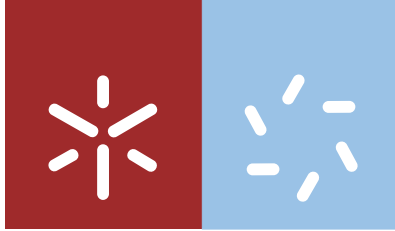Universidade do Minho
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Genotoxic, phytotoxic and protective effects of Portuguese propolis

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Dissertação de Mestrado
Mestrado de Biologia Molecular, Biotecnologia e Bioempreendedorismo em Plantas

Trabalho realizado sob a orientação da
Professora Doutora Ana Cunha
e do
Professor Doutor Rui Oliveira
e da
Professora Doutora Cristina Aguiar

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Declaração

Nome: Hélder Ricardo Cardoso Pereira

Endereço eletrónico: heldercpereira@yahoo.com **Telefone**: 938590214

Número do Bilhete de Identidade: 13739051

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Orientadores: Prof. Doutora Ana Cunha, Prof. Doutora Cristina Aguiar e Prof. Doutor Rui Oliveira

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

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Abstract

Propolis is a mixture produced by bees (*Apis mellifera* L.) from various plant sources with a diverse chemical composition including many bioactive phenolic compounds and terpenes characteristic of plant secondary metabolism. The bees use propolis mainly as a sealant for cracks in the beehive, but it has been used in human folk medicine for several millennia. A renewed interest in its study emerged in recent years due to the increasing popularity of natural products in foods, beverages and medicines.

Propolis extracts have been associated with varied biological activities, like antioxidant, antimicrobial and anti-inflammatory among others, and indicated for the treatment of several pathologies such as cancer or neurodegenerative diseases. Portuguese propolis, usually regarded as a by-product without any value in apiaries, remains insufficiently studied, hence the need for a better characterization of its properties.

In this study Portuguese propolis samples from different apiaries and different collection dates were selected and ethanolic and *n*-hexane extracts were prepared and tested using a yeast (*Saccharomyces cerevisiae*) and *in vitro* flax (*Linum usitatissimum*) cultures as models. Viability assays with yeast cells and plant growth analysis were performed to assess extracts toxicity. The yeast model was used to investigate mechanisms of cytotoxicity by the mitochondrial membrane potential-targeting fluorochrome rhodamine 123 approach and to evaluate genotoxicity by the comet assay. The *in vitro* plant model was used to evaluate extracts effects at the multicellular developmental level as well as in the photosynthetic function by pulse amplitude modulated fluorometry. Both photoinhibitiory and photoprotective potential against oxidative damage of the extracts were investigated using plants grown under high light intensities. Extracts antioxidant properties were also studied in yeast by flow cytometry using the redox-sensitive fluorochrome dichlorofluorescein diacetate.

Our results suggest that ethanolic extracts from Pereiro and Póvoa do Varzim apiaries are among the most toxic for both yeast and plants. They decrease yeast viability and mitochondrial membrane potential and dramatically affect early plant development inhibiting particularly root growth, photosynthetic efficiency and increasing non-photochemical quenching in a dose-dependent manner. Significant genotoxicity was found only in ethanolic extracts from Pereiro collected in 2010, also one of the most toxic in both models tested. However, some ethanolic extracts were also able to revert oxidative-induced damage. They reduced intracellular oxidation induced by hydrogen peroxide in yeast, and greatly recovered the total chlorophyll content reduced by high-light-induced photooxidative stress. Here, also *n*-hexane extracts were effective.

Globally our results are in line with the antioxidant properties revealed by propolis worldwide but also underline the strong toxicity in different cellular models suggesting that eukaryotic universal mechanisms/structures may be the most affected, possibly mediated by the production of reactive oxygen species. These effects are promising for different applications namely in food industry as preservative, agro-chemical as bioherbicide or pesticide and in pharmaceutical industry as a source of new drugs.

Resumo

O própolis é uma mistura produzida pelas abelhas (*Apis mellifera* L.) a partir de várias plantas, possuindo uma constituição química diversa que inclui numerosos compostos fenólicos e terpenos bioativos característicos do metabolismo secundário vegetal. O própolis é usado pelas abelhas como selante para fendas na colmeia, mas tem sido também usado na medicina humana há vários milénios. Um interesse renovado no seu estudo surgiu, em tempos recentes, devido à popularidade dos produtos naturais em alimentos, bebidas e medicamentos.

Os extratos de própolis estão associados a atividades biológicas variadas: antioxidante, antimicrobiana, anti-inflamatória e indicados para aplicação na terapia de várias patologias tais como cancro ou doenças neuro-degenerativas. O própolis português, frequentemente considerado um sub-produto sem valor na apicultura nacional, permaneceu até aos nossos dias insuficientemente estudado, daí a necessidade de realizar uma melhor caracterização das suas atividades.

Para estudar os extratos portugueses de própolis foram escolhidas várias amostras de vários apiários e períodos de coleta, que foram testadas numa levedura (*Saccharomyces cerevisiae*) e na planta do linho *in vitro* (*Linum usitatissimum*), utilizados como modelos biológicos. Ensaios de viabilidade com levedura e análise do crescimento do linho foram realizados para avaliar a toxicidade. O modelo levedura foi usado para investigar mecanismos de citoxicidade com o fluorocromo rodamina 123, que tem como alvo a membrana mitocondrial interna, e a genotoxicidade com o ensaio cometa. O modelo vegetal *in vitro* foi usado para avaliar os efeitos dos extratos ao nível do desenvolvimento multicelular, bem como na função fotossintética por fluorometria. Tanto o potencial fotoinibitório como fotoprotetor dos extratos contra danos oxidativos foram investigados com plantas expostas a intensidades luminosas elevadas. Novamente em levedura, realizou-se citometria de fluxo para aferir a atividade antioxidante com o fluorocromo fluoresceína diacetato e os efeitos ao nível mitocondrial com rodamina 123.

Os resultados obtidos sugerem que os extratos etanólicos de Pereiro e Póvoa são os mais tóxicos quer em levedura quer em linho. Diminuem a viabilidade celular e potencial de membrana mitocondrial em levedura, e afetam dramaticamente o desenvolvimento das plantas, inibindo o crescimento radicular, eficiência fotossintética e aumentando o quenching não fotoquímico. A genotoxicidade do extrato etanólico de Pereiro 2010 foi confirmada por ensaio cometa. Contudo, alguns extratos etanólicos também reverteram danos oxidativos. Reduziram a oxidação intracelular em levedura e recuperaram o teor de clorofilas reduzido por stresse fotooxidativo. Também, aqui os extratos de n-hexano foram eficazes.

Globalmente, os resultados estão em linha com as propriedades antioxidantes reportadas para o própolis, mas também sublinham a forte toxicidade em diferentes modelos, o que sugere que mecanismos universais dos eucariontes possam ser o alvo, possivelmente através da produção de espécies reativas de oxigénio. Estes efeitos são promissores para diferentes aplicações nomeadamente na indústria alimentar como conservante, agroquímica como bioherbicida ou pesticida, ou farmacêutica como fonte de novas substâncias.

