two times more potent than the CE ($IC_{50s}$ of 1.3 μg/ml and 2.2 μg/ml, respectively), whereas BoA was about four times more potent than BiA ($IC_{50s}$ of 0.8 μM and 3.9 μM, respectively). The anticancer effects of the extracts/compounds were shown to be dependent both on inhibition of cell proliferation, as shown by the induction of cell cycle arrest assessed by flow cytometry, and on induction of cell death, as measures by the PI staining. At the higher concentrations tested, apoptosis was a contributor to the cell death. Interestingly, contrarily to their $IC_{50s}$, BiA was a more potent inducer of apoptosis than BoA. Apoptosis was triggered by the intrinsic mitochondria pathway, probably through JNK activation but not through p53, since its levels were remarkably decreased by all the extracts/compounds. At lower doses of *E. nitens* extracts and tested triterpenoids, a non-apoptotic cell death was present, which could be mediated through a metabolic crisis, due to the significant activation of the AMPK energy-sensing regulator. This work shows the potential use of the wasted bark of *E. nitens* as an interesting source of potent natural anticancer triterpenoids against colorectal cancer cells.

## Potencial Anticancerígeno da Fração Triterpénica de Extratos da Casca de Eucalipto em Células do Carcinoma Colorectal


### RESUMO

O cancro é uma das maiores causas de morte mundial, sendo o cancro colorectal o terceiro tipo de cancro com maior ocorrência nos países desenvolvidos. A procura de compostos anticancerígenos naturais promissores está a aumentar exponencialmente, com a exploração de novas fontes que eram desconsideradas. Em Portugal as plantações de eucalipto são sobretudo utilizadas pelas indústrias de polpa e papel, e uma vez que a casca desta árvore não é utilizada no processo, produzem-se quantidades substanciais de resíduos que não são utilizados para fins com alto valor económico. Estes resíduos podem, no entanto, ser uma fonte interessante de compostos triterpénicos. Neste estudo, o potencial efeito anticancerígeno de extratos lipofílicos de *Eucalyptus nitens*, um bruto (CE) e um fraccionado ($F_2$) mais enriquecido em triterpenóides, bem como os seus compostos principais ácido betulínico (BiA) e ácido betulónico (BoA), foram estudados nas células HCT116 do carcinoma colorectal. Todos os extratos/compostos testados demonstraram possuir efeitos anticancerígenos potentes, tal como observado nos ensaios de viabilidade celular, de formação de colónias e de migração celular. Foi demonstrado que o extrato $F_2$ é duas vezes mais potente que o CE ($IC_{50s}$ de 1.3 μg/ml e 2.2 μg/ml, respectivamente), enquanto que o BoA foi cerca de quatro vezes mais potente que o BiA ($IC_{50s}$ de 0.8 μM e 3.9 μM, respectivamente). Também se verificou que os efeitos anticancerígenos dos extratos/compostos são dependentes quer da inibição da proliferação celular, como demonstrado pela indução de interrupção no ciclo celular avaliado por citometria de fluxo, quer da indução da morte celular, medido pela marcação por iodeto de propídio. Nas concentrações mais altas testadas, houve uma contribuição da apoptose para a morte celular encontrada. Contrariamente aos valores de $IC_{50s}$, o BiA foi um indutor de apoptose mais potente que o BoA. A apoptose foi desencadeada pela via de sinalização intrínseca mitocondrial, provavelmente através da ativação da via JNK mas não através do p53, visto que os seus níveis foram marcadamente diminuídos pelos extratos/compostos. A doses mais baixas dos extratos de *E. nitens* e dos triterpenóides testados, também ocorreu morte celular mas de uma forma independente de apoptose, a qual pode ter sido mediada por uma crise metabólica, em virtude da ativação significativa observada da via AMPK. Os resultados deste trabalho sugerem uma potencial utilização valorizada da casca de *E. nitens* como uma fonte de potentes triterpenóides naturais com atividade anticancerígena contra células do carcinoma colorectal.

# INDEX

## ABBREVIATIONS

| | |
|---|---|
| **5-FU** | 5-Fluorouracil |
| **AIF** | Apoptosis-inducing factor |
| **Akt** | Protein kinase B |
| **AMPK** | 5' adenosine monophosphate-activated protein kinase |
| **ANOVA** | Analysis of variance |
| **Apaf-1** | Apoptotic protease activating factor 1 |
| **APC** | Antigen-presenting cell |
| **$\beta$-TrcP** | Beta-transducin repeat containing E3 ubiquitin protein ligase |
| **BAD** | Bcl-2-associated death promoter |
| **BAK** | Bcl-2 homologous antagonist killer |
| **BAX** | Bcl-2-associated protein |
| **Bcl-2** | B-cell lymphoma 2 |
| **Bcl-Xl** | B-cell lymphoma-extra large |
| **BH3** | Bcl-2 homology 3 |
| **BiA** | Betulinic acid |
| **Bid** | BH3 interacting-domain death agonist |
| **Bik** | Bcl-2-interacting killer |
| **BoA** | Betulonic acid |
| **BRAF** | V-raf murine sarcoma viral oncogene homolog B1 |
| **BSA** | Bovine serum albumin |
| **CD95** | Cluster of differentiation 95 |
| **CE** | Crude extract |
| **COX2** | Cyclooxygenase |
| **CRC** | Colorectal cancer |
| **DCC** | Deleted in colorectal cancer |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DR** | Death receptor |
| **ERK** | Extracellular-signal-regulated kinase |
| **$F_2$** | Fraction 2 |

| | |
|---|---|
| **FADD** | Fas-associated protein with death domain |
| **FAP** | Familial adenomatous polyposis |
| **FBS** | Fetal bovine serum |
| **HDM2** | Human double minute 2 homolog |
| **HIV** | Human immunodeficiency virus |
| **HNPCC** | Hereditary nonpolyposis colorectal cancer |
| **IAP** | Inhibitor of apoptosis |
| **JNK** | c-Jun N-terminal kinase |
| **KRAS** | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| **LT** | Lupane triterpenoids |
| **MAPK** | Mitogen-activated protein kinase |
| **MAPKK** | MAPK kinase |
| **MAPKKK** | MAPKK kinase |
| **MCC** | Mutated in colorectal cancer |
| **MMP** | Mitochondrial membrane permeabilization |
| **NF-kB** | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| **Noxa** | Phorbol-12-myristate-13-acetate-induced protein 1 |
| **OA** | Oleanolic acid |
| **OMM** | Outer mitochondrial membrane |
| **p53** | Protein 53 |
| **PARP1** | Poly (ADP-ribose) polymerase 1 |
| **PBS** | Phosphate-buffered saline |
| **PI** | Propidium iodide |
| **PI3K** | Phosphoinositide 3-kinase |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **PTEN** | Phosphatase and tensin homolog |
| **Puma** | p53 upregulated modulator of apoptosis |
| **RIPA** | Radioimmunoprecipitation assay |
| **RPMI** | Roswell park memorial institute medium |
| **RTK** | Receptor tyrosine kinase |
| **SAPK** | Stress activated protein kinases |
| **SDS** | Sodium dodecyl sulfate |

| | |
|---|---|
| **SEM** | Standard error of the mean |
| **SMAC** | IAP-binding mitochondrial protein |
| **SMAD4** | SMAD family member 4 |
| **TGFβ-RII** | Transforming growth factor beta receptor II |
| **TRAIL** | TNF-related apoptosis-inducing ligand |
| **TREN** | Triterpenic-enriched *E. nitens* extracts |
| **UA** | Ursolic acid |

## LIST OF FIGURES AND TABLES

# INTRODUCTION

## 1. CANCER

Cancer is one of the leading causes of death in the world. In 2005, the global cancer incidence was of 11 million with over than 7.6 million of deaths, and it is prospected that by 2030 the incidence grows to 15.5 million with 11.5 million of deaths (Amin *et al.*, 2009).

Although being a very common disease, cancer is mainly preventable (Amin *et al.*, 2009). Only 5 to 10% of all cancers are completely hereditary, while several external and environmental factors are known to increase significantly the risk of this disease, such as smoking, poor diet (fatty foods and alcohol), exposure to radiation, obesity, excessive sunlight exposure, viruses, environmental pollutants and certain infections (Anand *et al.*, 2008).

Cancer is a complex disease that can develop in human beings over a number of years (Dzubak *et al.*, 2005). In the development of cancer a normal cell is transformed in a cancer cell through a process called carcinogenesis. During this process it is necessary that the genes regulating cell differentiation and growth undergo failure or alteration (Dzubak *et al.*, 2005). These genes are divided into oncogenes, which are responsible for the promotion of cell growth and proliferation; and into tumour suppressor genes, like p53, that inhibit cell division and survival. The carcinogenic process is characterized by three phases of development, which ultimately leads to cancer formation and growth. These steps are initiation/mutation, promotion and progression/metastasis. The normal cell suffers damage, leading to mutation of DNA which no longer undergoes DNA repair or undergoes defective DNA repair resulting in unrestrained proliferation (initiation) (Dzubak *et al.*, 2005). The continuous proliferation facilitates the occurrence of even more mutations originating a mass of cells that no longer have their normal abilities and only proliferate (promotion). The growth of the tumour is aided by angiogenesis, which not only provides nutritients to the tumour, but also allows cancerous cells to migrate to other tissues via the circulatory system, metastasizing the tumour (progression), which normally is lethal. The most noticeable change in these cells is their ability to avoid programmed cell death (apoptosis), leading to the tumour growth (Dzubak *et al.*, 2005). Besides the resistance to apoptosis, sustainable cell proliferation without stimulating signals, promotion of angiogenesis as well as the ability to tissue invasion and establishment of metastasis, other characteristics ("hallmarks") of cancer include evasion to growth suppressors, limitless proliferative potential, local promotion of inflammation, genome instability and mutation, evasion of immune system

and deregulation of metabolic pathways (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Chemotherapy is a standard treatment to cancer, especially after surgical tumour removal (Saxena *et al.*, 2006; Drag *et al.*, 2009). Considering that chemotherapy's main aim is to eliminate the remaining cancer cells present in the organism, most of the times the success of this treatment determines the final success of the recovery (Drag *et al.*, 2009). The efficacy of this treatment has improved greatly in the last decade, but the treatment for this disease still faces a very high mortality rate (Santos *et al.*, 2011). Also, the toxic adverse effects of the used drugs lead to severe life quality threats and 80% of the patients undergoing this treatment die due to the resistance of cancer cells to drugs. Therefore, the development of new potent, non-toxic and non-resistant anticancer agents is crucial to more effective therapies (Fulda and Debatin, 2000; Jung *et al.*, 2007; Drag *et al.*, 2009; Santos *et al.*, 2011).

## 1.1. COLORECTAL CANCER (CRC)

Colorectal cancer (CRC) is the third most occurring cancer (8.5% of all cancers (Jung *et al.*, 2007)) in the world and one of the leading causes of death in the developed countries (with 65.500 deaths worldwide (Rajendran *et al.*, 2008)), being uncommon in most non-developed countries (Jung *et al.*, 2007; Rajendran *et al.*; 2008; Xavier *et al.*, 2009a; Chintharlapalli *et al.*, 2011). Therefore, the incidence rates of CRC are highly variable in different regions of the world and the differences of the occurrence of this disease in migrants suggests that environmental factors and diet play a major role in the development of this cancer (Chintharlapalli *et al.*, 2011).

CRC can be divided into sporadic, familial and inherited. The sporadic represents 50 to 60% of CRC and there is no evidence of the disease in family history, being more common in individuals with age over 50. The familial account for 30 to 40% of all the cases, and normally the patient has a history of CRC in the family (Souglakos, 2007). The inherited CRC represents 4 to 6% and can be further divided depending on whether there are or not colonic polyps: familial adenomatous polyposis (FAP) on the presence of those and hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch Syndrome on their absence (Rustki, 2007).

The initial event of carcinogenesis in the colon epithelium is mostly the accumulation of a genetic alteration in the antigen-presenting cell (APC) gene, which is part of the Wnt signalling pathway (Fig.1). This mutation disables the production of the APC protein leading to accumulation of the β-catenin protein, which translocate to the nucleus, leading to the activation of transcription of genes that, at high levels, cause cancer (Souglakos, 2007). However, mutation in this pathway are not the only ones necessary for the cell to become cancerous; other mutations must take place, such as in the TP53 gene that is responsible for killing the cell if there is any defect in the Wnt pathway. Genetic alteration in the transforming growth factor $\beta$ receptor II (TGF$\beta$-RII), phosphatase and tensin homologue (PTEN), cyclooxygenase 2 (COX2), deleted in colorectal cancer (DCC), mutated in colorectal cancer (MCC), SMAD4, phosphoinositide 3-kinase (PI3K), v-raf murine sarcoma viral oncogene homolog 1 (BRAF), v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) and Bcl-2-associated x protein (BAX) genes have also been found to play a role in CRC (Takami *et al.*, 1995; Soreide *et al.*, 2006; Souglakos, 2007).

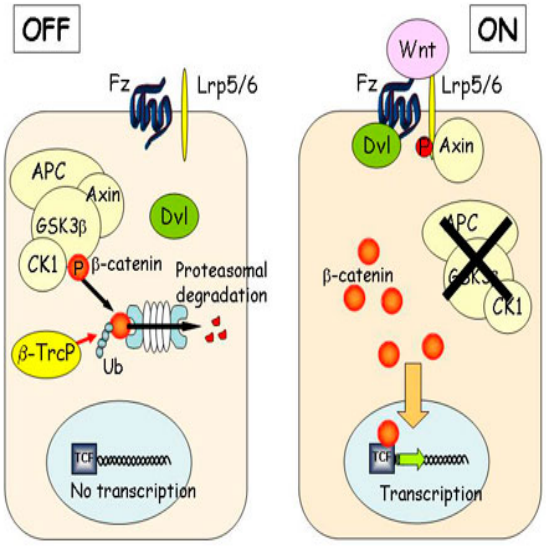


**Figure 1** – APC/β-catenin signalling in CRC. (OFF) The APC protein is synthesised, β-catenin is phosphorylated and occurs ubiquitin-mediated degradation of β-catenin by the beta-transducin repeat containing E3 ubiquitin protein ligase ($\beta$-Trc)P. (ON) The APC protein is not produced, occurring accumulation of β-catenin which enters the nucleus, enabling transcription. [http://www.umcutrecht.nl]

Complete remission of CRC by sirurgical removal is possible. Nevertheless, approximately 50% of the cases eventually develops incurable metastesis, normally in the liver, even when coupled with adjuvant treatment (Jung *et al.*, 2007; Rajendran *et al.*, 2008). Therapies for gastrointestinal cancers have many defficiencies since the general response to treatment is low to moderate, the life extension is, on most cases, only of two to three months, there is a high toxicity to normal cells, high percentages of relapse cases and multiple drug resistances observed (Rajendran *et al.*, 2008). Chemotherapy based on 5-fluorouracil (5-FU) is the standard treatment over the last forty years to extend patients survival when the CRC is

metastasized. However, the response rate of CRC metastasis to 5-FU is only of approximately 20% mainly due to cancer cell resistance to this drug (Jung *et al.*, 2007; Rajendran *et al.*, 2008; Xavier *et al.*, 2011). To overcome this resistance two new drugs were developed, Irinotecan and Oxalipatin, that, combined with 5-FU, are able to overcome some of the drug resistance, increasing the survival of the patients (Rajendran *et al.*, 2008; Xavier *et al.*, 2011). Nevertheless, this improvement of efficiency is not observed in all patients (Xavier *et al.*, 2011).

## 2. CANCER CELL SIGNALING

The process of carcinogenesis encompasses a series of genetic mutations that leads to modifications in very important biological pathways related with cell proliferation, survival and cell death, leading to dramatic changes in the cell.

Some of them that are frequently activated in CRC are the MAPK/ERK and PI3K/Akt signalling pathways (Fig.2) which also determine treatment response. These pathways are usually activated by mutations either on KRAS or BRAF genes. Mutation on KRAS gene can lead to the activation of MAPK/ERK and/or PI3K/Akt pathways, whereas mutations on BRAF gene activates MAPK/ERK pathway only. These mutations are found in over 50% of CRC cases and occur alternately with 32% of cases being due to KRAS gene mutation and 14% due to BRAF gene mutation (Xavier *et al.*, 2009a; Xavier *et al.*, 2009b; Xavier *et al.*, 2012).

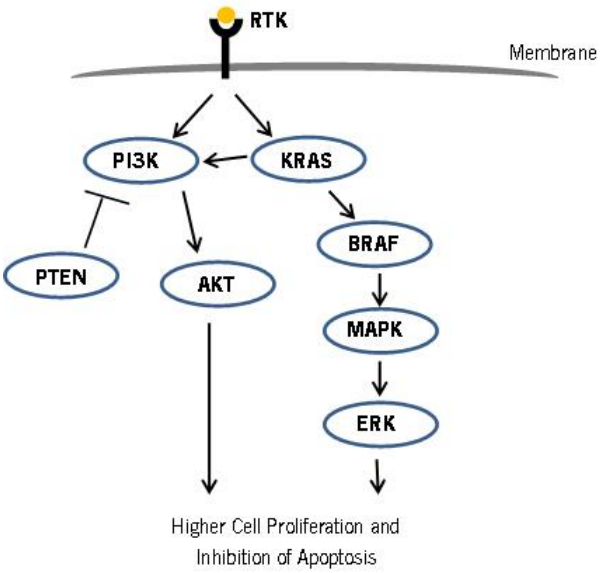


**Figure 2** – Schematic representation of MAPK/ERK and PI3K/Akt pathways in relation with cell proliferation and apoptosis. RTK – receptor tyrosine kinases

## 2.1 MAP KINASE PATHWAYS

Mitogen-activated protein kinases (MAPK) are a family of ubiquitous proline-directed, protein-serine/threonine kinases, which participate in signal transduction pathways that control several intracellular events (Pearson *et al.*, 2001), including embryogenesis, inflammatory response, cell differentiation, cell proliferation and cell death (Chen *et al.*, 2001). They help mediate diverse processes from transcription of proto-oncogenes to programmed cell death (Cobb, 1999). The three major and best-known subfamilies of MAPKs are the extracellular-signal-regulated kinase (ERK) 1/2, which conveys growth signals from the RAS/RAF pathway and receptor kinases, and the c-Jun N-terminal kinase (JNK) 1/2/3 and the p38 MAP kinase (p38) $\alpha/\beta/\gamma/\delta$, which relay various stress signals.

The MAPK are catalytically inactive in their base form, and require, in order to become active, phosphorylation events in their activation loops. The activity is regulated by a cascade of activations where MAPKs are phosphorylated by the MAPK kinase (MAPKK), which is, in turn, phosphorylated by MAPKK kinase (MAPKKK) (Cobb, 1999; Chen *et al.*, 2001; Pearson *et al.*, 2001; Cuevas *et al.*, 2007). The MAPKKK are not specific to one MAPKK, being able to regulate multiples MAPKKs, and leading to the activation of different subfamilies of MAPK, like the mentioned ERK, JNK and p38 (Cuevas *et al.*, 2007).

### 2.1.1. ERK PATHWAY

The MAPK/RAF/ERK pathway is one of the most important signalling networks that control proliferation, differentiation and cell survival (Kolch, 2000), and this cascade is activated by a variety of receptors involved in growth and differentiation (McCubrey *et al.*, 2006). When ERK is improperly activated it contributes to malignant transformation.

Raf is a serine/threonine protein that can be found in three forms: a-Raf, b-Raf and c-Raf, that are recruited to the membrane, bind to Ras, being

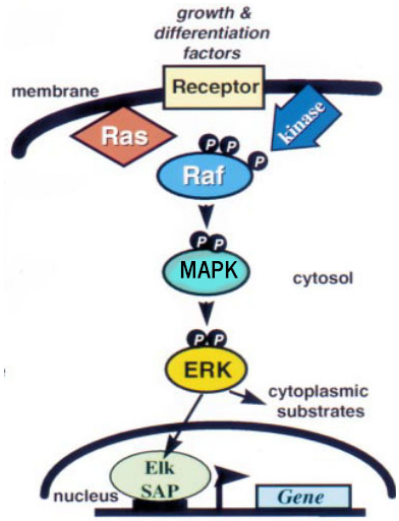


**Figure 3** – Schematic representation of the ERK pathway. [Image from Kolch, 2000]

subjected to phosphorylations and dephosphorylations promoting the activation of Raf. After Ras activation, a MAPKKK phosphorylates two MAPKK proteins, MAPK 1 and 2, which activates the MAPK ERK 1 and 2 (Fig.3). The activated ERK acts on cytosol by activating NF-kB, and on the nucleus by promoting the phosphorylation of several transcription factors.

### 2.1.2. JNK AND P38 PATHWAYS

JNK and p38 are protein kinases (also called stress-activated protein kinases – SAPKs) that are activated by environmental stresses, inflammatory cytokines, growth factors, mitogens, oncogenes and inducers of cell differentiation and morphogenesis (Bogoyevitch *et al.*, 2010). These two pathways are frequently deregulated in cancer, including in CRC (Xavier *et al.*, 2012). They function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival and migration (Wagner and Nebreda, 2009).

The JNK protein kinase play a critical role in the extrinsic as well as in the intrinsic (mitochondrial) apoptotic pathways, by upregulating pro-apoptotic genes (Dhanasekaram and Reddy, 2011; Xavier *et al.*, 2013). They are encoded by three genes: JNK1, JNK2 and JNK3. The transcripts of these genes are alternatively spliced to yield four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms. JNK1 and JNK2 are products of alternative splicing of a single gene and so are expressed in several tissues, while JNK3 is specifically expressed in the brain (Wagner and Nebreda, 2009). The three JNKs play a vital regulatory role in the responses to several stresses, neural development, inflammation and apoptosis (Dhanasekaram and Reddy, 2011).

The p38 MAPK subfamily is involved in cell differentiation, cell migration inhibition of cell cycle progression, apoptosis and autophagy (Zarubin and Han, 2005; Ashwell et al., 2006). It has been identified four p38 MAPK: p38-$\alpha$, p38-$\beta$, p38-$\gamma$ and p38-$\delta$, which are encoded by MAPK14, MAPK11, MAPK12 and MAPK13, respectively. The p38-$\alpha$ is the most abundant one, being present in most cell types (Wagner and Nebreda, 2009).

## 2.2. APOPTOSIS

Apoptosis, which in Greek literally means "falling away", is an intrinsic programmed cell death process highly conserved in different species, that occurs in multicellular organisms as a natural and organized process, which plays an important role in the embryonic development and in the balance of human tissues by adjusting the involved physiologic processes (Fulda, 2008; Fulda and Kroemer, 2009; Wu *et al.*, 2010; Yadav *et al.*, 2010). Through apoptosis, the organism is able to maintain homeostasis, by the elimination of damaged or unnecessary cells without local inflammation due to leakage of cells' contents. Therefore, any cell that has abnormalities such as DNA damage, oncogene activation, nutrition deficiency or hypoxia, can be eliminated without damage to the surrounding cells (Yadav *et al.*, 2010). Apoptosis is characterized by distinct morphological characteristics such as cellular shrinkage, chromatin condensation, plasmatic membrane blebbing, oligonucleosomal DNA fragmentation and collapse of the cell in smaller units (formation of apoptotic bodies) (Liu *et al.*, 2004; Santos *et al.*, 2011).

Since apoptosis is involved in the regulation of many physiological processes, a deficient apoptosis signalling may contribute to a variety of different pathological conditions (Fulda and Kroemer, 2009). Cancer is one of such pathologies, since cancer cells are able to escape apoptosis, allowing tumours to grow rapid and uncontrollably (Yadav *et al.*, 2010).

There are two different pathways through which apoptosis can be initiated: the intrinsic pathway that involves the mitochondria and the extrinsic pathway that involves the plasmatic membrane via death receptors (DR), both of them inducing activation of proteolytic enzymes called cysteine aspartic acid specific proteases (caspases) (Fulda, 2008; Fulda and Kroemer, 2009).

### 2.2.1. THE INTRINSIC MITOCHONDRIAL PATHWAY

The intrinsic pathway (Fig.4) can be activated by different stress stimuli, such as heat shock, ultraviolet radiation or DNA damage. The permeabilization of the mitochondrial membranes is frequently the decisive event that delimits the boundary between survival and death (Fulda *et al.*, 1997; Fulda and Debatin, 2000; Liu *et al.*, 2004; Fulda and Kroemer, 2009). The B-cell lymphoma 2 (Bcl-2) family proteins are regulators of apoptosis and comprise both anti-

apoptotic members, such as Bcl-2, Bcl-XI and McH that inhibits the permeabilization of the mitochondrial membrane, and pro-apoptotic member, such as BAX, BAK, BAD, Bik, Bid, Puma, Noxa and BH3 that permeabilize the mitochondrial membrane (Fulda *et al.*, 1997; Yadav *et al.*, 2010). Bax and Bak proteins can be upregulated by the tumour suppressor protein p53 (Yadav *et al.*, 2010), be inserted in the outer mitochondrial membrane (OMM), permeabilizing it and causing the release of cytochrome c, apoptosis inductor factor (AIF) and second mitochondria derived activator of caspases (SMACs) (Fulda and Debatin, 2000; Dzubak *et al.*, 2005; Fulda, 2008; Fulda and Kroemer, 2009; Yadav *et al.*, 2010). Imbalances in the ratio of the anti-apoptotic versus pro-apoptotic Bcl-2 proteins regulate the permeabilization of the OMM (Fulda *et al.*, 1997; Rzeski *et al.*, 2006; Fulda and Kroemer, 2009; Wu *et al.*, 2010).

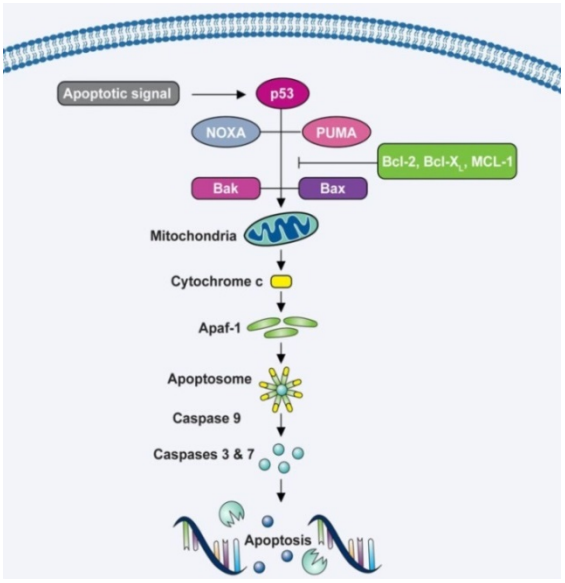


**Figure 4** – Scheme of the Intrinsic Mitochondrial Pathway, with release of cytochrome c. [Image from http://www.biooncology.com]

After its release, cytochrome c interacts with the apoptotic protease activating factor-1 (Apaf-1) and recruits pro-caspase-9 to stimulate the formation of the apoptosome, which will lead to the cleavage and activation of caspases 9 that will mediate the activation of caspases 3, 6 and 7 to promote apoptosis (Fig.4). The SMAC promotes activation of caspases by deactivating inhibitors of apoptosis proteins (IAPs), allowing apoptosis to continue; and the AIF induces apoptosis without caspases involvement (Fulda, 2008; Fulda and Kroemer, 2009; Yadav *et al.*, 2010).

### 2.2.2. THE EXTRINSIC DEATH RECEPTORS PATHWAY

In the extrinsic pathway (Fig.5), apoptosis is triggered by activation of pro-apoptotic death receptors, such as CD95 or TNF-related apoptosis-inducing ligand (TRAIL) receptors through its ligands cognate. The stimulation of the DR leads to receptor trimerization, recruitment of adaptor molecules, such as fas-associated protein with death domain (FADD), and activation of caspase 8

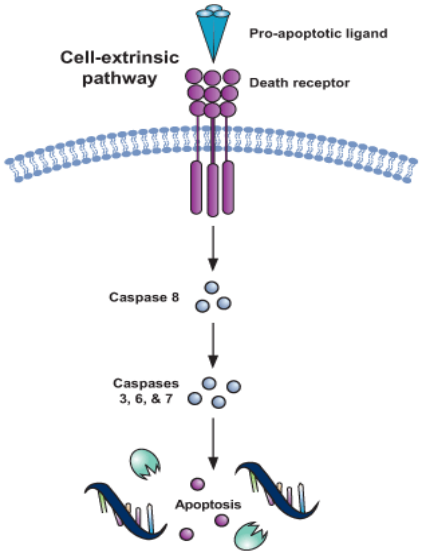


**Figure 5** – Scheme of the Extrinsic Death Receptor Pathway. [Image from http://www.biooncology.com]

initiator, which spreads the death signal to effector caspases like caspases 3, 6 and 7 (Fulda, 2008; Yadav *et al.*, 2010).

At this point, both intrinsic and extrinsic pathways converge with the activation of the same caspases. Also, caspase 8, through its mediated cleavage, activates the BH3 interacting-domain death agonist (Bid) which is translocated to the mitochondria to promote cytochrome c release (Fulda, 2008).

### 2.2.3. ROLE OF P53 IN APOPTOSIS

The protein 53 (p53) is a tumour suppressor protein whose activity stops the formation of tumours, being considered the "guardian of the genome". It mediates critical functions in cells, such as inhibition of proliferation by induction of cell cycle arrest and induction of apoptotic cell death. In normal cells, p53 is usually present at very low levels and it can be activated in response to stress, like hypoxia, heat shock and DNA damage agents. Its stability is regulated by the E3 ubiquitin ligase and human double minute 2 homolog (HDM2), which mediates the ubiquitination of p53 and allows its degradation by the proteosome, maintaining the p53 levels low (Chari *et al.*, 2009).

When activated, p53 translocates to the nucleus where it binds to specific DNA sequences elements within the regulatory regions of target gene promoters regulating the transcription of several genes related with apoptosis and/or cell cycle (Chari *et al.*, 2009). Furthermore, this protein can also activate the apoptotic mechanism through interaction with the members of the anti- and pro-apoptotic Bcl-2 family proteins to induce the intrinsic mitochondrial pathway (Vaseva and Moll, 2009).

## 3. *EUCALYPTUS* SPP.

*Eucalyptus* spp., from the family Myrtaceae, are evergreen flowering trees (Russell *et al.*, 1997). This genus consists of approximately 600 species and has a physiological plasticity (it supports tropical and temperate climes) that allows some species to propagate in different regions of the world (Moura *et al.*, 2012). In spite of this, only fifteen species occur outside Australia, and only nine do not occur there, thus eucalyptus is mostly native to Australia. The eucalyptus tree has high value mainly because it is a major source of cellulose for paper manufacture, being the most common short fibre source for pulpwood in Portugal, Spain, Brazil, Chile, South Africa, Japan, among other countries (Pereira *et al.*, 2005; Domingues *et al.*, 2010; Domingues *et al.*, 2011b; Moura *et al.*, 2012). It is also very sought for its essential oil, which, despite being highly toxic if ingested pure, is a good antiseptic, anti-malarial, industrial solvent and insect repellent as well as used as additive for food industry (Russell *et al.*, 1997; Pereira *et al.*, 2005; Moura *et al.*, 2012). Eucalyptuses are also valuable for apiculture and the resulting honey being studied for their antibacterial activity (Irish *et al.*, 2011). Furthermore, the lipophilic extracts from the bark of some eucalyptus (namely *Eucalyptus globulus*, *Eucalyptus camaldulensis* var. *obtusa*, *Eucalyptus grandis*, *Eucalyptus maidenii*, *Eucalyptus urograndis* and *Eucalyptus nitens*) are profoundly rich in triterpenic compounds, specially triterpenic acids like oleanolic, 3-acetyloleanolic, ursolic, 3-acetyl-ursolic, betulinic, 3-acetylbetulinic and betulonic acids, that are valuable bioactive compounds (Siddiqui *et al.*, 2000; Kibblewhite *et al.*, 2001; Pereira *et al.*, 2005; Domingues *et al.*, 2010; Domingues *et al.*, 2011a;Domingues *et al.*, 2011b; Santos *et al.*, 2011b; Domingues *et al.*, 2012).

### 3.1. *EUCALYPTUS NITENS*

*Eucalyptus nitens* (Fig. 6) is a species of fast growth and great development, with a natural distribution in small and normally isolated populations. It is a tall to very tall forest tree, normally with heights between 40 to 70 meters being able to reach 90 meters and diameter of 1 to 2 meters. Its juvenile leaves are smooth, orbicular and very fragrant; adult leaves are alternate, long, curved, flexible and they set up a high silvery crown when grown in plantations (Tibbits *et al.*, 1997). *Eucalyptus nitens* is also known as Shining Gum, since *nitens* in Latin means shining/bright and this refers to their leaves, buds, bark and fruits that have a distinct glossy.

*Eucalyptus nitens* is mostly planted for industrial purposes, given that most of their crops are used to the pulp and paper industry (Domingues *et al.*, 2011) due to its final pulp properties (Domingues *et al.*, 2011a). The most meaningful plantations are in Chile (45%), Australia (30%) and South Africa (17%) and can be also found in New Zealand, Northern Spain and Portugal. This species is normally planted in unsuitable sites for *E. globulus* (the leading eucalyptus planted for the pulp and paper industry), because it can tolerate a climate much colder and also grows very well at high altitudes, two characteristics that *E. globulus* does not have (Tibbits *et al.*, 1997).

**Figure 6** – *E. nitens* in an industrial plantation in Australia. [Adapted from CSIRO Forestry and Forest Products]

It has been reported that the outer bark of *E. nitens* contains significant quantities of triterpenoids and acetyl derivatives, like ursolic, 3-acetylursolic, betulinic, oleanolic, 3-acetyloleanolic and betulonic acids (Domingues *et al.*, 2011a; Domingues *et al.*, 2012).