Index

Introduction

1. Propolis: nature, composition and biological activities

1.1   Nature and composition of propolis

Propolis is a natural mixture produced by bees (*Apis mellifera* L.) from various plants' exudates, being abundant in resins and waxes with a myriad of compounds such as flavonoids. Characteristically, it is hard and brittle when cold but soft, pliable, and very sticky when warm, being also known as bee glue. This product is employed mainly as a sealant material on the beehive but also as an antiseptic. The very origin of the word *propolis* stems from the Greek language words *pro* – which means *for* or *in defence of* – and *polis – city*, in this particular case the beehive (Sforcin, 2007).

Besides this natural usage by bees, propolis has been used as medicine for over several millennia - with records dating from the ancient civilisations of Egyptians, Arabs or Greeks -, but in recent years a renewed scientific interest emerged with many studies focusing its biological activities, mainly envisaging pharmacological but also other applications, partially due to the fact that the majority of its components are considered safe substances for human ingestion, as most of its components are natural constituents of food (Grange and Davey, 1990; Lofty, 2006; Sforcin*,* 2007; Falcão *et al.*, 2010). For instance, given its known antibacterial and antioxidant activities, the use of propolis as a food preserver or as a food supplement to improve human health has already been tested (Banskota *et al.*, 2002; Moreira *et al.*, 2008; Fokt *et al.*, 2010).

Propolis' compounds originate from three main sources: plant exudates, bee's metabolic secretions and other materials added during propolis elaboration (Marcucci, 1995). Bees mix resins with their own salivary secretion, which contains ß-glucosidases that hydrolyse glycosyl flavonoids into flavonoid aglycones (Pereira *et al.*, 2002). Propolis composition is extensive and diverse, varying geographically with the flora from which bees collect the raw materials, the time period when such collections are made and also with the different behavioural patterns exhibited by different bee communities. In the Northern Hemisphere, bees collect in the end of spring, during the summer and in the beginning of autumn, while in some countries of the Southern Hemisphere such as Brazil the collection is made throughout the year (Bankova *et al.,* 1998). In Europe, poplar (*Populus* sp.) buds are the main source for the bees, hence the "poplar type" propolis.

2

Hundreds of compounds have been identified in propolis worldwide and range from polyphenols, phenolic aldehydes, sesquiterpenes, quinones, coumarins, amino acids and steroids to inorganic compounds. Propolis from temperate regions is rich in galangin, chrysin, pinocembrin, caffeic acid, ferulic acid and cinnamic acid (Marcucci, 1995; Falcão *et al.*, 2010). Other compounds such as isosakuranetin or kaempferide are characteristic of "poplar type" samples, but not typically present in large proportions (Marcucci, 1995; Park *et al.*, 2002; Falcão *et al.*, 2010). Portuguese propolis contains the most common compounds of the temperate zones already mentioned, but also several other phenolic components such as methylated, sterified or hydroxylated derivatives of already described flavonoids, rare forms of pinocembrin and *p*-coumaric ester derivative dimers (Falcão *et al.*, 2010). Generally, flavonoids from the resins and other phenolic compounds constitute approximately 50% of the mixture, while beeswax, pollen, and others represent, respectively, 30, 10 and 5% (Grange and Davey, 1990; Lofty, 2006; Sforcin *et al.*, 2007; Falcão *et al.*, 2010).

1.2   Biological activities of propolis

Due to the very complex chemical composition of propolis samples, different solvents must be used to extract and isolate the fractions/compounds before testing for biological activities. Propolis extracts, also known as balsams, are usually obtained with ethanol, methanol, dimethyl sulfoxide (DMSO), *n*-hexane, glycerol or even water, though many non-standard solvents can be used as well (Marcucci, 1995; Cunha *et al.*, 2004; Najafi *et al.*, 2007; Fokt *et al.*, 2010).

Antibacterial activity of propolis extracts has been reported against a wide range of Gram-positive bacterial strains of cocci or rods like *Streptococcus* or *Bacillus*, but only limited activity against Gram-negative bacilli (Grange and Davey, 1990; Mirzoeva *et al.*, 1997; Menezes *et al.* 1997). Growth inhibition of *Staphylococcus aureus* or *Escherichia coli* was described, albeit less significant for the latter. Antimicrobial activity varies depending on the type of propolis, the dosage and the solvents used (Lofty, 2006). Ethanolic extracts were found to be effective against anaerobic Gram-positive bacteria (Mirzoeva *et al.*, 1997), being this activity attributed to their polyphenol content. Propolis could inhibit bacterial growth by preventing cell division, disorganizing the cytoplasmic membrane and cell wall, and by inhibiting protein synthesis. What is interesting is that this plethora of actions does not match a single classic antibiotic mode of action (Grange and Davey, 1990; Takasi *et al.*, 1994; Lofty, 2006), foretelling a wide range of new antimicrobial molecules to unveil.

Virucidal activity of propolis extracts on several DNA and RNA viruses such as *Herpes simplex* type 1 and 2 (enveloped), poliovirus type 2 (non-enveloped), adenovirus type 2 (non-enveloped) were demonstrated *in vitro,* although the latter was less susceptible than herpes and polioviruses. This virucidal activity has been attributed to the high content in flavonoids like galangin and chrysin (Amoros *et al.,* 1992).

Propolis has also demonstrated antifungal activities with effectiveness on *Candida* yeast strains, particularly on *Candida albicans* (Ota *et al.,* 2001; Lofty, 2006). Other species proved also to be susceptible, as *Penicillium notatum* or *Aspergillus flavus* growth was inhibited with concentrations of propolis extracts of 15 to 30 mg/ml (Pepeljnajak, 1982). Regarding antiprotozoal activity, it was reported that ethanolic and DMSO extracts of propolis were active against *Trypanosoma cruzi* (Pepeljnjak, 1982; Higashi and de Castro, 1995; Lofty, 2006).

Ethanolic extracts of propolis could also exhibit anti-inflammatory activity, which might be promising to treat diseases caused by chronic inflammation. Caffeic acid phenethyl ester (CAPE), a component of propolis, was shown to have this property. This phenolic compound is a potent inhibitor of early and late events of T-cell activation and their immune response in inflammatory processes (Park and Kahng, 1999; Lofty, 2006).

Extracts from Brazilian propolis samples have shown cytotoxicity and the ability to inhibit the proliferation of human malignant tumour cells, possibly due to the compound artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) (Kimoto *et al.,* 1998). The cytotoxic effects of artepillin C were most noticeable in carcinoma and malignant melanoma by apoptosis, abortive mitosis and massive necrosis, as suggested by histological observations. Besides tumour growth suppression, the immune system was activated, with increased numbers of helper T cells. Propolis and artepillin C also appear to inhibit lipid peroxidation (Kimoto *et al.,* 1998, 2001). Besides artepillin C, other cinnamic acid derivatives, such as baccharin or drupanin, a common and abundant component of bee propolis, induce tumour cell death with less genotoxicity to haemopoetic cells than normal anticancer drugs (Lofty, 2006). Portuguese propolis extracts have also shown antitumoral activities (Carvalho, 2013). There is some evidence that propolis could block tumour angiogenesis. CAPE, already mentioned for its anti-inflammatory properties, could also be effective in tumour angiogenesis inhibition as was demonstrated by studies with chick embryo chorioallantoic membrane as an animal model (Song *et al.,* 2002).

Propolis has also been described as a protective agent of liver, heart and brain (Irmak *et al.,* 2003; Fuliang *et al.,* 2005; Lofty, 2006). Regarding liver protection, effectiveness was assessed with rat hepatocytes pre-treated with propolis extract and on which hepatotoxicity was induced by acetaminophen, yielding a decrease in mortality and severity of hepatocyte necrosis (Seo *et al.,* 2003).

It was also shown that propolis is able to inhibit the action of the enzyme hyaluronidase, which degrades hyaluronic acid, a major constituent of the extracellular matrix that contributes to cell proliferation (Starr and Engleberg, 2006). This enzyme has a role in infection by some pathogens like *Staphylococcus aureus*, being produced by the pathogen to obtain hyaluronic acid that is used as carbon source (Starr and Engleberg, 2006; Moreira *et al.,* 2008).

Several human pathologies result from an increased level of reactive oxygen species (ROS) and propolis extracts as well as some of its compounds have already been reported to have a role in reducing ROS levels and thus on the related disorder symptoms (Ilhan *et al.*, 1999; Lofty, 2006). For instance, oxygen-derived free radicals have been implicated in the pathogenesis of cerebral injury after ischaemia – a restriction of blood supply to tissues that causes oxygen deprivation. The subsequent restoration of circulation can cause additional damage on ischaemic tissues as the sudden supply of oxygen leads to the formation of many free radicals, and CAPE, which also possess antioxidant properties and can act as free radical scavenger, demonstrated relevant activity in alleviating the symptoms of this disease (Lofty, 2006). Also, cardiomyopathy is the consequence of oxidative stress through the action of free radicals, when in the presence of the cancer treatment drug doxorubicin. The effects of propolis on doxorubicin-induced cardiomyopathy were studied in rats by intraperitoneal administration and it was observed that propolis had a protective effect towards the cardiac muscle comparable to that of rutin, a well-know cardioprotective flavonoid (Lofty, 2006).

2. Oxidative stress: sources of reactive oxygen species, damage and repair

2.1 Reactive oxygen species formation and scavenging mechanisms

Reactive oxygen species (ROS) are formed through the reduction of molecular oxygen to water by the transfer of four electrons, a process that all aerobic organisms perform during the accumulation of energy through the electron transport chain or metabolism of exogenous compounds. Many environmental factors induce ROS formation in cells (Fig. 1A). The mitochondria (Fig. 1B) and chloroplast (Fig. 1C) are the main sources of ROS in eukaryotic cells, due to the electron transport chain of oxidative phosphorylation and of photosynthesis, respectively. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are examples of common and noxious ROS that affect cellular components like nucleic acids, proteins and lipids, inflicting severe damage to cells and to the organism. These ROS are known causes of degenerative diseases such as cancer, epilepsy or neurodegenerative diseases and integral part of the biological ageing process (Harman, 1991, 2006; Malinska, 2010; Mubarakshina and Ivanov, 2010).
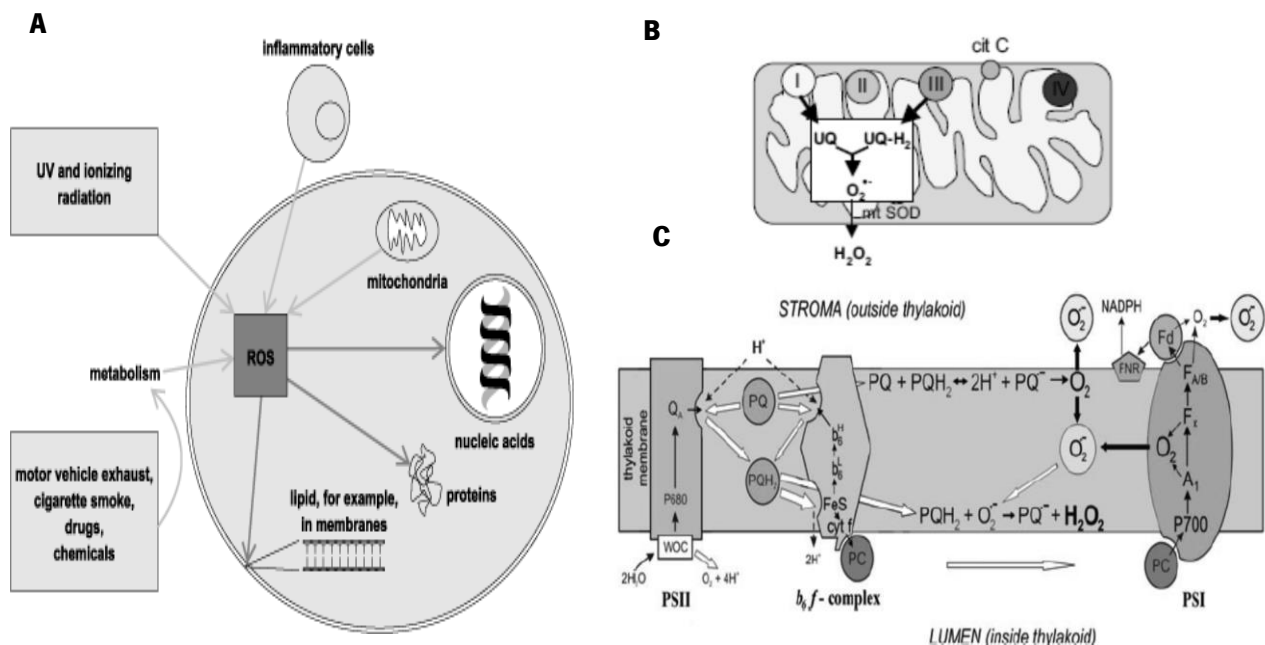

Figure 1. Potential sources and targets for reactive oxygen species in animal cells. **A)** Enviromental factors and intracellular targets of ROS; **B)** Oxidative phosphorylation on mitochondria; **C)** Electron transport chain on chloroplast (Cooke and Evans, 2013; Novo and Parola, 2005; Mubarakshina and Ivanov, 2010).

The presence of intracellular H_2O_2 can oxidise cysteine and methionine residues of iron-sulfur proteins, leading to the formation of free radicals by the Fenton reaction (Fig. 2) catalysed by transition metals such as iron or copper.

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + \dot{O}H + OH^-$$
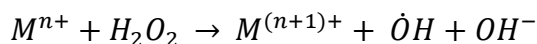
Figure 2. The generic reaction mechanism as first described by Fenton, on which transition metals convert hydrogen peroxide into a more harmful hydroxyl free radical and hydroxide anion.