## 3.2. EUCALYPTUS PULP RESIDUES EXPLOITATION

There is a growing interest of performing an integrated exploitation of plant biomass as a source of materials, chemicals, fuels and energy in the concept of biorefinery. This approach has attracted the interest of agro-forest industries in order to take the maximum value out of their crops. In particular to the pulp and paper industries, because it produces substantial quantities of residues, especially bark, leaves and fruits, which are simply burned to energy production or left behind, this is an example where this biorefinery concept may reach a big success (Pereira *et al.*, 2005; Domingues *et al.*, 2010; Domingues *et al.*, 2011b).

These byproducts of the pulp and paper industries may have high valuable compounds in their composition and they can be exploited without affecting the industry production and minimizing residues formation, being promising sources of bioactive natural chemicals (Domingues *et al.*, 2010; Domingues *et al.*, 2011b). So, a more profound study of these non-used byproducts can contribute to increase the economic value of crops and retrieving valuable

compounds that would be otherwise discarded (Pereira *et al.*, 2005; Domingues *et al.*, 2011b; Santos *et al.*, 2011b). The exploitation of high value low molecular mass compounds, such as phytosterols (namely $\beta$-sitosterol), lignans and triterpenoids, from industrial byproducts is a strategy already implemented in some pulp industries (Domingues *et al.*, 2010), as some of these compounds, specially triterpenoids, can be of great interest as anticancer agents.

The bark of eucalyptus species is among the residues with most interest (Domingues *et al.*, 2011b). The bark of different eucalyptus residues left behind by pulp and paper industries contains very valuable compounds, namely triterpenoids with great biological activities. The compounds extracted from these different species are basically the same, although with different proportions. So, *E. camaldulensis* var. *obtusa*, *E. nitens*, *E. globulus*, *E. maidenii*, *E. urograndis* and *E. grandis* are highly rich in triterpenic acids with lupane, ursane and oleane skeletons namely ursolic, 3-acetylursolic, oleanolic, 3-acetyloleanolic, betulinic and betulonic acids (Kibblewhite *et al.*, 2000; Siddiqui *et al.*, 2000; Pereira *et al.*, 2005; Domingues *et al.*, 2010; Domingues *et al.*, 2011a; Domingues *et al.*, 2011b; Santos *et al.*, 2011). Besides triterpenic compounds, it can also be found small quantities of fatty acids, fatty alcohols and aromatic compounds (Domingues *et al.*, 2011b).

## 4. NATURAL COMPOUNDS

Traditional medicine and diet have been serving humanity throughout the centuries as a means of preventing and treating most of chronic diseases (Fulda, 2008; Fulda and Kroemer, 2009; Yadav *et al.*, 2010; Yi *et al.*, 2010). Natural compounds have been extensively used in the treatment of multiple diseases and are of great value for the scientific community, either in its natural form or as models to synthetic modification (Rajendran *et al.*, 2008; Yadav *et al.*, 2010). These compounds used nowadays in modern medicine exhibit a great chemical diversity and, along with its analogues and many others natural products, show the great importance of these compounds in the efforts of discovering new drugs (Fulda, 2008; Fulda and Kroemer, 2009; Yadav *et al.*, 2010; Yi *et al.*, 2010). 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. Furthermore, they have high yield, easy obtainment, favourable physiological functions, antioxidant activity and are generally accepted as dietetic

supplements (Amin *et al.*, 2009; Yadav *et al.*, 2010; Yi *et al.*, 2010), being considered a fascinating strategy as therapeutic agents (Bishayee *et al.*, 2011). Over the last decade, many bioactive compounds have been identified in plants and human diet and are being developed as chemopreventive agents to inhibit, slow down or even reverse the progression of several cancers (Amin *et al.*, 2009; Yadav *et al.*, 2010; Bishayee *et al.*, 2011). In fact, the great majority of anticancer agents are derived from natural compounds or their analogues (Fulda, 2008; Fulda and Kroemer, 2009; Santos *et al.*, 2011). The anticancer activity of natural compounds has been partially explained by its ability in triggering cellular death pathways, including apoptosis (Fulda and Kroemer, 2009).

## 4.1. TERPENIC COMPOUNDS

One of the groups of natural compounds are terpenes (or terpenoids or isoprenoides) that can be divided, according with the number of structural molecules of isoprenes, into mono-, sesqui-, di-, sester-, tri-, tetra- and poly-terpenes which create extensive isoprenoides groups. With almost 40.000 different terpenes isolated from plants, animals and microbial species they are widely found in Nature. They are the biggest natural compound group found in plants (Dzubak *et al.*, 2005; Bishayee *et al.*, 2011). Terpenes and its metabolites play a very important role in plants defence mechanisms; they protect plants from the constitutive and induced defensive responses against insects and environmental stress (Yadav *et al.*, 2010). Among terpenes, the triterpenes have recently emerged as an unique group of phytochemicals with multi-functional activities (Bishayee *et al.*, 2011).

### 4.1.1. TRITERPENIC COMPOUNDS

Triterpenes, or triterpenoids, are isopentenyl pyrophosphate oligomers metabolites that are chemically related to squalene, which is a big group of compounds that have thirty carbon atoms arranged in five rings with several oxygen atoms connected. Triterpenes are part of the biggest plants compounds group, the Saponins (Yadav *et al.*, 2010; Bishayee *et al.*, 2011). It is estimated that there are over 20.000 triterpenes in Nature, and they are predominantly found in plants and fruits, being considered one of the most important natural compounds' class (Dzubak

*et al.*, 2005; Kommera *et al.*, 2011; Bishayee *et al.*, 2011; Csuk *et al.*, 2011). Thousands of new structures have been described with hundreds of new derivatives being found each year (Cichewicz and Kouzi, 2004). The variability of triterpenes in Nature is the result of the evolution of a big family of terpene synthase (Yadav *et al.*, 2010). The triterpenes can be subclassified in diverse groups including the cucurbitanes, cycloartanes, dammaranes, euphanes, friedelanes, holostanes, hopanes, isomalabaricanes, lanostanes, limonoids, lupanes, oleananes, protostanes, squalenes, tirucallanes, ursanes and other compounds (Bishayee *et al.*, 2011). In a biological perspective the most important triterpenoids structures are the oleananes, ursanes, lupanes, dammaranes and euphanes (Dzubak *et al.*, 2005), emphasizing the lupane group that are recognized as promising compounds for the development of new bioactive agents (Cichewicz and Kouzi, 2004; Kommera *et al.*, 2011; Domingues *et al.*, 2011b).

Even though for a long time the triterpenes were considered biologically inactive, the accumulating evidences of its wide pharmacological activities spectrum along with its low toxicity aroused a new interest about its potential (Bishayee *et al.*, 2011). The triterpenes, widely used in medicine in Asian countries, have a range of unique biological effects and have been studied for its anti-inflammatory, hepatoprotector, analgesic, antimicrobial, antimicotic, virostatic, immunomodulatory, tonic, anticancer, antiangiogenesis, analgesic, antipyretic, antioxidant, antiallergic, spasmolytic, anti-HIV and antimalarial effects (Dzubak *et al.*, 2005; Kommera *et al.*, 2011; Yadav *et al.*, 2010; Bishayee *et al.*, 2011). Therefore, they can be considered a promising and expanding platform to biological active natural compounds whose potential is only partially explored by the pharmaceutical industry.

#### 4.1.1.1. BETULINIC ACID

Betulinic acid (BiA), 3β,hydroxy-lup-20(29)-en-28-oic acid (Fig.7) is part of the lupane group, a pentacyclic triterpene, and it can be found in several plants throughout the world like in *Ziziphus* spp., *Amenone* spp., *Lycopodium* spp., *Syzygium* spp., *Betula* spp., *Tryphyllum* spp., *Ancistrocladus* spp., *Eucalyptus* spp., *Diopsyros* spp., *Paeonia* spp. and *Tetracera* spp.,

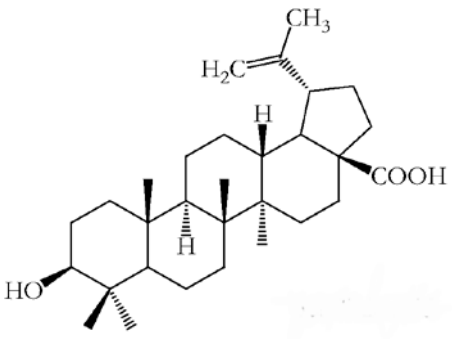


**Figure 7** – Chemical structure of betulinic acid.

being highly available in the white-barked birch tree (from the genus *Betula*) (Cjowdhury *et al.*, 2002; Amin *et al.*, 2009; Drag *et al.*, 2009; Kommera *et al.*, 2010a; Kim *et al.*, 2011; Soica *et al.*, 2011). Betulinic acid 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 (Cichewicz and Kouzi, 2004; Fulda and Kroemer, 2009). Depending on the extraction method, betulin comprehends over than 90% of the total isolated products and BiA around 1 to 5% (Drag *et al.*, 2009).

Betulinic acid, a white crystalline solid, is a bioactive compound that possess a wide range of pharmacological effects like anti-inflammatory, antimicrobial, antiplasmodial, anti-HIV, anti-diabetic, cardiovascular, anti-atherosclerosis, anti-obesity, anti-proliferative, anti-angiogenic, cytotoxic, anticancer, antiparasitic, antimalarial, anti-viral, antioxidant, hepatoprotector, anti-allergic and anti-tuberculosis effects as well as spasmogenic, anthelmintic and antinociceptive activities (Ryu *et al.*, 1994; Cichewicz and Kouzi, 2004; Dzubak *et al.*, 2005; Fulda and Kroemer, 2009; Wu *et al.*, 2010; Bishayee *et al.*, 2011). It has been postulated that many of these effects may be due to its ability to modulate immune functions, being an important immunomodulator (Yi *et al.*, 2010).

The Native Americans used the bark of white birch tree (rich in BiA) as a potent folk medicine to treat skin diseases and as an anti-inflammatory (Fulda and Kroemer, 2009). The cytotoxic activity of BiA was first described in 1976 by Trumbull and collaborators, but it was only in 1995 that Pisha *et al.* published an important paper reporting the cytotoxic effects of BiA in a human melanoma cell line, causing an increasing interest in this compound. It was first thought that this compound was cytotoxic specifically to melanoma cell lines (Fulda, 2008; Fulda and Kroemer, 2009), but since then its anticancer activity has been reported against several other human cancers, including neuroblastoma, glioblastoma, colon, breast, liver, lungs and prostate carcinomas, among others (Cichewicz and Kouzi, 2004; Ehrardt *et al.*, 2004; Fulda, 2008; Amin *et al.*, 2009; Eichenmuller *et al.*, 2009; Fulda and Kroemer, 2009; Chintharlapalli *et al.*, 2011).

Many mechanisms of action have been published for explaining its anticancer effect. One of them is the ability of BiA to induce cell death by apoptosis (Drag *et al.*, 2009; Eichenmuller *et al.*, 2009; Santos *et al.*, 2011), which is suggested to be mediated by the increase of mitochondrial membrane permeability, meaning through the intrinsic mitochondrial apoptosis pathway (Fulda *et al.*, 1997; Dzubak *et al.*, 2005; Fulda and Debatin, 2000; Zuco *et al.*, 2001;

Ehrhardt *et al.*, 2004; Liu *et al.*, 2004; Jung *et al.*, 2007; Fulda, 2008; Eichenmuller *et al.*, 2009; Fulda and Kroemer, 2009; Wu *et al.*, 2010; Bishayee *et al.*, 2011; Kommera *et al.*, 2011; Santos *et al.*, 2011). Betulinic acid has also been proven to be a topoisomerase I and II inhibitor (Chowdhury *et al.*, 2002; Wada and Tanaka, 2005) as well as being responsible by the downregulation of the transcription factors specificity protein 1 (Sp1), 3 (Sp3) and 4 (Sp4) (Drag *et al.*, 2009; Chintharlapalli *et al.*, 2011), which regulates the expression of a vast number of genes involved in many cellular functions from differentiation, proliferation and apoptosis (Deniaud *et al.*, 2009). An interesting and clinically important fact is that BiA has a higher effect in an environment with pH lower than 6.8, which is the pH at which almost every tumours develops (Cichewicz and Kouzi, 2004; Dzubak *et al.*, 2005).

A very important characteristic that has been reported for this compound is its ability to trigger apoptosis in cancer cells resistant to drugs, suggesting that BiA may circumvent some forms of resistance in cancer patients that show resistance to chemotherapy (Fulda and Debatin, 2000; Jung *et al.*, 2007; Drag *et al.*, 2009). Furthermore, despite its cytotoxicity against a variety of cancers, normal cells and tissues appear relatively resistant to BiA, taking up to 100 mg/kg in animal studies without showing great toxicity, which points to a very good therapeutic window (Zuco *et al.*, 2002; Dzubak *et al.*, 2005; Rzeski *et al.*, 2006; Csuk *et al.*, 2011). 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 BiA will eventually be accepted as an adjuvant therapeutic in the treatment of cancer cells. However, BiA is a highly lipophilic molecule with limited water solubility (being limitedly soluble in organic alcohols and highly soluble in pyridine and acetic acid) which can lower its in vivo uptake. So, developing specialized formulations/carriers, such as liposomes, may help to augment its in vivo efficiency as an anticancer agent (Cichewicz and Kouzi, 2004;).

Tough BiA is widely available in Nature, their sources may not be enough to a possible increase in the demand of this compounds. But, fortunately, the BiA's precursor betulin is highly available in several species of plants with significant yields over 20%, and BiA is easily prepared from betulin in a simple two-step process (Chintharlapalli *et al.*, 2011; Soica *et al.*, 2011). Also, due to its outstanding characteristics, a number of new BiA derivatives are being synthetized (Kim *et al.*, 2001; Liu *et al.*, 2004; Rajendran, 2008; Kommera *et al.*, 2010a; Nakagawa-Goto *et al.*, 2010; Kommera *et al.*, 2011; Santos *et al.*, 2011).

#### 4.1.1.2. BETULONIC ACID

Betulonic acid (BoA), 3-oxolup-20(29)-en-28-oic-acid (Fig.8), is also part of the lupane group and a pentacyclic triterpene, which can be found in *Syzigium* spp, *Eucalyptus* spp., *Prunella* spp., *Ziziphus* spp. and *Betula* spp. (Ryu *et al.*, 1994; Symon *et al.*, 2005; Domingues *et al.*, 2010; Kommera *et al.*, 2010a). Betulonic acid can also be obtained from betulinol or from betulin. The yield obtained from the extraction of BoA from betulinol is aroun 90%, whereas from betulin is only about 60% (Saxena *et al.*, 2 006).

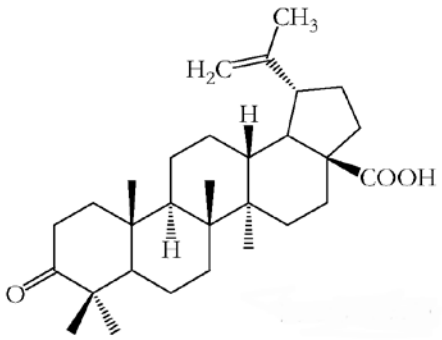


**Figure 8** – Chemical structure of betulonic acid.

Betulonic acid is a white crystalline solid, which, until the year 2000, was only focus of interest as a precursor of BiA (Melinokova *et al.*, 2012). However, it was discovered that it was by itself a very valuable bioactive compound with great pharmacological activities such as anti-viral, anticancer, anti-inflammatory, anti-malarial, anti-angiogenic, anti-HIV, cytotoxic, hepatoprotector, antioxidant, antimicrobial, and immunomodulatory (Pavlova *et al.*, 2003; Sorokina *et al.*, 2004; Sorokina *et al.*, 2006; Vabilevsky *et al.*, 2009; Melinokova *et al.*, 2012; Semenov *et al.*, 2012). In fact, it has been reported that in many of its effects, BoA is much more active and potent inhibitor than BiA, being immensely potent against several tumour cell lines (Symon *et al.*, 2005; Wada and Tanaka, 2005). Betulonic acid is also known for being a powerful topoisomerase II inhibitor (Ryu *et al.*, 1994).

As mentioned, BoA has a remarkable cytotoxic activity against cancers such as melanoma and prostate, neck and head, ovary/cervix, lung, colon, breast, thyroid and liver carcinomas (Saxena *et al.*, 2006; Shintyapina *et al.*, 2007; Kommera *et al.*, 2010a).

Due to its very low solubility in aqueous medium (being only soluble in organic solvents), it has not been yet possible to determine in all its extent BoA's powerful activity (Saxena *et al.*, 2006). Also because of this, there is not yet literature that clarifies exactly the mechanisms of action of the anticancer activity of BoA, but it has been reported that it may up-regulate p53 activity (Zhang *et al.*, 2008). Also, it has been reported that BoA may be a powerful agent that

improves cytostatic effects of drugs (as a bioregulator to decrease the organism resistance to toxic cytostatic effects) (Sorokina *et al.*, 2004).

As with BiA, BoA seems to be little toxic to normal fibroblast cells (Saxena *et al.*, 2006). There has been also a number of BoA derivatives that are being synthesized (Vabilevsky *et al.*, 2009; Semenov *et al.*, 2012).

#### 4.1.1.3. URSOLIC ACID

Ursolic acid (UA), 3β-hydroxy-urs-12-en-28-oic acid, (Fig. 9) is a natural pentacyclic triterpene carboxylic acid. 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, and in eucalyptus (Liu, 1995; Domingues *et al.*, 2010).