The resulting oxidised metals can be re-reduced by molecular oxygen free radical on the Haber-Weiss reaction so that these metals can be reused once again in the Fenton reaction. The conjugation of hydrogen peroxide, molecular oxygen radicals and transition metals make for a very dangerous predicament for the cells. The formation of ROS like superoxide or singlet oxygen in the mitochondria and in the chloroplast are well-documented facts (Novo and Parola, 2005; Malinska, 2010). Singlet oxygen particularly is produced in the photosystem II of the chloroplast (Pospíšil, 2012). Enzymes such as catalases or peroxidases catalyse the scavenging of hydrogen peroxide, being one important line of defence on living organisms. Cells need to maintain a reducing intracellular state, in spite of a largely oxidising extracellular environment, allowing them to perform many functions such as the proper folding of proteins (Smirnoff, 2005; Drakulic *et al.*, 2005).

In a state of homeostasis, ROS production is counter-balanced by enzymatic processes, as above mentioned, and antioxidant compounds present in the cells. These molecules are in balance, so it must be accepted that a "normal" level of ROS always occurs causing minimal damage. In the event of a loss of homeostasis caused by increased free radical production or failure of antioxidant defences, it is said that cells are under oxidative stress and subject to cellular damage (Izawa *et al.,* 1995; Munné-Bosh and Alegre, 2003; Smirnoff, 2005; Collins, 2009).

While ROS molecules are undoubtedly harmful in high concentrations, they are also needed at low concentrations as inter- and intracellular signalling molecules. The ROS signal transduction network is an evolutionary conserved pathway on all aerobic organisms. Molecules such as singlet oxygen, superoxide and hydrogen peroxide act as signal transductors to control a large array of biological processes ranging from the regulation of development and growth to responses to biotic and/or abiotic stimuli. In plants, ROS signaling was shown to be involved in processes of seed after-ripening, lignification, root hair formation, closure of stomata or programmed cell death. The key to this phenomenon seems to be the cells ability to maintain a low steady-state level of ROS molecules, while allowing for its accumulation in specific subcellular locations

that act as signals (Izawa *et al.,* 1995; Munné-Bosh and Alegre, 2003; Smirnoff, 2005; Collins, 2009; Suzuki *et al.*, 2011).

2.2 DNA integrity, toxicity and repair

DNA integrity is essential for the viable and normal function of organisms. Numerous endogenous and exogenous agents such as ROS molecules produced in the electron transport chain in the mitochondria and chloroplasts, ionizing radiation, exogenous toxic compounds or inflammation compromise that integrity and are sources of genotoxicity. A popular hypothesis, albeit not unanimously accepted, is that oxidative stress is directly connected to the ageing process (Harman, 1991). This postulate assumes that our cells accumulate damage in its constituent biomolecules slowly over time causing organs to deteriorate over the years, in an unavoidable obsolescence. DNA can be damaged through single and double-stranded breaks, base and sugar replacements, apurinic/apyrimidinic lesions or DNA-proteins binding (Collins, 2009). Cells have some defensive mechanisms, including superoxide dismutase, catalase, several peroxidases and antioxidants such as ascorbate, tocopherol, uric acid, β-carotene and glutathione that allow for the elimination of ROS deleterious effects (Izawa *et al.*, 1995).

3. Plant secondary metabolites and protection against environmental challenges

3.1 Coping with high light stress conditions in the chloroplast: a tough "iron-arm"

Plants are exposed to a wide array of environmental stress conditions, ranging from low water availability, temperature fluctuations, nutrient deprivation and high light exposures. These stresses lead up to an imbalance between the amount of reactive oxygen species and the antioxidant defences (Smirnoff, 1993; Pastori and Foyer, 2002; Xiong *et al.*, 2002).

The photosynthethic pathway allows the plant to gather sunlight and generate enough chemical energy to proceed with overall thermodynamically unfavourable reactions of the Calvin cycle, involved in sugar synthesis from CO_2. Molecular diatomic oxygen is a by-product of photosynthesis that diffuses passively between cells and leaves source organs through stomata. However, when exposed to light, the O_2

concentrations may reach high levels and an imbalance between the electron transfer flow and the recycling rate of photochemical products in Calvin cycle renders the chloroplasts particularly susceptible to oxidative damage by ROS formation (Smirnoff, 2005). In addition, exposure to higher than normal light intensities may lead to higher intracellular amounts of ROS due to increased rates of molecular oxygen photoreduction in photosystem II but also to and increased flux of H_2O_2 in peroxisomes via photorespiration (Niyogi, 1999; Mittler, 2002; Heldt, 2005; Smirnoff, 2005; Pospíšil, 2012).

Plants have several mechanisms to cope with this variable oxidative challenge conditions and maintain homeostasis, but when the rate of light absorption far exceeds the capacity of their photosynthetic apparatus, this frequently leads to the repression of photosynthesis in a process known as photoinhibition.

Indeed, while ROS are needed at low concentrations for cell signaling, at higher concentrations they cause severe damage at several organization levels of the plant cells such as the chloroplasts. Apart from the enzymatic (superoxide dismutase, catalase and peroxidases) and non-enzymatic antioxidant compounds already mentioned above, the plant cells have other mechanisms that also protect the chloroplast from oxidative damage like the xanthophyll cycle (XC) and photorespiration (Doke, 1997; Munné-Bosh and Alegre,. 2003). Carotenoids, tocopherol, ascorbate and glutathione help maintaining the integrity of the photosynthetic membranes under oxidative stress (Havaux, 1998; Smirnoff and Wheeler, 2000; Munné-Bosch and Alegre, 2003). Tocopherol and β-carotene have also been shown to act in singlet oxygen scavenging in lipid membranes and in photosystem II protection (Munné-Bosch and Alegre, 2003).

The de-epoxidised carotenoids of the XC zeaxanthin and antheraxanthin coupled with a low thylakoid lumen pH, which results from high light conditions, and a minor light harvesting complex protein named PsbS, can act to dissipate the excessive electron energy from photosynthesis as heat, in a process known as non-photochemical quenching (NPQ). The XC consists on the interconversion of violaxanthin, antheraxanthin and zeaxanthin during the period of high light exposure when the violaxanthin accumulated on leaves starts to be converted into the other two (Fig.3) (Young *et al.* 1997; Smirnoff, 2005; Baker, 2008).
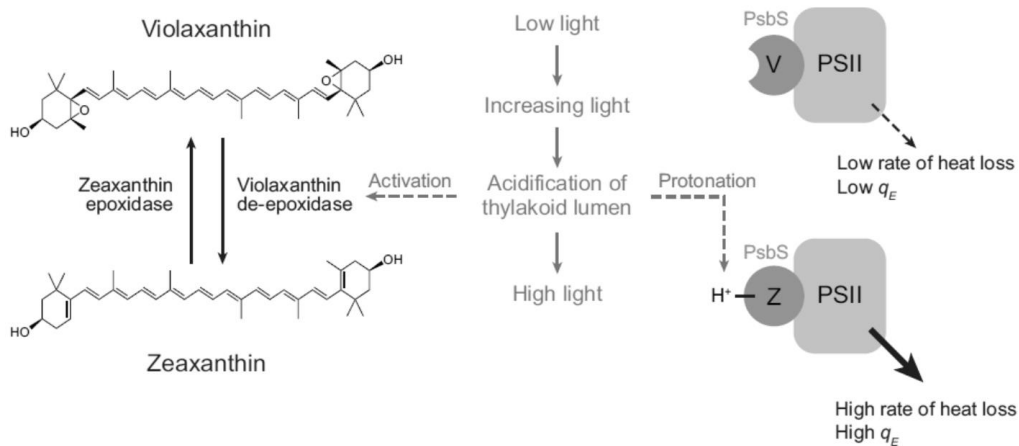
Fig. 3 Interconversion of xanthophyll cycle plant carotenoids, and the mechanism of heat dissipation by non-photochemical quenching (Baker, 2008).