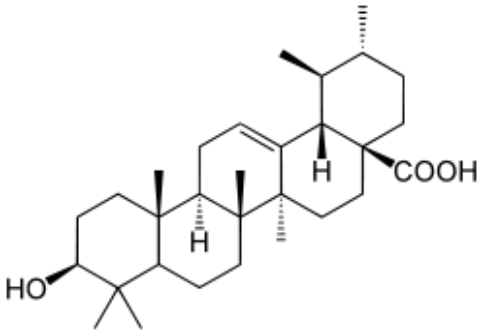


**Figure 9**– Chemical structure of ursolic acid.

UA is also a bioactive compound and has numerous effects such as anti-inflammatory, anticancer and hepatoprotective effects (Liu, 1995; Xavier *et al.*, 2009b; Xavier *et al.*, 2013), as well as it is able to provide protection against oxidative damage in DNA (Ramos *et al.*, 2008; Ramos *et al.*, 2010). It also presents low toxicity to normal cells (Xavier *et al.*, 2009b). It has been reported that UA's anticancer effect is due to its capacity of modulating important signalling pathways, such as PI3K, inhibiting cell proliferation and inducing apoptosis (Liu, 1995 Xavier *et al.*, 2009b; Xavier *et al.*, 2012).

#### 4.1.1.4. OLEANOLIC ACID

Oleanolic acid (OA), 3β-hydroxy-olea-12-en-28-oic acid, (Fig.10) is a natural occurring triterpenoids that is found in Phytolacca spp., Syzygium spp., Eucalyptus spp., in garlic and other medicinal plants (Liu, 1995; Domingues *et al.*, 2010).

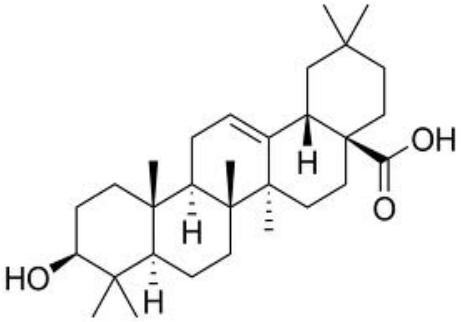


**Figure 10** – Chemical structure of oleanolic.

OA is a bioactive compound and possesses interesting effects such as hepatoprotector, anticancer, anti-HIV and antiviral activities, being little toxic to normal cells and tissues (Liu, 1995). It has been reported that, besides being less potent in inducing cell death than UA, it may be used in cancer treatment in specific physiological conditions, such as under metabolic stress (Duarte, 2012).

## OBJECTIVES OF THE WORK

Portugal is one of the leading countries in the production of eucalyptus for the pulp and paper industry. However, this sector is responsible for the production of tons of bark residues that does not have a valuable use. The present work intends to give a contribution to the exploitation of this byproduct of the pulp and paper industry as possible source of high-added-value bioactive compounds, a study that is under the scope of the European project AFORE (FP7: CP-IP 228589-2 AFORE) that intends to develop novel applications for forest residues.

Therefore, the objective of this work was to study the potential anticancer effect of the triterpenic fraction of extracts obtained from the bark of *E. nitens* in colorectal cancer cells. An enriched triterpenic fraction ($F_2$) in BiA and BoA was also tested and, in addition, these two isolated compounds.

For that, the anticancer potential of the triterpenic extracts/compounds was studied by the MTT assay, the anchorage-dependent colony forming assay and by the cell migration assay (wound healing). Induction of cell death and apoptosis was studied by the PI staining and the presence of nuclear condensation, respectively. In addition, effects on cell cycle were studied by flow cytometry. Finally, the involvement of several signalling pathways, related with proliferation and death, in the effects of the triterpenic extracts/compounds were analysed by western blot.

With this work we intend not only to establish the importance of triterpenoids compounds as anticancer agents, but also to test an important source of these compounds that are being disregarded as residues from crops used in the pulp and paper industry.

# Material and Methods

## 1. CHEMICALS

Propidium iodide (PI), quercetin, RNAse A, 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640, antibiotic/antimicotic solution, bovine serum albumin (BSA), N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was bought from Biochrom AG (Berlin, Germany). All the other compounds were of analytical grade.

## 2. *EUCALYPTUS NITENS* EXTRACTS AND TRITERPENOIDS

Betulinic acid and betulonic acid were purchased from Molekula Ltd (Gillingham, Dorset, United Kingdom) and Chemos GmbH (Regenstauf, Germany), respectively. *Eucalyptus nitens* crude extract (*E. nitens* CE) and the triterpenic enriched fraction 2 (*E. nitens* $F_2$) were kindly provided by Professor Armando Silvestre from the Department of Chemistry, University of Aveiro. The extracts were prepared by Domingues *et al.* through extraction of *E. nitens* bark with dichlorometane as previously described (Domingues *et al.*, 2011). The CE and $F_2$ extracts of *E. nitens* were then analysed by GC-MS and the main chemical composition present in Table 1 (Domingues *et al.*, 2012, unpublished data). Extracts and isolated compounds were dissolved in dimethyl sulfoxide (DMSO) in stock solutions, in order that DMSO concentration in cell culture was no higher than 0.5% (v/v). Controls received vehicle only.

## 3. ANTIBODIES

The primary antibodies were purchased from the following sources: anti-phospho-ERK, anti-PARP-1, anti-phospho-JNK, anti-Bcl-2 and anti-p53 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-caspase 9, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-AMPK$\alpha$ (Thr172) and anti-phospho-Akt (Ser473) from Cell Signalling (Danvers, MA, USA); anti-caspase 3 from Calbiochem (San Diego, CA, USA); and anti-$\beta$-actin from Sigma-Aldrich. Secondary antibodies anti-rabbit and anti-mouse were purchased from Santa Cruz Biotechnology.

**Table 1** – Chemical composition (based on % of weight from the total extract weight) of the *E. nitens* crude extract and of the triterpenic enriched fraction 2 (Domingues *et al.*, 2012, unpublished data).

| Compounds | Crude extract (%) | Fraction 2 (%) |
|---|---|---|
| Fatty acids | 1.2 | 4.2 |
| Long chain aliphatic alcohols | 0.9 | 0.2 |
| Sterols | 1.2 | 0.0 |
| Triterpenoids | 70.7 | 93.3 |
| β-amirin | 0.3 | 0.0 |
| Lupeol | 0.3 | 0.0 |
| Betulonic acid | 7.0 | 24.2 |
| Oleanolic acid | 20.8 | 15.5 |
| Betulinic acid | 19.0 | 32.5 |
| Ursolic acid | 10.2 | 15.5 |
| 3-acetyloleanolic acid | 3.2 | 0.0 |
| 3-acetylursolic acid | 1.8 | 0.6 |
| Unidentified triterpenoids | 8.1 | 4.9 |
| Other compounds | 1.9 | 0.0 |
| Unidentified compounds | 24.1 | 2.3 |

## 4. CELL CULTURE

HCT116 cells (human colorectal carcinoma cell line) were kindly provided by Professor Raquel Seruca from IPATIMUP, Porto. The cell line was maintained in culture in 25 $cm^2$ polystyrene flasks (TPP, Switzerland) with RPMI 1640 medium, containing 6% FBS, 1% antibiotic–antimicotic solution, 0.1 mM sodium pyruvate, 10 mM HEPES and 2 g/L sodium bicarbonate under an atmosphere of 5% $CO_2$ and 95% air at 37°C.

## 5. MTT REDUCTION ASSAY

MTT reduction assay was performed to evaluate the potential of the tested compounds/extracts to decrease the number of viable cells as previously described (Lima *et al.*, 2011). Briefly, HCT116 cells were plated in 24-multiwell culture plates at $8x10^4$ cells per ml and allowed to grow for two day. Cells were incubated with test compounds/extracts at different concentrations and for different time periods. One hour before the end of incubation, 50µL of

MTT (final concentration of 0.5mg/ml) was added to each well. When the incubation time ended, the medium was removed and discarded, and the formazan crystals (formed by the cell's capacity to reduce MTT) were dissolved with a 50:50 (v/v) DMSO:ethanol solution. The absorbance was measured at 570nm, with background subtraction at 690nm. The results were expressed as percentage relative to the control. The concentration of each test compound/extract that decreases the number of viable cells to half ($IC_{50}$) was calculated using mathematical modelling with the program GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

## 6. ANCHORAGE-DEPENDENT COLONY FORMING

The anticancer potential of tested compounds/extracts was also tested by their ability to inhibit the formation of cell colonies. HCT116 cells were plated in 6-multiwell culture plates at 500 cells per ml for two days for cell attachment. Cells were then incubated with different concentration of test compounds/extracts dissolved in new culture medium for six days (without changing the medium). After this incubation time, medium was removed from the wells, washed with PBS and cells fixed with ice cold absolute ethanol for 15 minutes. Then, cells were stained with Giemsa working solution (0.4% w/v) for 5 minutes and the wells washed with tap water. The plate wells were photographed and colony forming ability estimated by quantifying colony area intensity using appropriate image analysis system (Adobe Photoshop CS3 Adobe Systems Incorporated, San Jose, CA, USA).

## 7. CELL DEATH ANALYSIS BY PROPIDIUM IODIDE (PI) STAINING

PI staining was performed to evaluate cell death induced by the tested compounds/extracts. This DNA fluorophore is impermeable to live cells, but will stain strongly the DNA of dying and death cells due to their compromised or permeabilized plasma membranes (Rieger *et al.*, 2011) The HCT116 cells were plated in 6-multiwell culture plates at $8x10^4$ cells per ml, for two days for cell attachment and growth, before incubating them with test compounds/extracts dissolved in new culture medium. After treatment with different concentrations and incubation times, cells were collected (both floating and attached cells) washed with cold PBS containing 6% (v/v) FBS, and incubated with PI added to a final

concentration of 0.25mg/ml for 10 minutes. The percentage of cells positive for PI (death cells) was quantified by flow cytometry using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc., Miami, FL, USA) counting at least 40,000 cells per sample. An example of quantification of cell death by flow cytometry with PI staining is given in the Figure 11.

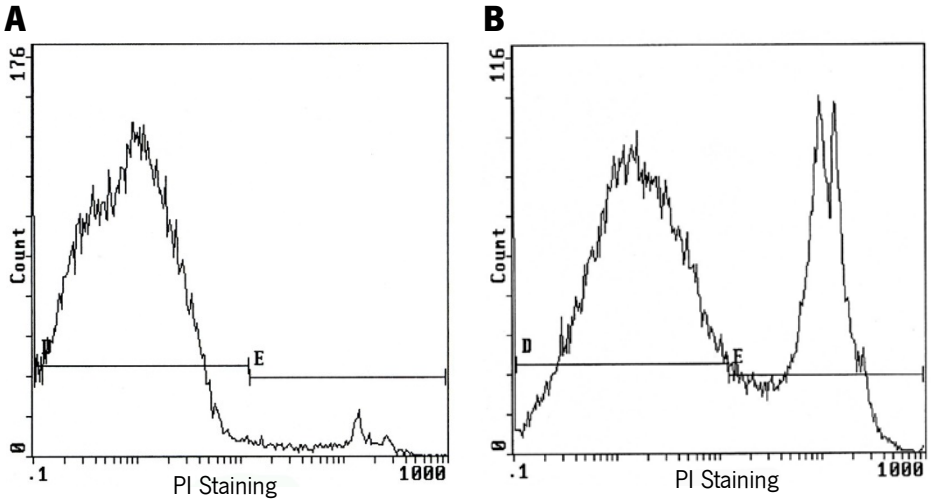


**Figure 11 –** Representative images of flow cytometry for analysing cell death by PI staining, of a control sample (A) and a treatment condition with induction of cell death (B). Represented in the plots are life cells – cells at gate D with red autofluorescence (PI negative), and dead cells – cells at gate E with PI red fluorescence (PI positive). The percentage of cell death is given by the formula $\frac{\mathbf{E}}{\mathbf{D+E}} \times \mathbf{100}$.

## 8. NUCLEAR CONDENSATION

Nuclear condensation was performed in order to estimate cell death by apoptosis. The HCT116 cells were plated in 12-multiwell culture plates at $8x10^4$ cells per ml, for two days before incubating them with different concentrations of test compounds/extracts dissolved in new culture medium. After 48h of treatment, cells were collected (both floating and attached cells) and washed with PBS. Cells were then fixed resuspending them in paraformaldehyde (PFA) 4% (w/v) in PBS for 20 minutes, and washed again with PBS. Cells were attached into a polylysine treated slide using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA), and incubated with Hoechst (final concentration of 5µg/ml) for DNA staining. Nuclear morphology was visualized on a fluorescent microscope and photos taken from different fields of each sample. The percentage of apoptotic cells were calculated from the ratio between cells with nuclear

condensation typical of apoptosis and the total number of cells, from a count higher than 500 cells per sample.

## 9. CELL CYCLE ANALYSIS

The analysis of cell cycle was done by flow cytometry using PI for staining DNA of permeabilized cells. For that, HCT116 cells were plated in 6-multiwell culture plates at $8x10^4$ cells per ml for two days for cell attachment and growth, before incubating them with the test compounds/extracts dissolved in new culture medium. After treatment for 48h with different concentrations of compounds/extracts, cells were collected (both floating and attached cells) and washed with cold PBS. Then, cells were fixed and permeabilized in ice cold ethanol 70% (v/v) for 15 minutes and washed again with PBS. Finally, cells were incubated with staining solution (50 µg/ml PI and 20 µg/ml RNase A in PBS) at 37°C for 15 minutes. Analysis of cell cycle progression by flow cytometry was done using a Coulter Epics XL Flow Cytometer (Beckman Coulter Inc.) counting at least 40,000 single cells per sample. Phases of cell cycle were fitted using the mathematical Watson Pragmatic model with the FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA).

## 10. MIGRATION ASSAY (WOUND HEALING)

Migration assay was performed to evaluate the effect of tested compounds/extracts to affect the extent of wound closure. For that, HCT116 cells were plated in 12-multiwell culture plates at $2.5x10^5$ cells per ml, and allowed to grow for two days until cell confluence was reached. Cell layer was then wounded in an x shape with an 100 µl tip, and a photo of the wound site was taken. Cells were incubated with the test compounds/extracts for 24h and a new photo in the central wound site taken. The extend of wound closure was given as the percentage of covered area after 24h in relation to the initial uncovered area, using the following formula:

$$\text{Extent of wound closure} = \frac{\left(\text{Initial uncovered area-Final uncovered area}\right)}{\text{Initial uncovered area}} \times 100$$

and used to estimate the cells' migration ability.

## 11. WESTERN BLOTTING

To measure the expression of different proteins involved in cell signalling and apoptosis western blot was used using total cell homogenates. HCT116 cells were plated in 6-multiwell culture plates at $8x10^4$ cells per ml for two days for cell attachment and growth. Cells were treated with different concentrations of tested compounds/extracts for 24h or 48h and a total cell homogenate obtained incubating cells for 15 minutes at 4°C with ice cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris-HCl (pH 8), 2mM EDTA) containing 1mM PMSF, phosphatase inhibitors (20mM NaF, 20mM $Na_2V_3O_4$) and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was quantified by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and BSA used as protein standard. For Western Blot, 20 µg of protein was resolved in SDS-polyacrilamide gel and then electroblotted to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked in TPBS (PBS with 0.05% of Tween-20) containing 5% (w/v) of non-fat dry milk, washed in TPBS and incubated with primary antibody overnight. After washing, membranes were incubated with the secondary antibody for 1h, and membranes washed again. The immunoreactive bands were detected using the Immobilon solutions (Millipore) under a chemiluminescence detection system, the Chemidoc XRS (Bio-Rad Laboratories). Band area intensity was quantified using the Quantity One software from Bio-Rad. $\beta$-actin was used as loading control.

## 12. STATISTICS

Data expressed as the mean ± SEM of at least 3 independent experiments. Statistical significances among data groups were analysed by one-way ANOVA followed by the Dunnett's multiple comparison test, or by the Student's *t*-test, as appropriate, using GraphPad Prism 5.0 software (San Diego, CA, USA). Differences between groups were considered statistically significant when $P \leq 0.05$.

# Results and Discussion

The pulp and paper industries are one of the most important industries in Portugal contributing positively to the country's export (Pereira *et al.*, 2005; Domingues *et al.*, 2010; Domingues *et al.*, 2011b; Moura *et al.*, 2012). Eucalyptus wood is the main source of fibbers for the production of paper. However, it generates large amounts of residues, mainly bark, which can be a source of valuable compounds of otherwise burned or discarded material (Doningues *et al.*, 2011). Therefore, the exploitation of this byproduct for the production of interesting added-value new products is being viewed by the pulp and paper industry as a way to increase their revenue and to implement the biorefinery concept in their production chain (Domingues *et al.*, 2011a; Mota *et al.*, 2012). Previously, it was reported that lipophilic extracts from the bark of *Eucalyptus* species can be an important source of high-value triterpenic compounds in view of their high content and attributed bioactive properties (Domingues *et al.*, 2011a).

In the present work, the potential anticancer effect of lipophilic extracts enriched in triterpenic acids obtained from the bark of *E. nitens* was studied in the colorectal HCT116 cancer cells. Although *E. nitens* is not the main species of eucalyptus in Portugal, it possess high content of triterpenic acids like the most abundant species *E. globulus*, but contrarily to this one, where ursane acids dominates, it is the richest in oleanane and lupane acids (Domingues *et al.*, 2011a).

For this work, two extracts of *E. nitens* were used (see Table 1, in Materials section): a lipophilic crude extract (CE) with about 70% (w/w) of triterpenoids, where the main ones are oleanolic acid (20.8%) followed by betulinic acid (19%), ursolic acid (10.2%) and betulonic acid (7%); and, a fraction ($F_2$) of the CE more enriched in triterpenoids (about 93% of total weight), specially in lupane acids, since the main ones are betulinic acid (32.5%) followed by betulonic acid (24.2%), ursolic acid (15.5%) and oleanolic acid (15.5%). In addition, the above lupane acids were also tested alone for comparison.

## 1. TRITERPENIC ACIDS-ENRICHED EXTRACTS FROM *E. NITENS* POSSESS ANTICANCER ACTIVITY AGAINST HCT116 CELLS

In order to study the anticancer potential of the two *E. nitens* extracts, HCT116 cells were incubated for 48h with different concentrations of CE or $F_2$ extracts, and cell viability evaluated by the MTT assay (Fig. 12). As shown in Fig. 12A & 12C, both extracts presented high anticancer activity, decreasing significantly the number of viable cells in a concentration dependent manner. Based on these results, their $IC_{50}$ (concentration of extract necessary to decrease by half the number of viable cells as compared with negative control) were calculated and presented as an example for the *E. nitens* CE in Fig. 13A. The results showed that the *E. nitens* $F_2$ extract was more active than the *E. nitens* CE extract, with $IC_{50s}$ of 1.3 µg/ml and 2.2 µg/ml, respectively.

Considering the high content of these extracts in triterpenoids, especially in betulinic, betulonic, oleanolic and ursolic acids, the main compounds were also tested alone. In a previous work of our group (Duarte, 2012), the anticancer potential of both UA and OA were evaluated in HCT116 cells using the same culture conditions than here. Whereas for UA a good anticancer activity was obtained with an $IC_{50}$ of 8 µM, for OA no significant decrease of cell viability was observed until the highest concentration tested (50 µM), meaning that the $IC_{50}$ is much over this value. In the present work, the remaining two main triterpenoids of the *E. nitens* extracts, BiA and BoA, were tested. Both compounds exhibit a high capability to decrease the number of viable cells, also in a concentration-dependent manner (Fig. 12D and E). Interestingly, BoA presents a 4 times lower $IC_{50}$ concentration than BiA, with 0.8 µM and 3.9 µM, respectively, and a 10 times lower than UA.

Converting the $IC_{50}$ of the isolated compounds from µM to µg/ml, and taking into account the percentage of each compound in both extracts, the concentration range of the isolated compounds are in the same order of magnitude than that of the extracts. However, a compound alone does not explain the total extract activity, which suggests that all the three main active triterpenoids BoA, BiA and UA contribute to the extracts' activity. Interestingly, taking into account only these three triterpenoids, *E. nitens* $F_2$ extract has about two times more of their quantity than *E. nitens* CE (see Table 1, in Materials section), and the $IC_{50}$ of the former is about half of the latter. This observation corroborates that the activity of the *E. nitens* extracts dependes mainly from the three active triterpenoids (OA is non-active until 50 µM).

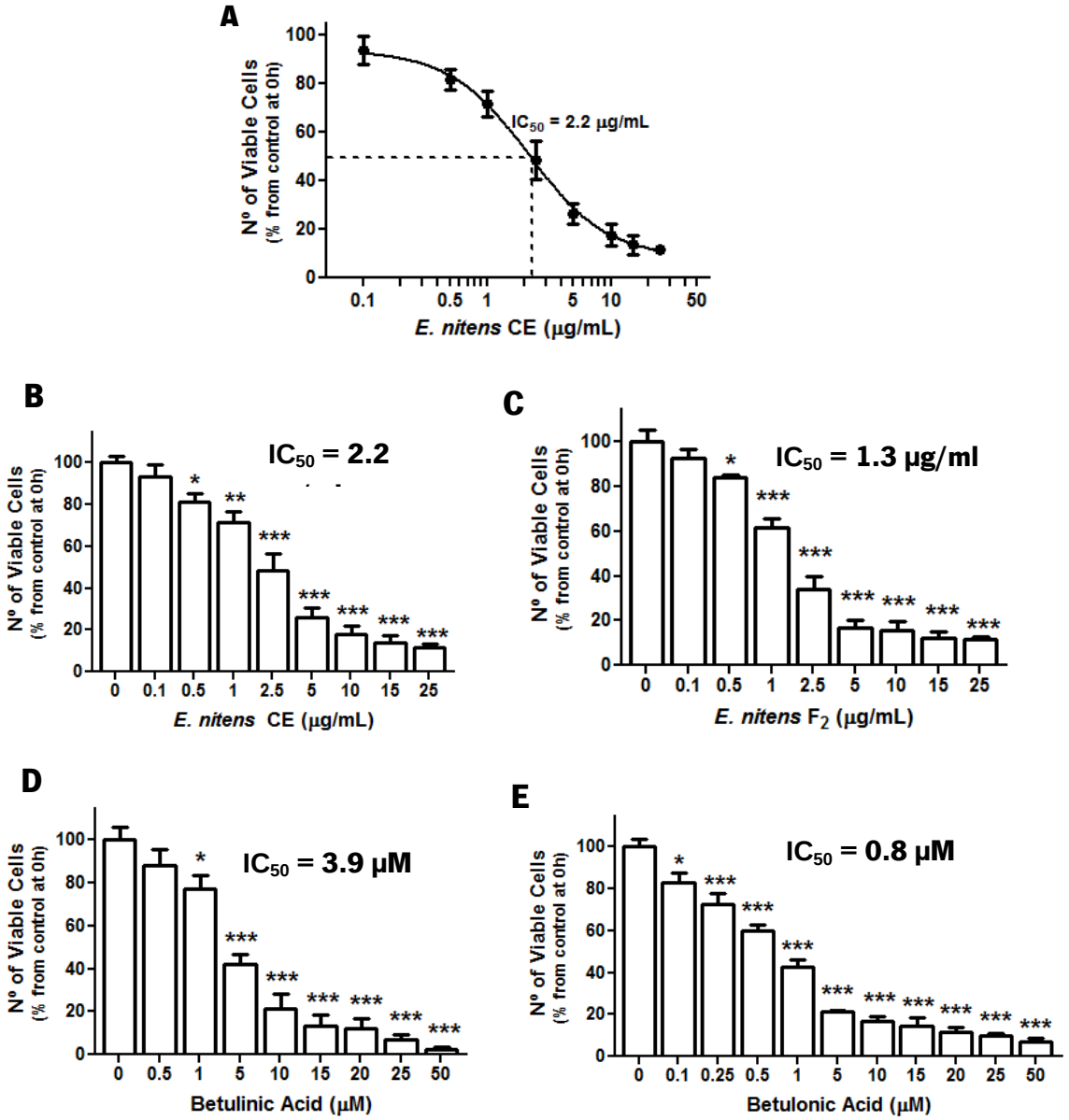


**Fig. 12** – Effects of *E. nitens* CE (**B**) and $F_2$ extracts (**C**), as well as the isolated compounds betulinic acid (**D**) and betulonic acid (**E**) in cell viability of HCT116 cells after 48h of incubation, as measured by the MTT assay. In (**A**) is presented, as an example, data of (**B**) with a sigmoid mathematical modelling with the program GraphPad Prism for calculation of the $IC_{50}$ value. The same metod was used to calculate the $IC_{50}$ given in C, D and E. Values are mean ± SEM of at least three independent experiments. * P≤0.05, ** P≤0.01, *** P≤0.001, when compared with respective control, analysed by one-away ANOVA with Dunnett's multiple comparison test.

The morphologic changes observed in HCT116 cells after being incubated for 48h with eucalyptus extracts (Fig. 13A) or isolated lupane acids (Fig. 13B), also agrees to their potent anticancer activity. Along with the increasing concentration of the extract/compound, the number of adherent (live) cells decreased and the floating (dead) increased. The anticancer activity of eucalyptus extracts has been poorly investigated. Recently, polyphenolic-rich extracts from *E. globulus* bark demonstrated some capacity to reduce human breast cancer cell proliferation (Mota *et al.*, 2012). In addition, Islam *et al.* (2012) showed the anti-tumour activity of a lipophilic extract of *E. camaldulensis* against Ehrlich ascites carcinoma celsl in mice. In the present work, it was demonstrated a high anticancer potential of triterpenic acids-rich extracts from *E. nitens* bark in colorectal cancer cell, with $IC_{50}$ of about 2 µg/ml.

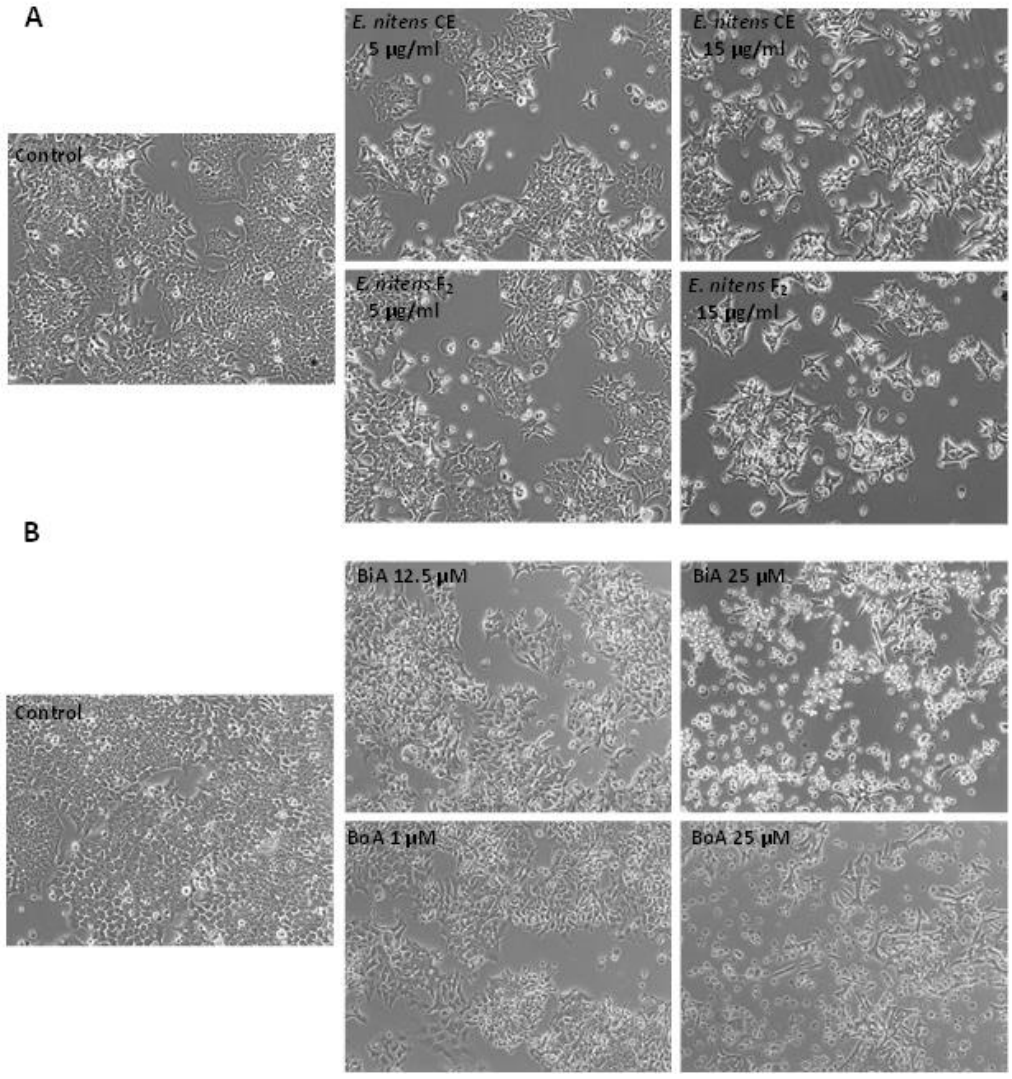


**Fig. 13 –** Representative images of the effect of different concentrations of *E. nitens* crude extract (CE) and *E. nitens* fraction 2 ($F_2$) extract (**A**), and test compounds betulinic acid (BiA) and betulonic acid (BoA) (**B**) on the morphology of HCT116 cells after 48h incubation. Photos were taken with an objective of 10 times magnification in an inverted microscope (Olympus IX71 – Lisboa, Portugal) with phase contrast.

The anticancer potential of their main triterpenoids are, however, described in the literature. Many studies (Cichewicz and Kouzi, 2004; Ehrardt *et al.*, 2004; Symon *et al.*, 2005; Wada and Tanaka, 2005; Fulda, 2008; Amin *et al.*, 2009; Eichenmuller *et al.*, 2009; Fulda and Kroemer, 2009; Chintharlapalli *et al.*, 2011), including our owns (Ramos *et al.*, 2008; Xavier *et al.*, 2009b; Ramos *et al.*, 2010; Xavier *et al.*, 2012), demonstrated the anticancer activity of these triterpenoids against several types of tumours cells. Many mechanisms of action, mainly for UA (Liu, 1995 Xavier *et al.*, 2009b; Xavier *et al.*, 2012) and BiA (Deniaud *et al.*, 2009), were also depicted. Considering that OA is non-active in HCT116 cells and that the lupane acids were more active than UA, along with the *E. nitens* extracts, the work was proceeded with BiA and BoA.

The observed decrease in the number of viable cells induced either by the extracts or by the isolated compounds could have been a result of inhibition of cell proliferation and/or induction of cell death. As to better clarify that, some concentrations of extracts and compounds were chosen and their effect on cell viability along with different times of incubation (6, 24, 48 and 72h) was measured by MTT reduction assay (Fig. 14A, C, E and G). Furthermore, cell death was followed by flow cytometry by measuring the ability of live cells to exclude PI after different times of incubation (24, 48 and 72h) (Fig. 14B, D, F and H).

As shown in Fig. 14A, cells incubated with 1 or 2.5 µg/ml of *E. nitens* CE accompanied the growth of control cells until 24h of incubation, but decreased significantly after 48h. On other hand, cells incubated with 10 µg/ml of CE did not grow in the first 24h but did not induce cell death (Fig.13B). With the PI staining assay, cell death was only observed after 48h of incubation and in a concentration-dependent manner (Fig. 14B). At 72h, the same results were obtained, but with a higher magnitude of cell death. The pattern of results of *E. nitens* $F_2$ (Fig. 14C and D) was similar to the ones of CE. However, the magnitude of effects was higher at lower concentrations, which, once more, shows that the $F_2$ has higher anticancer activity when compared to the CE. Regarding the isolated compounds, cell death was already visible at 24h of incubation with the higher tested concentration of each compound, 15 µM for BiA and 50 µM for BoA, being of notice that even tough BiA's concentration is significantly lower than BoA's, it shows more cell death (Fig. 14F and H). This result seems contradictory to the $IC_{50}$ value calculated for BiA when compared with BoA. However, at 48h of incubation, BoA at lower doses induces higher cell death as compared with BiA, which agrees with the MTT assays (Fig. 14E, F,