A major cause for photoinhibition is the overexcitation of photosystem II reaction centre, which leads chlorophyll molecules to attain a triplet state (^{3}Chl.) and resulting in the formation of singlet oxygen ($^1O_2$) (Fig.4). The triplet excitation energy of some chlorophyls at specific sites of the light harvesting complex can be effectively quenched by lutein, another carotenoid, but others are not and in the presence of molecular oxygen contribute to singlet oxygen formation. Some carotenoids are able to revert the triplet state of chlorophyll and the singlet state of oxygen to their ground fundamental state, forming a triplet carotenoid that dissipates its energy as heat via non-photochemical quenching.

Plants have other mechanisms to cope with light stress besides scavenging, dissipating and repairing. The avoidance of excessive photosynthetic-dependent ROS production and thence oxidative damage is a way to escape stress effects. The well-known chloroplast movement observed under high light conditions, which consists of the relocation of chloroplast from the cell surface to the side cell walls parallel to sunbeams (Heldt, 2005), is an important example.
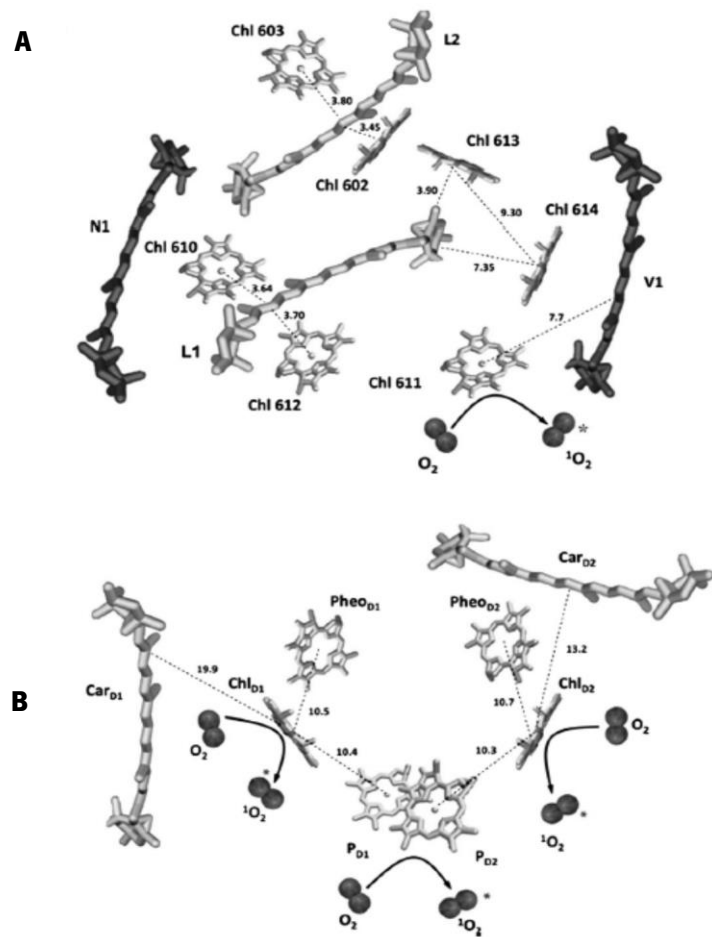
Figure 4. Singlet oxygen generation in the light harvesting complex **(A)** and the reaction center of PSII **(B)** (Pospíšil, 2012).

3.2 Plants chemical wealth: defence against abiotic stress and communication in war and peace

Terpenes, or isoprenoids, are a major class of secondary metabolites in plants. Terpenes are highly diverse, both in functions and activities as well as in their chemical structures, but they share the same method of sequential assembly from a couple building blocks, each of which consists of a branched five-carbon atoms chain. The two building blocks are the interconvertible isopentenyl pyrophosphate (IPP) and

dimethylallyl pyrophosphate (DMAPP), which are condensed together in a sequential way through enzymes called prenyltransferases. The major families of terpenes are mono-, di-, tri- and sesquiterpenes. All the carotenoids are in fact diterpenes, synthesised from building blocks of the precursor molecule geranyl-geranyl pyrophosphate (GGPP) and the sterols like cholesterol are triterpenes (Humphrey and Beale, 2006; Crozier *et al.*, 2006).

Limonoid triterpenes have biological activities against insects and are used to develop commercial insecticides (Isman *et al.*, 1997). Terpenes are constituents of many plant species essential oils with reported antimicrobial activities (Delaquis *et al.*, 2001). There are also terpenes with anti-cancer potential, for instance well know anti-cancer drug, paclitaxel, is a terpene metabolite (Humphrey and Beale, 2006).

In plants, the phenylpropanoid metabolism constitutes a major pathway of secondary metabolism leading to the synthesis of phenolic compounds. These compounds are a wide group of molecules characterised for possessing at least one aromatic ring with one or more hydroxyl groups attached. Phenolic compounds can be classified in two groups, flavonoids or non-flavonoids. Flavonoids are a family of diverse, over 9000 compounds, which comprise a large portion of the secondary metabolism of plants. The main classes of flavonoids include the flavones, flavonols, isoflavones, chalcones, coumarins and anthocyanidins. In plants, flavonoids could have diverse roles such as UV protection, pigmentation, in the stimulation of nitrogen fixing and disease resistance. Flavonoids can act as singlet oxygen quenchers and although mainly distributed on the leaf surface and epidermal cells, they are also present in chloroplasts (Crozier *et al.*, 2006; Hernández *et al.*, 2008). The phenylpropanoid pathway is frequently induced by stress conditions of a wide range, such as high light radiation, high temperature or pathogens. Many phenolic compounds of the plant secondary metabolism such as caffeic acid, kaempferol or apigenin have reported antioxidant activities (Zheng and Wang, 2001). The production of ROS is also upregulated at periods of stress and phenylpropanoids such as coumarins, flavonoids, phenolic acids or stilbenes have been for a long time associated with several stress related function, most remarkably protection against photoinhibition and scavenging of ROS (Young *et al.*, 1997; Li *et al.*, 2000; Smirnoff, 2005; Crozier *et al.*, 2006). Some flavonoids like galangin and 7-hydroxyflavanone can act also as pro-oxidants besides their superoxide scavenging activities (Dewick, 2002).