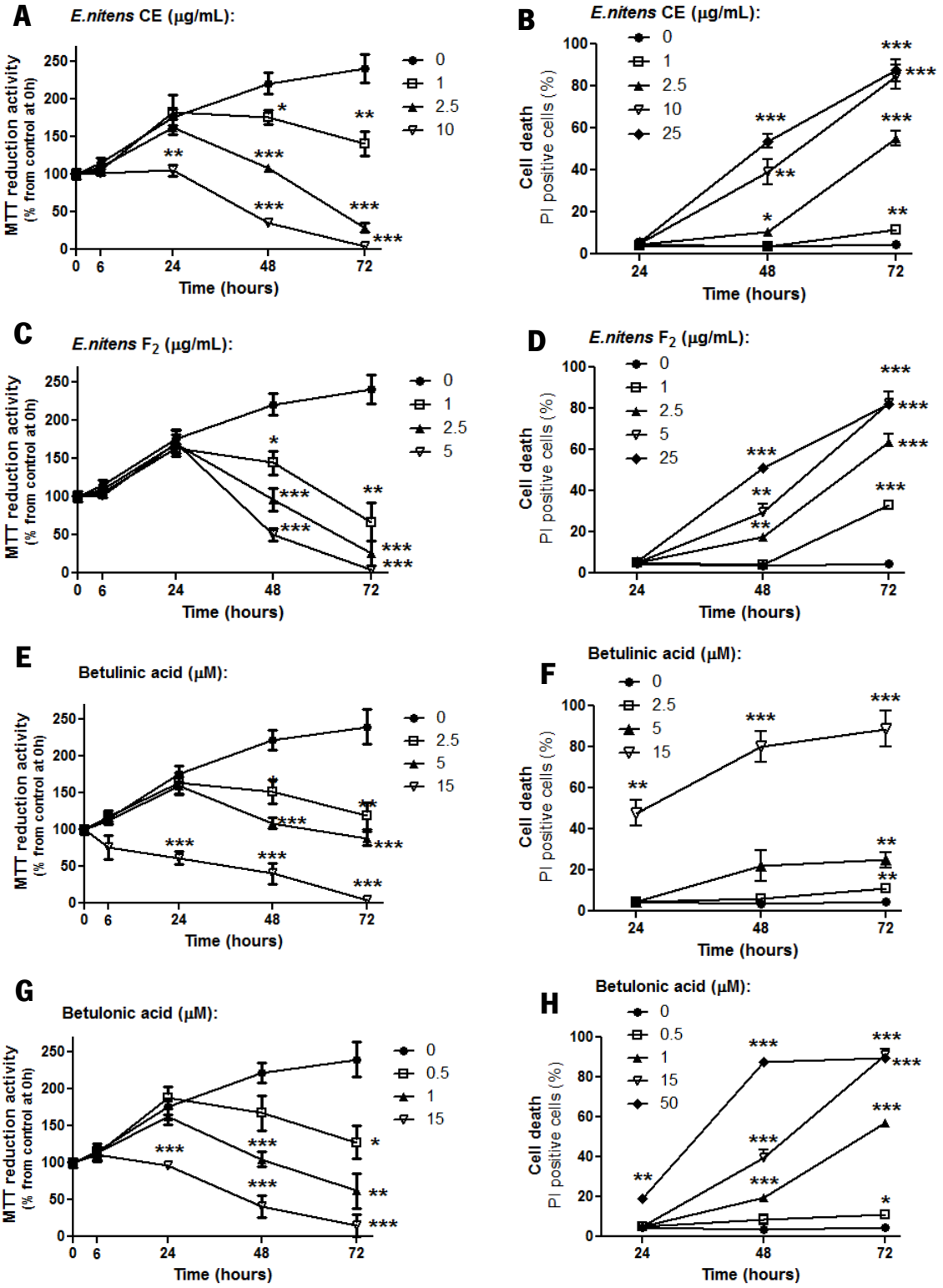


**Fig. 14 –** Effect of *E. nitens* crude extract (CE), *E. nitens* fraction 2 ($F_2$), betulinic acid and betulonic acid at different concentrations in cell viability of HCT116 cells along the time. **(A, C, E and G)** Number of viable cells measured by the MTT assay. **(B, D, F and H)** Cell death evaluated by the PI staining as measured by flow cytometry. Values are mean ± SEM of at least three independent experiments. * P≤0.05, ** P≤0.01, *** P≤0.001, when compared with respective control, analysed by the Student's t-test.

G and H). All of these results seem to indicate a contribution of both inhibition of cell proliferation and induction of cell death in the observed reduction of viable cells by the extracts and the compounds.

In order to confirm the anticancer potential of the triterpenic acid-enriched *E. nitens* extracts and its main compounds, an anchorage-dependent colony formation assay and cell migration assay was performed. As shown in Figure 15A, the two tested extracts and compounds exhibited a marked inhibition of colony formation and growth in a concentration-dependent manner. The observed effects resulted mainly from inhibition of cancer cell colonies growth and colony size – Fig. 15B – since concentrations of extracts/compounds that induced cell death, in the conditions used in this assay (concentrations higher than that present in Fig. 15), were discarded. In accordance with the results of the cell viability assay, *E. nitens* $F_2$ extract was shown to be more potent than the CE, having the concentrations of the first (2.5 and 5 μg/ml) achieved the same results of the latter at 5 and 10 μg/ml (Fig.15). However, for the triterpenoids BiA and BoA a similar inhibition of colonies growth were obtained for the same concentrations. As expected, the positive control – starved cell (grown in medium with 0.6% (v/v) FBS) – resulted in marked colonies growth inhibition.

For the migration assay, the capacity of cells to wound healing/closure were evaluated in the presence of different concentrations of extracts/compounds for 24 hours. As a positive control, cells were incubated in starvation medium (medium with 0.6% (v/v) FBS), and representative images of this and control cells are present in Figure 15B. In the migration assay, the wounds were made in a confluent cell layer, in order that the wound closure would be dependent on cell migration and not on cell proliferation. When cells reach confluence, they acquire a quiescent state, where cell cycle pauses at G0 state (Coller *et al.*, 2006).

Figure 16A shows that the lower concentrations of either the extracts or the compounds do not have any effect on the cell migration ability, being their values very similar to those of the control. However, with a higher concentration we did obtain migration inhibition in cells treated with the extracts and the compounds. Rzeski and collaborators (2006) had previously reported that cultures treated with BiA had a significant lower number of cell migrating to the wounded area. Taking into account the two *E. nitens* extracts, it was observed again that half the concentration used for the CE obtain the same result for $F_2$. The same also happens with the isolated compounds, where 5 μM of BoA has the overall same result of 10 μM of BiA (Fig. 16A),

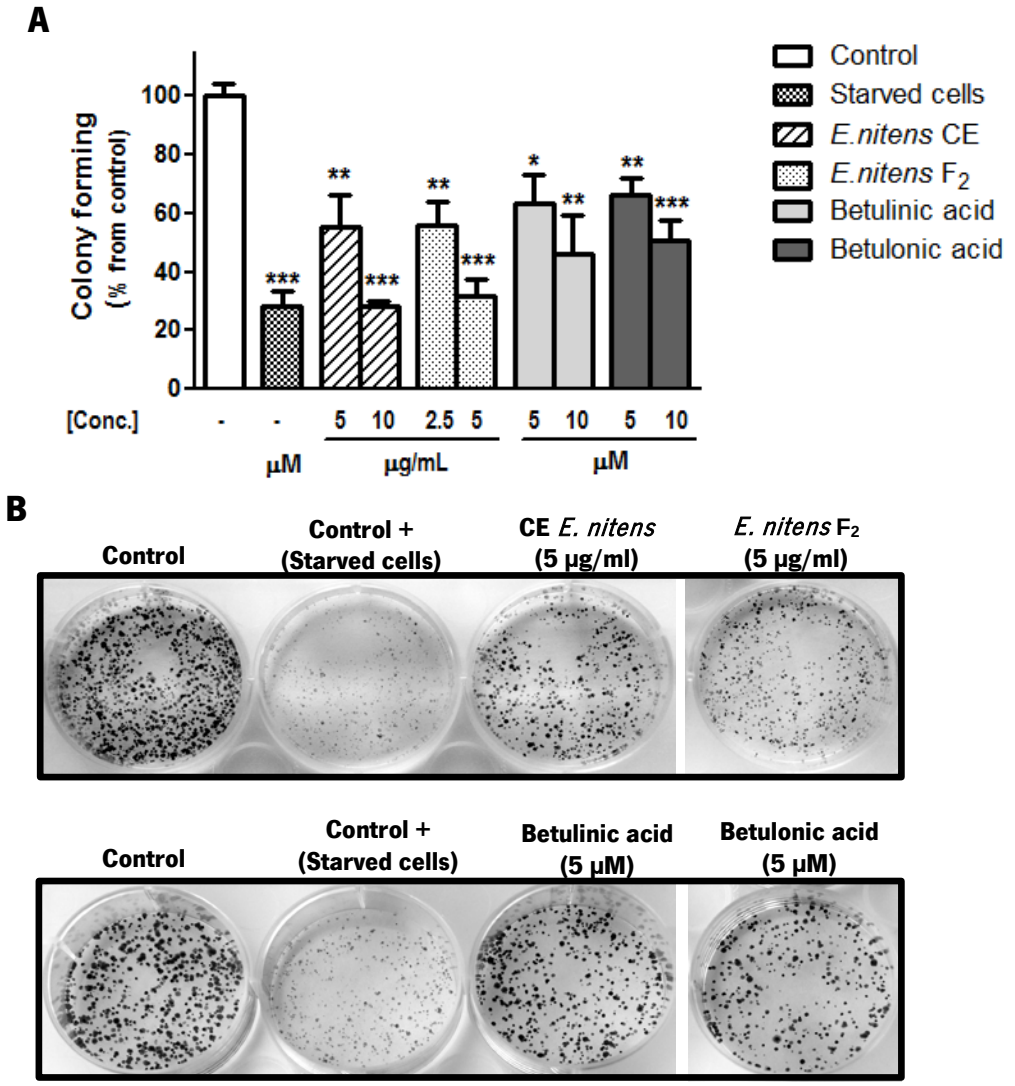


**Fig. 15 –** Effect of *E. nitens* crude extract (CE), *E. nitens* fraction 2 ($F_2$), betulinic acid and betulonic acid in the ability to inhibit colony forming in HCT116 cells. Cells were incubated with test compounds/extracts (controls received vehicle only – DMSO; starved cells received 0.6% (v/v) FBS-containing medium) for six days before being fixed and stained with Giemsa. **A)** Values are expressed as the mean area intensity of colonies and are mean ± SEM of the mean area intensity of colonies of at least three independent experiments. * P≤0.05, ** P≤0.01, *** P≤0.001, when compared with respective control, analysed by the Student's t-test. **B)** Representative images of the anchorage-dependent colonies formation in HCT116 cells under the effect of tested compounds/extracts for six days.

demonstrating, once more, that BoA is a more potent compound than BiA. The effect of higher concentrations of extracts/compounds than that shown in Figure 16 was not shown, since inhibition of wound closure did not result from inhibition of cell migration, but noticeably from induction of cell death, in the conditions used in this assay.

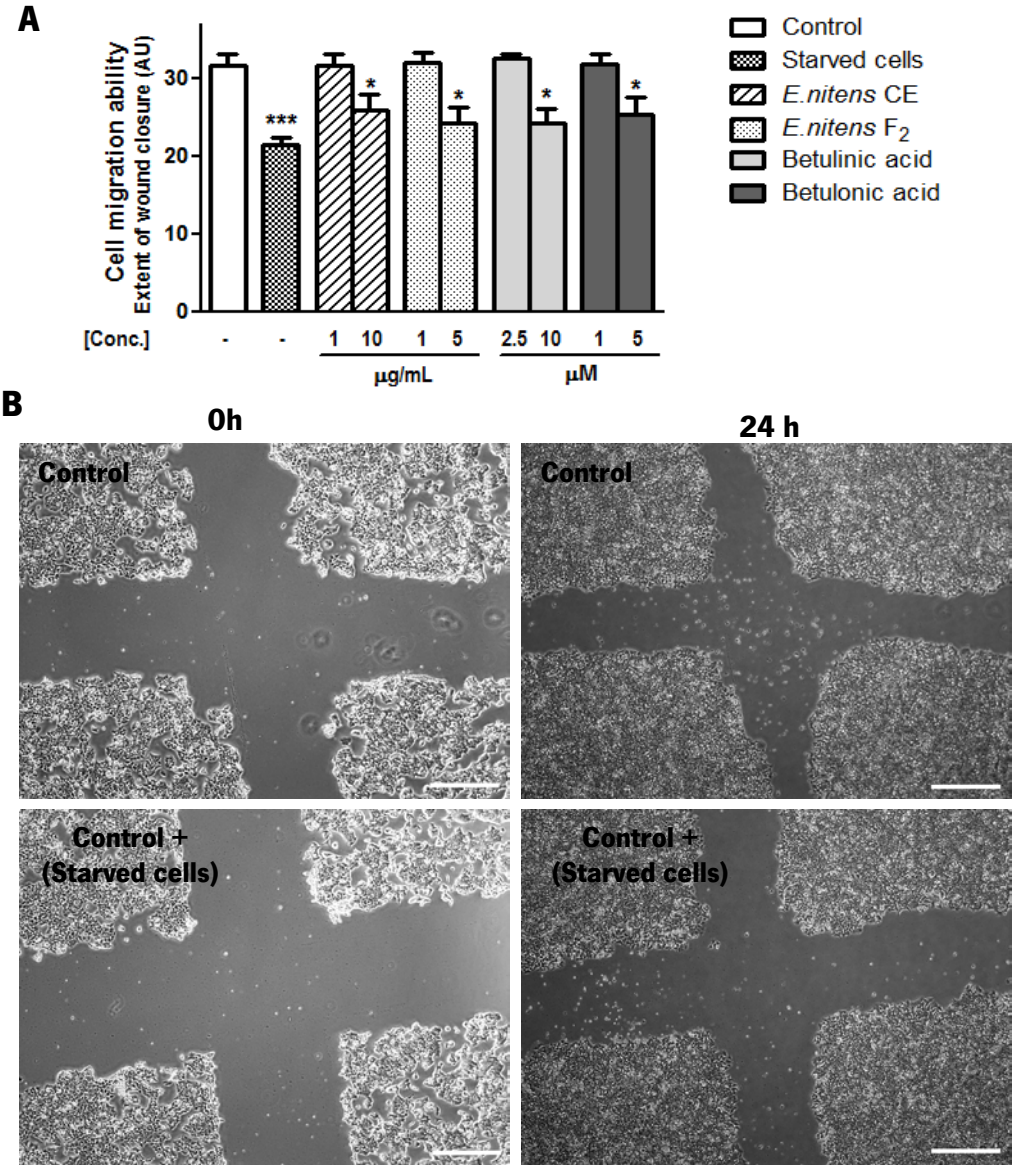


**Fig. 16 –** Effect of *E. nitens* crude extract (CE), *E. nitens* fraction 2 ($F_2$), betulinic acid and betulonic acid in the ability of HCT116 cells to migrate. Cells were incubated with test compounds/extracts (controls received vehicle only – DMSO; starved cells received 0.6% (v/v) FBS medium) for 24 hours. **A)** Values are given as mean ± SEM of the percentage of covered area after 24h in relation to the initial uncovered area, of at least three independent experiments. * P≤0.05, *** P≤0.001, when compared with respective control, analysed by the Student's t-test. **B)** Representative images of the wound before (0h) and after 24 hours of incubation for the negative control (complete medium with vehicle) and the positive control (starved cells) (bar **–** 500 µm).

All the present results show that BoA is the most active anticancer compound for HCT116 cells between the tested triterpenoids. *Eucalyptus nitens* bark can be an interesting source of extracts rich in bioactive triterpenoids, namely BoA, BiA and UA, with potent anticancer activity against HCT116 cells.

## 2. APOPTOSIS CONTRIBUTES TO THE CELL DEATH INDUCED BY *E. NITENS* EXTRACTS AND THEIR MAIN LUPANE ACIDS IN HCT116 CELLS

Considering the cell death induced by the tested extracts and compounds, we wondered whether it was apoptosis dependent. During apoptosis, DNA is fragmented and condensed in order to be included in the apoptotic bodies (Elmore, 2007). Therefore the ability of the *E. nitens* extracts and tested isolated compounds to induce nuclear condensation in HCT116 cells after 48h of incubation, as a marker of cell death by apoptosis, were studied. As shown in Figure 16, apoptosis occurs in cells treated with either the extracts or the compounds, but only at the higher tested concentrations. Among the *E. nitens* extracts, $F_2$ seems to be more potent inducing apoptosis than CE, since the first has about five times more apoptosis at 10 μg/ml than control cells, whereas the latter has no effect. Interestingly, BiA, which has a higher $IC_{50}$ than BoA at 48h, has a higher ability to induce cell death by apoptosis at lower doses (Fig.17A). Some intermediate concentrations of the tested extracts or compounds that already induced cell death as assessed by the PI staining (Fig.14), did not, however, induce apoptosis. So, nuclear condensation assay was also performed after 72h of incubation with these concentrations of extracts/compounds that induce cell death as observed with PI staining, but no induction of apoptosis was observed (data not shown).

These results indicate that our tested extracts and compounds are able to induce cell death by apoptosis only at high concentrations. At lower or intermediate concentrations cell death occurs, but is not as a result of apoptosis but through some kind of non-apoptotic type of cell death.