3.3 Propolis as a blend of allelochemicals

Plant allelochemicals, plant secondary metabolites with communication roles between species, include an array of compounds such as phenols, terpenes, alkaloids, quinones, saponines, tannins, fatty acids or peptides (Crozier *et al.*, 2006). Allelopathy, an important ecological phenomenon, consists on the production and release of allelochemicals by certain plant species that have effects on other plants species physiology and development or even in other type of organisms. Some plants produce phenolics compounds such as rutin or chlorogenic acid that are toxic to certain insects' larvae that predate on plants foliage (Isman and Duffey, 1982, Medeiros, 1990; Delachiave *et al.*, 1999). Many plant phenolic compounds like *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, catechol and protocatechuic acid have allelopathic effects against mycorrhizal fungi such as *Laccaria laccata* and *Cenococcum graniforme* (Pellissier, 1993). Also, many plant species used as source of raw materials by bees to produce propolis have reported biological activities and allelopathic effects. Propolis extracts from Bulgaria, whose plant source is *Populus nigra* similar to Portuguese propolis display antimicrobial activities against fungi and bacteria (Salomão *et al.*, 2004). It is known, for instance, that Brazilian propolis is made from exudates of *Baccharis dracunculifolia*, a plant with recognised allelopathic potential against other plant species (Gusman, 2008).

Plant allelochemicals are likely present in the composition of propolis, and considering the biological activities attributed to secondary metabolites like alkaloids, phenolic compounds and terpenes, namely the antioxidant potential, it is reasonable to hypothesize a protective effect of propolis against induced oxidative stress on living cells. Indeed, some phenolic compounds present in propolis extracts are known to play *in vivo* the role in maintaining homeostasis and counter oxidative stress damage, thus explaining the antioxidant properties of those propolis samples (Kasai, 2002; Humphrey and Beale, 2006; Collins, 2009).

As referred before, many plant secondary metabolites like phenylpropanoids or terpenes have toxic activities that protect the plant against pathogenic microorganisms or herbivores. These compounds can act as natural pesticides, which in some plants can account for 10% of its dried biomass. In response to microbial infections plants synthesise substances called phytoalexins, which comprise many of the above-discussed chemical classes such as flavonoids, isoprenoids or stilbenes. Many of these phytoalexins like psoralen, xanthotoxin or bergaptol have demonstrated antibiotic activity against a broad spectrum of pathogenic fungi and bacteria. Psoralens in particular have phototoxic effects, that is, their toxicity is activated by UV light exposure (Pathak and Fitzpatrick, 1992; Manderfeld *et al.*, 1997; Hendt, 2005).

Although there is a huge amount of accumulated knowledge about phytochemicals and related bioactivities, about the wide infochemical web related to species-species communication at the ecological level, and many studies focusing on propolis composition and bioactivities, we believe that there are many interesting biological and other properties still to uncover. Two examples are propolis potential genotoxicity and genoprotection and phytotoxicity. To address these topics two powerful techniques were used in this work, and in this sense some detailed information will be provided in the next two sections.

4. Assessing effects on photosynthesis by pulse amplitude modulated fluorometry

Light energy absorbed by the leaves, and ultimately by the photoreceptors of photosystems I and II, remain only very transiently in the excited pigments. There are three main competitive pathways by which this excitation energy will decay: most of it is relayed to the electron transport chains (the photochemical pathway or photochemical quenching), some can be dissipated as heat (the non-photochemical quenching) or emitted back as fluorescence, as light causes the transient closure of some PS reaction centres and hence limits the photochemical pathway (White and Critchley, 1999; Baker, 2008). The proportion of energy that is channelled by each of the pathways will depend on the light conditions, light adaptation status of the plant and stress conditions that may affect different aspects of the photosynthetic machinery.

The pulse amplitude modulated (PAM) fluorometry is a very useful method to study the effect of different factors - environmental, biotic, abiotic, extracts, compounds - on photosynthesis. While being rapid and very sensitive, it can be also non-intrusive and used on intact leaves as well as isolated chloroplasts or subchloroplast particles (White and Critchley, 1999; Schreiber *et al.*, 1995), but virtually in all photoautotrophic organisms or samples. Concerning only plant photosynthesis, this technique has been widely used to evaluate the effects of many types of stresses, like water deficit in crop species (Carvalho *et al.,* 2011) or light stress (Dixon and Paiva, 1995; Hutin *et al.*, 2003), but also to study non-foliar systems (Breia *et al.*, 2013).

The PAM technique is based on the analysis of chlorophyll fluorescence yields of photosystem II (PSII) under different experimental controlled conditions, and makes use of short light saturation pulses (typically less than 1 s at several thousand μmol of photons m^{-2} s^{-1}) to transiently drive a very high proportion of PSII reaction centres to closure (virtually all), with Q_A (the primary quinone electron acceptor of PSII) at its most reduced state, thus unabling the photochemical pathway (Baker, 2008; Papagergiou and Govindjee, 2004)

what will result in maximum fluorescence yield. This method allows the determination of many important photochemical and non-photochemical parameters, and hence to take conclusions about the photosynthesis status. The most commonly used parameters are the maximum quantum yield of PSII (F_v/F_m), measured in dark-adapted samples, and the effective quantum yield of PSII (Φ_{II}) and non-photochemical quenching (NPQ) in samples under light conditions. F_v/F_m represents the intrinsic or maximum quantum yield of photosystem II (PSII) measured in dark-adapted samples; the effective quantum yield (Φ_{II}) of PSII represents the efficiency by which the absorbed energy is actually channeled to photochemistry and measured under light-adapted samples. The non-photochemical quenching (NPQ) represents the absorbed light energy that is dissipated by other processes (like in the form of heat) than photosynthesis.

Considering all this characteristics, this technique will be used to assess the effects of propolis extracts added to the culture medium in the photochemical and non-photochemical capacities of *in vitro* grown plantlets of flax.

Carotenoids have essential roles in photosynthesis as they contribute to light harvesting and are associated with the photosystem II reaction centre (Smirnoff, 2005). Carotenoids can contribute to photoprotection and act as antioxidants by quenching singlet oxygen and also by reacting with superoxide and other free radicals. If carotenoid synthesis is somehow inhibited or the degradation accelerated, the chloroplast undergoes rapid photo-oxidative damage. Zeaxanthin is a carotenoid noteworthy for its involvement in the non-photochemical quenching of excitation energy from PSII, in which the excess energy is transferred to zeaxanthin and freed as heat (Smirnoff, 2005; Havaux and Nyogi, 1999; Barry *et al.*, 1990).

5. Assessing cellular damage and protection against oxidative stress

5.1   DNA damage assessment by comet assay

To assess DNA damage in individual cells a technique such as single-cell gel electrophoresis can be employed. Single-cell gel electrophoresis, also known as the comet assay or microgel electrophoresis, is a widely used method for assessing damage of DNA, hence its usefulness for genotoxicity tests or DNA damage and repair studies amongst many others. Östling and Johanson reported this assay in 1984 as a technique for the direct visualisation of DNA damage in individual cells. Cells are embedded in an agarose microgel, lysed, electrophoresed and stained with an appropriate DNA binding fluorochrome. The electric current makes the negatively charged supercoiled DNA molecules migrate and the relaxation imposed by single strand breaks or

fragmentation by double strand breaks lead to higher displacement of DNA towards the anode. The cells with the staining dye and a clear one-directional path of leaked DNA outside resemble a comet and its tail and thus prompted the naming of the assay. The average tail length is taken as a measure for genotoxic damage. The original Östling and Johanson method, performed under neutral conditions, had limitations related with the sensitivity for single-stranded DNA breaks on supercoils only detecting double-strand breaks, but the assay was easily adapted by Singh *et al.* in 1988 to more stringent alkaline lysing conditions so that it also allows for the detection of the single-strand DNA breaks, by relaxing and unwinding the supercoils (Fairbairn *et al.*, 1995; Menke *et al.*, 2000; Collins, 2009).

The comet assay is nowadays ubiquitous in genotoxicity testing. It is simple and easy to perform and allows for rapid and visual assessment of DNA damage in individual cells (Dhawan *et al.*, 2009).

5.2 Flow cytometry as a mean to assess cell damage and protection in yeast

Flow cytometry is a laser-based technique that allows for the detection and counting of cells on suspension by using several fluorescent labels, the fluorochromes. Flow cytometry techniques offer several advantages over traditional culture-based techniques to quantify cells, the latter being often time consuming and not suited for non-culturable microorganisms (Veal *et al.* 2000).

Perhaps the most attractive proposition of flow cytometry cell measurement is the ability to obtain real-time *in vivo* information about the microorganisms. The two fluorochromes used in this work (fluorescein diacetate and rhodamine 123) for cell staining will allow for both the quantification of propolis-induced damage and to assess protective effects on yeast cells (Veal *et al.*, 2000) after an imposed oxidative challenge.

6. Biological models to study propolis biological activities

To evaluate a range of biological effects of propolis extracts at the developmental, physiological, cellular, and DNA level, two different eukaryotic models were used: the unicellular yeast *Saccharomyces cerevisiae* and the plant species *Linum usitatissimum* (flax). The use of different models allows comparing responses and also identifying specific effects and more transversally fundamental modes of action.

6.1   Yeast as a model of genotoxicity

The potential genotoxicity of a drug candidate such as propolis extracts or compounds can be assessed using a simple organism like yeast as a model. *Saccharomyces cerevisiae* is a prime example of a unicellular eukaryote that shares the essential cellular pathways with even the higher multicellular eukaryotes (Seioghe and Wolfe, 1999; Liu *et al.*, 2008) and thus is widely used to study complex physiological and molecular processes on metazoan cells.

Major benefits of *S. cerevisiae* include the rapid growth and tractability, being cheap and simple to maintain in culture. Adding to that, the availability of the full genome sequence of *S. cerevisiae* makes this organism a very interesting proposition for various fields of biology (Pabla *et al.*, 2006; Grzelak *et al.*, 2006), namely in the investigation of drug effects on particular molecular, metabolic or other cellular mechanisms.

Yeast cells adapt their growth and development depending on the nutrients available. Yeast can grow on a variety of compounds as carbon sources such as glucose, fructose, sucrose, raffinose or trehalose. However, yeast cells have a preference for glucose or fructose over all other mono-, di- or trisaccharides, and prefer fermentable carbon sources like all the aforementioned over nonfermentable compounds like ethanol, glycerol or acetate. These nonfermentable energy sources are not usable in anaerobic processes, only being catabolised by oxidative phosphorylation in the mitochondria. This conditional nutrient preference is regulated by several key enzymes in glycolysis and gluconeogenesis, in which glucose act as the repressor of the transcription of genes needed for less favourable energy sources catabolism. This repression by glucose is the basis of the Crabtree effect, allowing for the distinction between species that aerobically perform fermentation, such as *Saccharomyces cerevisiae*, from those that do not, like *Kluyveromyces sp.* (Broach, 2012). In this work the yeast model will be used to assess propolis effects on cytotoxicity, DNA damage and protection and also on cell redox status.

6.2   *In vitro* cultures of *Linum usitatissimum* as a plant versatile multicellular platform

Linum usitatissimum L., commonly known as flax, is a widely used, easily to cultivate angiosperm. This species is cultivated worldwide for the production of oil and fibre (Chakravarty and Srivastava, 1996; Millam *et al.,* 2005). Its high regeneration rate *in vitro*, short life cycle and small genome size are key attributes for researchers.

In vitro cultures of flax have been well established in the late 1990s (Cunha and Fernandes-Ferreira, 1996, 1999) and recently this plant system was successfully used in the evaluation of the effects of propolis extracts on different physiological and early plant developmental aspects (Pereira, 2008; Paulo, 2009; Oliveira, 2011; Amorim, 2011; Apresentação, 2012; Carvalho, 2012). Major benefits of this biological plant platform include its versatility – a wide variety of types of cultures (suspensions, *calli*, shoot and root cultures, plantlets) allowing to evaluate the effects of extracts and compounds at different organizational levels (from sub-cellular to plant developmental); sensitivity; the robustness and consistency of responses; as well as the ease and economy of culture maintenance. In this work, this model will be used to assess propolis effects at the plant developmental level and also in the photosynthetic function, evaluating both the inhibitory properties and protective effects against oxidative stress induced by high light.

7. Objectives and scope of this work

There is a renewed interest in natural products for their potential applications on several industries (pharmaceutical, cosmetic, agrochemical), which have intensifiedprospection efforts for active substances. Given the concerns often raised by the possible toxicity of chemically synthesised active compounds, persistence in the soils and effects on non-target organisms in the case of pesticides and herbicides, the generalized acquisition of resistance by the target organisms, as well as the often difficult and expensive process that is to synthesise the complex molecules that most of the bioactive metabolites are, there is a need to study sources of natural bioactive compounds. Also, the narrow range of chemical motifs and correspondent molecular target sites of the currently available herbicides, responsible for the increasing number of weed resistances (Duke, 2011), demand an urgent response.

Bee propolis is a well-known by-product of the beehive with a rich and complex chemical composition and many reported bioactivities and therefore it appears to constitute an excellent natural raw material to address these concerns for finding cheap, natural and safe substances. The polyphenols such as the flavonoids are often associated with biological activities namely antioxidant, antimicrobial, anti-inflammatory or anti-tumour (Mirzoeva *et al.*, 1997; Kimoto *et al.*, 1998; Park and Kahng, 1999; Lofty, 2006; Moreira *et al.*, 2008). In this context the present work was devised and thought to be of value by studying propolis activities in two different kinds of organisms: yeast and plant, choosing *Saccharomyces cerevisiae* as a model species and *in vitro* cultures of *Linum usitatissimum* as a model plant platform, respectively.