Whilst there is no scientific data as how *E. nitens* extracts or BoA induces apoptosis in cancer cells, it has been reported that BiA induces apoptosis in cancer cells through activation of the intrinsic mitochondrial pathway (Fulda and Debatin, 2000). Therefore, the levels of several proteins related with apoptosis were evaluated by western blot after treating HCT116 cells with

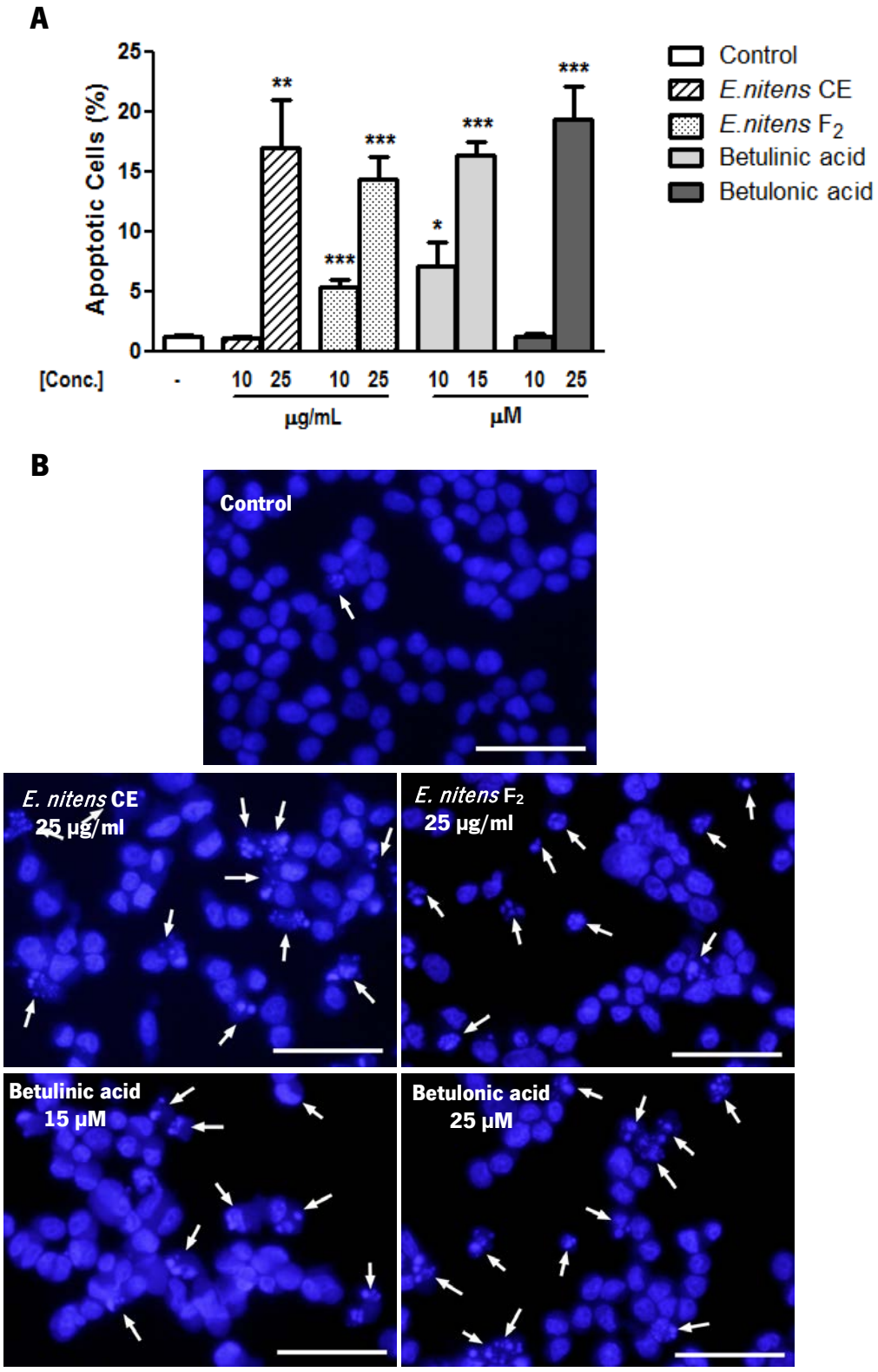


**Fig. 17 –** Effect of *E. nitens* crude extract (CE), *E. nitens* fraction 2 ($F_2$) extract, betulinic acid and betulonic acid in the in the presence of nuclear condensation in HCT116 cells for 48h, as a marker of apoptosis. **A)** Values are mean ± SEM of at least three independent experiments. * P≤0.05, ** P≤0.01, *** P≤0.001, when compared with respective control, analysed by the Student's t-test. **B)** Representative images of the induction of apoptosis in HCT116 cells by the tested compounds/extracts after 48h incubation; arrows indicate apoptotic cells with condensed and fragmented nuclear chromatin (bar **–** 50 µm).

either an eucalyptus extract or a tested triterpenoids compound. HCT116 cells possess a wild-type p53 (Kim *et al.*, 2012), and therefore are prone to p53-dependent apoptosis. Thus, the natural flavonoid quercetin was used in this work as a positive control, since it was previously shown to induce p53 and caspases-dependent apoptosis in colorectal cancer cells (Xavier *et al.*, 2011).

During apoptosis, caspases are the execution proteins responsible for the cell death program. Their sequential cleavage results in their activation that in turn will end in the cleavage of critical cellular substrates, including poly(ADP.ribose) polymerase-1 (PARP1) (Chaitanya *et al.*, 2010). In the intrinsic mitochondrial pathway of apoptosis, a suppression in the anti-apoptotic protein Bcl-2 and increase of the pro-apoptotic Bax protein result in the imbalance of the Bcl-2/Bax ratio, with the consequent permeabilization of the mitochondrial membrane, the release of cytochrome c and apoptosome assembly that leads to caspase 9-mediated caspase 3 activation (Fulda *et al.*, 1997; Fulda, 2008). The cleavage of caspases and PARP1 (inactivating it) are considered good markers of apoptosis (Chaitanya *et al.*, 2010).

As observed in Figure 18B, all the extracts and compounds at the higher concentrations tested induced the cleavage of caspase 9, the decrease of procaspase 3 levels (an indirect indication of cleavage of caspase 3) and the cleavage of PARP1 after 48h of incubation. These results corroborate that apoptosis are triggered by both eucalyptus extracts and by their main lupane triterpenoids acids, but that did not occur at intermediate concentrations where cell death already take place.

Among the triterpenoids, the western blot results also showed that BiA is more active than BoA, since cleavage of PARP1 were present at 10 μM and 15 μM in BiA but not in BoA (Figure 18), regardless the lower $IC_{50}$ for the later. Interestingly, BiA at 15 μM already induced PARP1 cleavage in HCT116 cells after 24h of incubation. It is important to note that, as seen on Figure 14F, this was also the concentration that, after 24h of incubation, has a significant percentage of cell death assessed by PI staining, unlike BoA and the two extracts that did not present significant death at time. These results confirm the superior ability of BiA to induce cell death by apoptosis, as compared to BoA. Previous results in literature also reported the ability of BiA to induce apoptosis in leukaemia and neuroblastoma cancer cells accompanied by cleavage of caspase 3 and PARP1 (Fulda and Debatin, 2000; Wu *et al.*, 2010). The levels of Bcl-2

decrease in a concentration-dependent manner, especially for *E. nitens* CE, BiA and BoA, even at concentrations that apoptosis was not present (Figure 18B).

Different types of stresses, including DNA damage, can trigger apoptosis, and the p53 tumour suppressor is an important regulator in the process (Chari *et al.*, 2009). Therefore, the levels of p53 were also measured by western blot and shown in Figure 18. As expected, the positive control quercetin (50 μM) induced the levels of p53 accompanied by cell death by apoptosis, as shown by the cleavage of caspase 9 and PARP1 as well as the decrease in the levels of procaspase 3 and Bcl-2 (Figure 18), and the induction of nuclear condensation (data not shown).

As shown in Figure 18A, contrarily to quercetin, both eucalyptus extracts, BiA and BoA, decrease the expression of p53 protein in a concentration-dependent manner after 24h of incubation. After 48h of incubation, the decrease of p53 levels was even stronger, especially for eucalyptus extracts and BoA at concentrations that cell death occurs independent of apoptosis (Figure 18B). Although p53 can interact with members of the Bcl-2 family to induce the mitochondrial pathway of apoptosis (Vaseva and Moll, 2009), these results may indicate that apoptosis induced by these extracts/compounds is not dependent in p53. However, that needs to be elucidated in further experiments. Interestingly, in a previous result in our laboratory, UA was also shown to decrease the levels of p53 in colorectal cancer cells (Xavier *et al.*, 2013), which may indicate that triterpenoids in general may be involved in reduction of p53 levels. The p53 suppression may be explained either by the interruption in its synthesis or by a fastest HDM2 activation that leads to the proteosome-related degradation of p53 (Zhang *et al.*, 2010). Although this needs to be clarified in following experiments, it was reported that, in fact, BiA is an inducer of proteosome to increase protein degradation (Huang *et al.*, 2007). Since these extracts and triterpenoids induce apoptosis apparently independent of p53, it would also be interesting to test in further experiments whether these extracts/compounds would be able to induce this type of cell death in apoptotic-resistant cancer cell lines with p53 mutations.

Overall, these results indicate that apoptosis mediated by the mitochondrial-caspase 9-caspase 3 axis contributes to the cell death induced by the eucalyptus extracts and their isolated compounds BiA and BoA in HCT116 cells, mainly at higher concentrations. At intermediate concentrations, a non-apoptotic type of cell death occurs together with loss of p53.

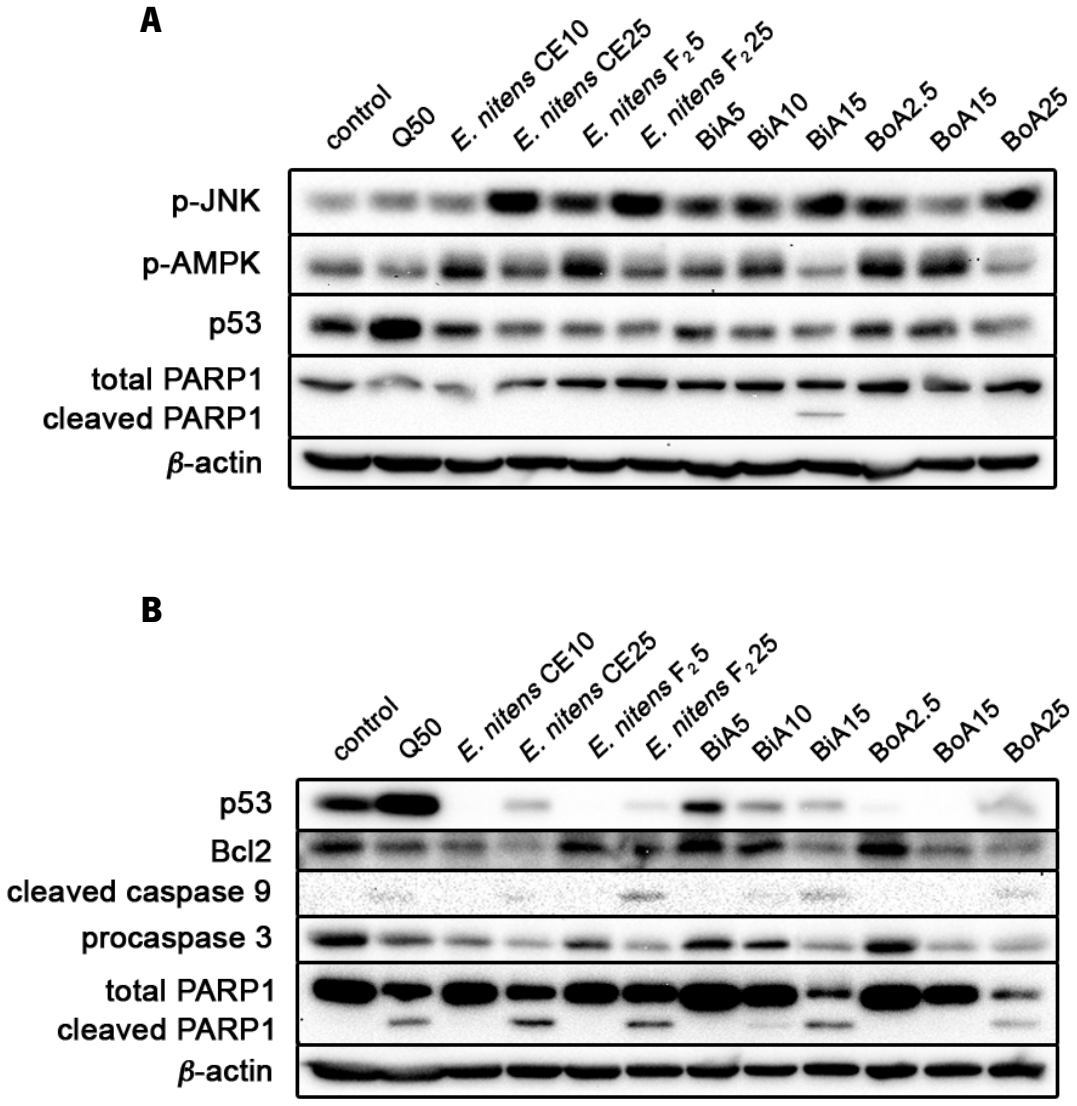


**Fig. 18 –** Effect of *E. nitens* crude extract (CE; at 10 and 25 μg/ml), *E. nitens* fraction 2 ($F_2$) extract (at 5 and 25 μg/ml), betulinic acid (BiA; at 5, 10 and 15 μM)) and betulonic acid (BoA; at 2.5, 15 and 25 μM)) on the levels of: **A)** phospho-JNK, phospho-AMPK, p53 and PARP1 after 24h of incubation; **B)** p53, Bcl-2, caspase 9, caspase 3 and PARP1 after 48h of incubation, using western blot. *β*-Actin was used as loading control. Images are representative of at least three independent experiments.

## 3. *EUCALYPTUS NITENS* EXTRACTS AND THEIR MAIN LUPANE ACIDS INDUCE JNK AND AMPK SIGNALLING IN HCT116 CELLS

Different signalling pathways play a fundamental role in cancer development. In pathways related with promotion of cell proliferation and inhibition of apoptosis, usually a kinase (proto-oncogene) is mutated resulting in the constitutive activation of the signalling pathway without external mitogenic signals (Orton *et al.*, 2005). The HCT116 cell line has an activation mutation in KRAS, which usually results in the activation of both MAPK/ERK and PI3K/Akt pathways.

In this work, the activity of these pathways was assessed by measuring the phosphorylation status of their end kinases – ERK and Akt, respectively – as previously described (Xavier *et al.*, 2009b). As expected for a KRAS-mutated cell line, phosphor-ERK levels were high even after 24h of incubation (time after medium change). However, both the extracts and isolated compounds BiA and BoA did not change (either induction or inhibition) significantly the phosphorylation levels (data not shown). In a previous study, Xavier *et al.* (2009b) also showed that the MAPK/ERK pathway did not mediate the anticancer effect of the triterpenoids UA. Unexpectedly, in view of the KRAS mutation, HCT116 cells did not show detectable levels of phospho-Akt after 24h of incubation. Probably, a functional PTEN is inhibiting the constitutive activation of the PI3K/Akt pathway due to KRAS mutation. Therefore, p-Akt levels were measured after 2h of incubation with the extracts/compounds and, although phosphorylation of Akt was observed, there were no significant differences when compared with control cells (data not shown). These results demonstrated that both PI3K/Akt and MAPK/ERK signalling pathways were not involved in the anticancer effects of *E. nitens* extracts and their main lupane acids.

The effect of test extracts/compounds on the stress-activated protein kinases p38 and JNK was also studied, in view of their involvement in the control of proliferation and induction of apoptosis (Ashwell et al., 2006; Dhanasekaram and Reddy, 2011). Although there was no significant effects on the phosphorylation levels of p38 (data not shown), both the *E. nitens* extracts and the lupane acids induced p-JNK levels in a concentration-dependent manner (Figure 18A). Differently from here, a previous work reported that both these kinases were induced in melanoma cells treated with BiA (Csuk *et al.*, 2011). However, it was recently shown that the triterpenoid UA induced apoptosis through JNK pathway in colorectal cancer cells (Xavier *et al.*, 2013). Since the tested triterpenoid-enriched extracts and their isolated compounds BiA and BoA

induce JNK signalling more strongly at the higher concentrations where apoptosis occur, it will also be interesting in future experiments to check whether apoptosis are dependent on JNK activation. These extracts/compounds at higher concentrations may be inducing stress in cells that leads to JNK activation that will result in apoptosis through the mitochondrial-caspase 3 pathway (Weston and Davies, 2007).

As discussed above, test extracts and compounds at low/intermediate concentrations were able to induce non-apoptotic cell death only after 24h of incubation. In fact, at this concentrations cells even grow as good as control cells during 24h and then the number of viable cells begin to decrease (see Fig. 14 A, C, E & G). Therefore, we asked whether these *E. nitens* extracts, BiA and BoA were inducing some kind of metabolic stress that precipitate cell to die. For that, the phosphorylation levels of AMPK were measured by western blot, since this kinase is a sensor of cell energy status (AMP/ATP ratio) and plays a fundamental role in the cellular energy homeostasis (Inoki *et al.*, 2012). As can be observed in Figure 18A, p-AMPK is markedly activated by both extracts/compounds after 24h of incubation, but, interestingly, mostly at intermediate concentrations where non-apoptotic cell death occur at later time (Fig. 14 and Fig.16). Another observation was that, at these concentrations where non-apoptotic cell death occur after 48h and 72h of incubation, cells morphologically lack their shining feature (contrarily to control cells) when observed under phase contrast in an inverted microscope. In addition, cells easily lose their adherence to the wells with a minimal physical impact, being left floating in the medium. With these results, we hypothesise that cells in the presence of low concentrations of triterpenoids are able to grow in the first 24h of incubation. Then, after the lacking of some important factor in the medium for energy production, cells undergo a reprogramming through the metabolic master regulator AMPK, such as by inducing autophagy and inhibiting protein synthesis through the mTOR pathway (Inoki *et al.*, 2012), in order to have energy for survival under starvations conditions. However, due to some interruption of this survival pathway, the AMPK is even more activated but, eventually with the energy loss, the cells end up in a sudden death due to a metabolic crisis. Interestingly, it was previously shown that the triterpenoids UA is able to inhibit autophagy in colorectal and breast cancer cells (Xavier, 2010; Xavier *et al.*, 2013), which is in accordance to the hypothesis outlined above. Furthermore, Duarte (2012) demonstrated that the triterpenoids OA induced non-apoptotic cell death in HepG2 cells grown under starvation condition, but no significant cell death occurs when grown in complete medium. In accordance to the above hypothesis, it was also shown that the transcription factors Sp1, Sp3

and Sp4 are downregulated in cells treated with BiA (Chintharlapalli *et al.*, 2011), and, as shown here, that the tested extracts/compounds induced a marked loss of p53 levels, which indicates that protein synthesis is maybe being interrupted. So, taking all this information into account, we can postulate that these intermediate concentrations of extracts/compounds induce mechanisms for cell survival, but due to some important defect cells does not even have the machinery to programme their death, dying of a metabolic failure.

## 4. *EUCALYPTUS NITENS* EXTRACTS AND THEIR MAIN LUPANE ACIDS INDUCE CELL CYCLE ARREST IN HCT116 CELLS

In order to evaluate if the decrease in the number of viable cells induced by the two *E. nitens* extracts and the two triterpenoids compounds was due to inhibition of cell growth, the cell cycle progression was studied by flow cytometry, after 48h of incubation with extracts/ compounds. Figure 19A shows that, in general, the extracts and the compounds induced a significant G2/M arrest, which indicates their ability to induce cell cycle arrest. However, in most of the cases, this effect does not seem to be concentration dependent, probably due to the high cell death already present in the higher doses tested. In the particular case of BiA at a concentration of 15 μM, besides G2 arrest it was also observed S phase arrest and a significant decrease in the percentage of cells at G1/G0 (Fig. 19 A e B). This effect of BiA is in accordance with the results reported by Wu et al. (2010) in leukaemia cells. Drag and collaborators (2009) reported that cell cycle arrest induced by BiA is dependent of the cell line. Overall, our data in the cell cycle arrest induced by the extracts/compounds confirm the results obtained in the anchora-dependent colony forming (Fig. 14), where we observed an inhibition of colony growth, there is colonies contained less cell number as compared with control condition.

In addition, with the cell cycle analysis, a concentration-dependent increase in the percentage of cells at sub-G1 was observed after treating cells for 48h with either one of the *E. nitens* extracts or lupane acids (Figure 19A). This indicates the ability of this extracts/compounds to induce DNA fragmentation, which is typical to what happens during apoptosis. These results confirmed the higher ability of *E. nitens* $F_2$ extracts and of BiA to induce cell death by apoptosis as compared with *E. nitens* CE and BoA, respectively, and mainly at higher concentrations (Figure 19).

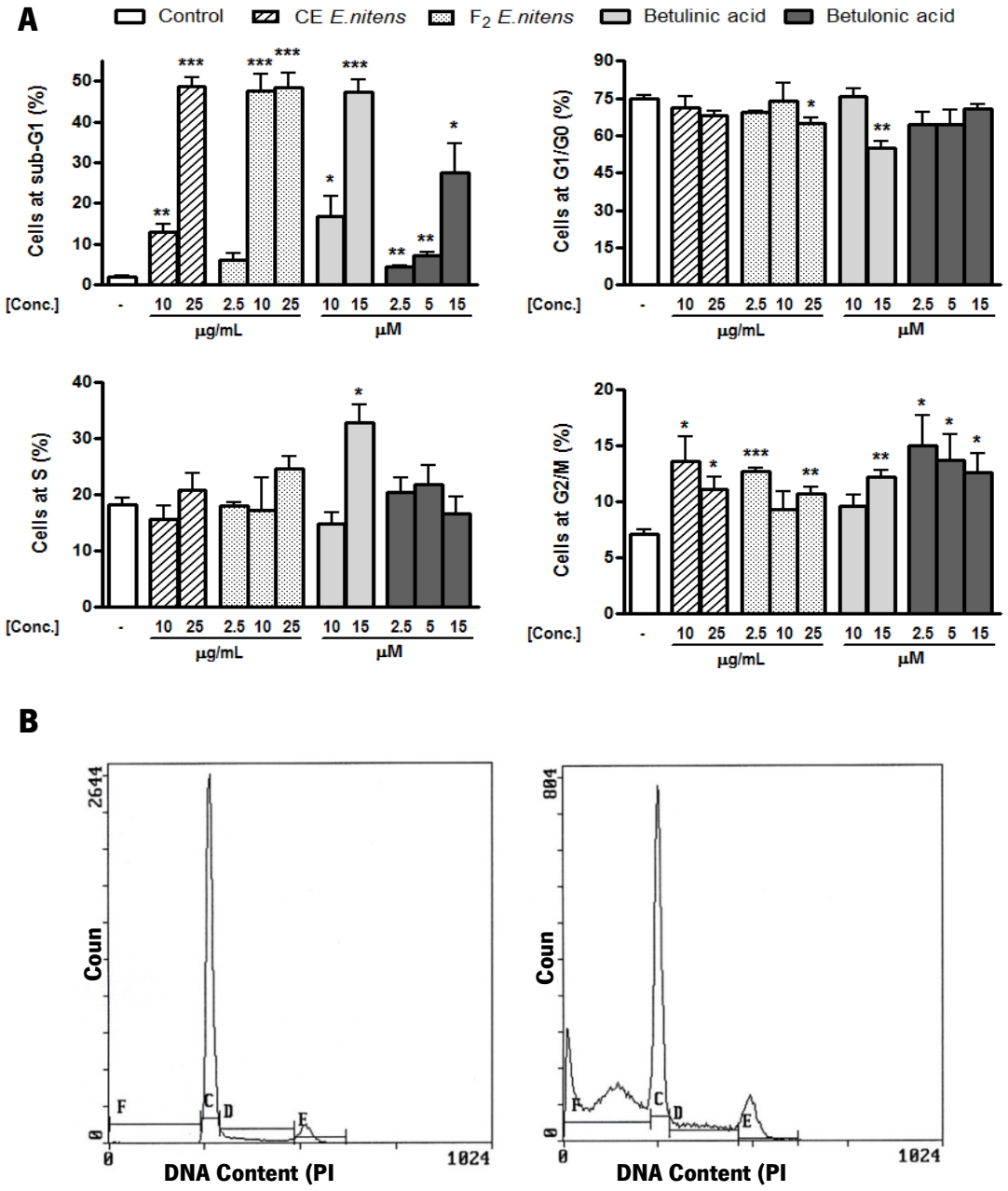


**Fig.19 –** Effect of *E. nitens* crude extracts (CE), *E. nitens* fraction 2 ($F_2$), betulinic acid (BiA) and betulonic acid (BoA) in the cell cycle of HCT116 cells after 48h of incubation. **A)** Values are mean ± SEM of at least three independent experiments. * P≤0.05, ** P≤0.1, *** P≤0.001, when compared with respective control, analysed by the Student's t-test. **B)** Representative images of cell cycle analysis in HCT116 cells in a control condition and treated for 48h with BiA 15 µM. Shown are cells at sub-G1 (gate F), and divided by the phases of cell cycle: G0/G1 (gate C), S (gate D) and G2/M (gate E). Clear in the images are the S and G2 arrest induced by BiA as compared with control, and a fraction of cell at sub-G1 typical of apoptosis.

# Conclusions and Future Perspectives

## 1. CONCLUSIONS

The ability to inhibit cell proliferation and to induce apoptosis are two of the major characteristics searched in new bioactive drugs for the treatment of cancer. In this study the effects of a crude lipophilic extract of *E. nitens* rich in triterpenic acids, and a fraction of this extract ($F_2$) even more enriched in this compound, was studied in HCT116 colorectal cancer cells. In addition, two of the major triterpenic constituents of the extracts, the betulinic and betulonic acids, were also evaluated for comparison.

Our findings suggest that all the extracts/compounds tested are biologically active, with potent anticancer effects. The *E. nitens* $F_2$ extract is more potent than the CE, with an $IC_{50}$ calculated based on cell viability of 1.3 μg/ml and 2.2 μg/ml, respectively, which correlates, therefore, with the extract content in triterpenoids, specially of BiA, BoA and UA. In respect to the tested isolated triterpenoids, BoA was shown to be 4 times more active than BoA, with an $IC_{50}$ of 0.8 μM and 3.9 μM, respectively. The anticancer potential of the extracts/compounds were confirmed by their ability to decrease significantly anchorage-dependent colonies formation and to inhibit migration of cells. This latter effect is a very important characteristic to be taken into account in cancer, since in later phases of disease development, cancer cells have the tendency to migrate from the tumour to metastasize other tissues in the body.

The anticancer effects of tested extracts/compounds are related in part to their inhibition of cell proliferation, since cell cycle arrest was observed. In agreement with that, inhibition of colony forming was correlated with the presence of colonies with less number of cells, as compared with control. It was also observed that induction of cell death contributes to the anticancer effects of the extracts/compounds, since PI-positive cells increase in a concentration dependent manner after 24h of incubation.

Our results also demonstrate that cell death induced by the extracts/compounds are in part apoptosis-related, especially at the higher concentrations tested, in view of the presence of nuclear condensation and a fraction of cells at sub-G1 phase. Interestingly, comparing the results between the lupane acids, although BoA is more potent decreasing cell viability, BiA is more potent inducing apoptosis. The apoptosis induced by *E. nitens* extracts and lupane acids seems to be mitochondrial and caspases-dependent, but may not involve p53 (Figure 20), since cleavage of caspase 9, caspase 3 and PARP1 was observed accompanied by a decrease of Bcl2

levels, whereas p53 levels were remarkably diminished. The PI3K/Akt, MAPK/ERK and p38 pathways were shown to not be involved in the anticancer effects of the tested extracts/compounds. On the other hand, the stress-related kinase JNK was induced in a concentration-dependent manner, which may be linked to the p53-independent induction of apoptosis by the mitochondrial-caspases pathway (Figure 20).

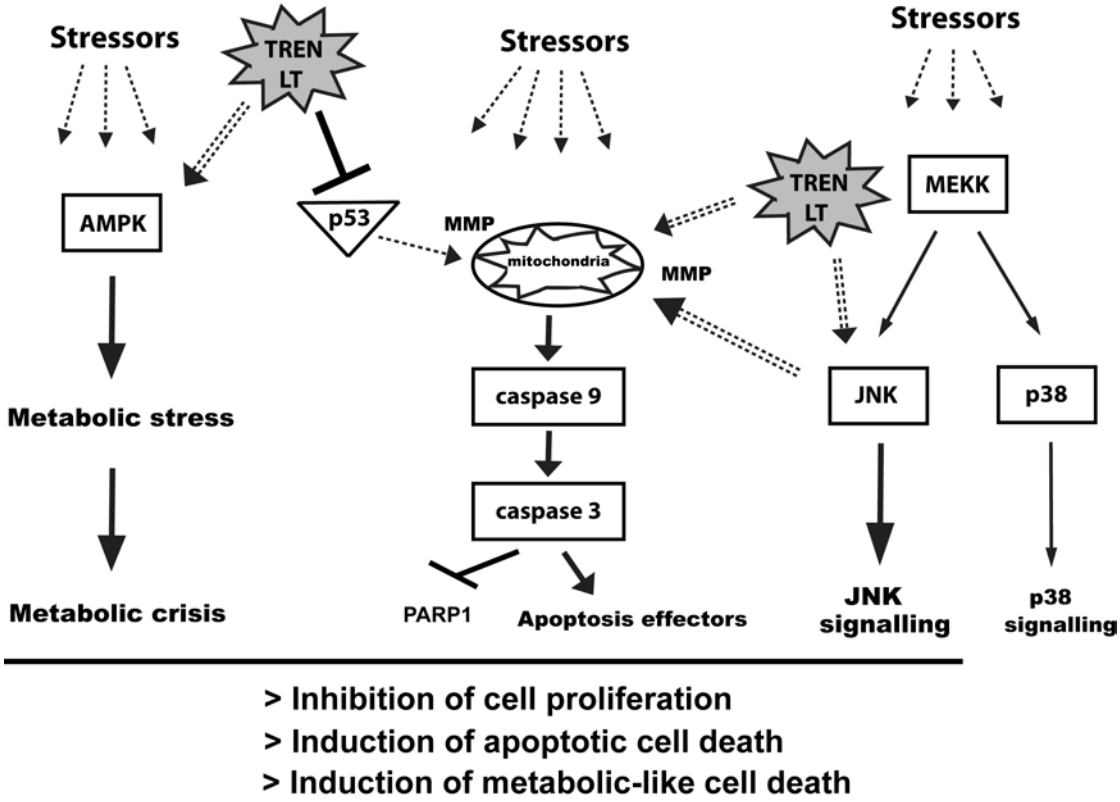


**Fig. 20 –** Scheme representing the possible mechanisms of action of the anticancer effect of the triterpenoids-rich *E. nitens* extracts (TREN) and the lupane triterpenoids (LP) betulinic acid and betulonic acid in HCT116 cells. MMP – mitochondrial membrane permeabilization.

However, apoptosis does not explain all the cell death induced by the *E. nitens* extracts and lupane acids. At low/intermediate concentrations, the extracts/compounds induce significant cell death that is not dependent in apoptosis, especially for *E. nitens* extracts and BoA. Interestingly, at these concentrations (but not at higher concentrations where apoptosis occurs), there was a significant activation of AMPK, as seen by its increased phosphorylation. Therefore, activation of this energy-sensing regulator, together with loss of p53, may be an indication of loss of energy for proper cell functions, such as for protein synthesis, that ultimately results in a metabolic crisis and cell death (Figure 20).

Summing up, the present results describes the potent anticancer effects of lipophilic extracts from *E. nitens* bark in HCT116 colorectal cancer cell, which can be an interesting source of bioactive natural triterpenoids used as potent anticancer drugs and/or for the semi-synthesis of new improved and more hydrophilic triterpenoids. Being the bark of *Eucalyptus* one of the main byproducts in Portugal from the pulp and paper industry, this work encourages the use of these residues to extract added-value compounds and to implement the biorefinery concept in their production chain.

## 2. FUTURE PERSPECTIVES

With this study it was demonstrated the potential anticancer effects of triterpenoids isolated from the bark of *Eucalyptus nitens*. They were able to trigger apoptosis accompanied by activation of the intrinsic mitochondrial-caspase 9 pathway, a decrease of Bcl-2 levels and the induction of JNK pathway. In addition, at lower doses, especially BoA was able to induce cell death independent of apoptosis and accompanied with the induction of AMPK activity and loss of p53. These novel results arise, however, many questions that remain unanswered that should be approached in future experiments.

Although the extracts/compounds induced apoptosis and activation of caspases, the present results do not prove that caspases are directly involved in apoptosis. By using pharmacological caspases inhibitors, or RNAi against caspases, it will clarify this issue. Also, it was hypothesised that apoptosis was triggered by JNK through induction of MMP, which could be depicted by using a pharmacological inhibitor against JNK and by measuring the mitochondrial membrane potential after treating cells with the *E. nitens* extracts or triterpenoids.

Because apoptosis was triggered by the triterpenoids and extracts accompanied with reduction of p53 levels, it may indicate that apoptosis was independent of p53. Making the same type of experiments in the isogenic HCT116 cells with p53 knockout, or by using RNAi against p53, would clarify if this tumour suppressor is really necessary for apoptosis induction by tested extracts/compounds. If this hypothesis is correct it is expected that these kind of compounds will induce cell death by apoptosis even in resistant cancer cells with p53 mutations, which would be an interesting source of anticancer drugs for decreasing cancer chemoresistance.

There are some studies that report the ability of some triterpenoids to induce proteasome activity, which may explain the decrease in p53 levels in this work and probably other proteins (like Sp transcription factors), which can lead to a non-apoptotic type of cell death. So, it would be interesting to measure the proteasome activity in the presence of these extracts/compounds and to test whether proteasome inhibition would prevent cell death induced by intermediate doses of BoA. That would explain the kind of "metabolic death" observed in this study due to a chronic degradation of vital proteins. However, the inhibition of their synthesis could be the cause of the reduction of these important transcription factors. In fact, AMPK activation, as observed here, is known to inhibit mTOR and, therefore, inhibit protein synthesis and to induce autophagy. However, it was recently reported that the triterpenoids UA inhibits autophagy at later steps (Xavier *et al.*, 2010; Xavier *et al.*, 2012b). If the same happens with BoA, cells would miss the acquisition of energy block from recycling cellular molecules, which would lead to a metabolic crisis and, eventually, death. This hypothesis should be explored in further experiments.

To have a broader perspective of its anticancer action, it would also be interesting to study the effects of the extracts/compounds in the inhibition of angiogenesis.

Also, due to the fact that betulinic and betulonic acids are structurally similar to cholesterol, those compounds may affect its cellular homeostasis and function, which will affect vital cellular processes, some already addressed above. So, investigating this relation could be very valuable.

All these possibilities would help clarify the full potential of the *E. nitens* bark as source of potent anticancer compounds, such as betulinic and betulonic acids.

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