The aim of this work is to investigate the potential genotoxic effects of Portuguese propolis, as well as the antioxidant potential and toxicity it may pose for plants and yeast. The main objectives for this work were therefore obtaining propolis extracts that can be sources of safe natural compounds with antioxidant activity; extracts that can inhibit plant growth and photosynthesis with potential for the development of new herbicides, and extracts that cause inhibition of yeast growth, DNA damage and protection.

To do so in yeast, drop test viability assays, comet assay and flow cytometry with Rhodamine 123 and fluorescein diacetate were carried on. On the plant front, *in vitro* culture medium incorporated with the selected propolis extracts were made to analyse effects on seedling growth and photochemical parameters by non-invasive PAM-fluorometry. Also, under photoinhibitory light conditions, photosynthetic pigments and photochemical efficiency were assessed to determine photo-protective effects from photooxidative stress induced by excess light.

Materials and Methods

1.   Preparation of propolis extracts

Portuguese propolis samples were collected during the summer of 2012 from apiaries in Gerês (Minho region) and Pereiro (Douro region). Each sample was extracted both with ethanol (Carlo Erba, analytical grade) and *n*-hexane (Merck, analytical grade). Approximately 15 g of each sample was incubated with 100 mL of solvent in an Erlenmeyer flask, under agitation (100 rpm), at 25 °C, in the dark. The solutions were filtered (Macherey-Nagel fast flow filter paper) using a Büchner funnel and Kitasato system coupled to a vacuum pump. The solid residues were further incubated with 80 mL and a third time with 50 mL of the respective solvent. The three filtrates obtained were mixed and the solvent separated in a rotary evaporator (Büchi Rotavapor RE 121) under low pressure, at 50 rpm and 35 °C (Büchi 461 water bath).

The propolis extracts obtained were named P12.EE and G12.EE for the Pereiro (P) and Gerês (G) ethanolic extracts (EE), and P12.HE and G12.HE for the Pereiro and Gerês *n*-hexane extracts (HE), respectively (Table I). Table I also includes other propolis samples/extracts obtained in previous investigations (Paulo, 2009; Oliveira, 2011; Amorim, 2011; Carvalho, 2012, da Apresentação, 2012) that were also tested in this work.

The extracts were stored in the dark at 4 °C until use. In subsequent experiments working solutions were freshly prepared at the necessary concentrations by diluting the extracts in the appropriate solvent (ethanol or *n*-hexane).

Table I: Designations of propolis extracts used in this work. The designations adopted took into consideration sample provenance, year of propolis collection and the solvent used for extraction, respectively in this order.

Location, year / Solvents	Pereiro 2010	Pereiro 2012	Póvoa 2009	Côa 2010	Gerês 2011	Gerês 2012
Ethanol	P10.EE	P12.EE	PV09.EE	C10.EE	G11.EE	G12.EE
n-hexane		P12.HE	PV09.HE		G11.HE	G12.HE

2.   Establishment of *in vitro* cultures of flax (*Linum usitatissimum* L.)

To test the effect of propolis extracts on plant development and photochemistry *in vitro* cultures of flax (*Linum usitatissimum*) were used. MS (Murashige and Skoog, 1962) basal medium (Duchefa) was prepared supplemented with 2% (w/v) sucrose (Fischer Scientific), and the pH adjusted to 5.8 prior to the addition of agar (VWR, Prolabo) (0.8% w/v). Volumes of 20 mL were dispensed into glass culture flasks with transparent polypropylene caps, autoclaved (121 °C, 20 min) and kept warm (*ca* 50 °C) for ulterior incorporation of propolis extracts. Working dilutions of propolis extracts were prepared at the concentrations needed for each experiment (ranging from 25 to 400 mg/mL), and 50 µl were added to each culture flask medium, by dropping and gently stirring, in a laminar flow chamber. Controls were prepared adding 50 µl of the correspondent solvent. Five replicates (flasks) per treatment were used.

Two independent *in vitro* plant cultures were established for this project: a first culture where all the extracts were tested at the concentration of 200 mg/mL diluted in 20 mL MS medium (0.5 mg/mL final concentration), and cultured under an average regular light intensity of 40 µmol m^{-2} s^{-1} (NL) – the screening experiment -, and a second culture where only selected extracts were tested at a range of concentrations and cultures grown under high light intensity (102 µmol m^{-2} s^{-1}) (HL) to evaluate photoinhibition and photoprotective capacities. The concentrations tested were the following: 25, 50, 100 and 200 mg/mL extract concentrations for ethanolic extracts and 50, 100, 200 and 400 mg/mL for the less active *n*-hexane extracts.

Flax seeds (kindly provided by the Banco Português de Germoplasma Vegetal, INIAV) were sterilized by immersion in 70% (v/v) ethanol for 2 min, followed by sodium hypochlorite (1.5% active chlorine) for 10 min and then washed thoroughly several times with deionized water. Seven seeds were plated per flask. The cultures were maintained in an acclimatized room at 25 °C, under a photoperiodic regime of 16 h and a mean light intensity of 40 µmol m^{-2} s^{-1} (OSRAM L35W/77).

3.   Plant growth and root microscopy analyses

The effects of propolis extracts in the early development of *in vitro* grown plantlets were analysed. Two weeks after seeding, flax plantlets were taken from the flasks with care and the main root, hypocotyl and epicotyl lengths were measured.

Particular morphological trait identified in the root apical region were examined by microscopy. The 2-3 mm apical segments of selected roots were excised, mounted in water and observed with a DM5000B fluorescence microscope equipped with an ebq100 light source (Leica).

4. Photochemical efficiency of PSII by PAM fluorometry

To evaluate the effects of propolis extracts in *in vivo* photosynthesis of plantlets, the technique of chlorophyll fluorescence analysis by pulse amplitude modulated fluorometry (PAM) was selected and a PAM-210 fluorometer device (Heinz Walz GmbH, 1997), controlled via the PAMWin software, was used. The emitter-detector unit consists of the following essential components: measuring light LED with short-pass filter (<690 nm), peak wavelength circa 650 nm; actinic LED, unfiltered, peak wavelength ca. 665 nm; far-red LED, long-pass filter (>710 nm), peak wavelength ca. 730 nm; PIN photodiode and dichroic filter, reflecting fluorescence at 90° towards the detector.

The photochemical parameter F_v/F_m, which represents the intrinsic or maximum quantum yield of photosystem II (PSII) measured in dark-adapted samples, which is calculated by the following formula $\frac{F_v}{F_m} = \frac{F_m - F_0}{F_m}$, and the effective quantum yield (Φ_{II}) of PSII, which represents the efficiency by which the absorbed energy is actually channelled to photochemistry and measured under light-adapted samples, were determined in independent well-developed leaves of two-week-old plantlets. The effective quantum yield was calculated by the following formula:

$$\Phi II = \frac{F'm - F}{F'm}$$, where F'_m represents the maximum fluorescence emitted by the sample under actinic light exposure after a short and intense saturation pulse (SP) and F the variable fluorescence emission before the application of the SP. The non-photochemical quenching (NPQ), a measure of the absorbed light energy that is dissipated by other processes than photosynthesis, is measured by the following equation:

$$NPQ = \frac{(Fm - F'm)}{F'm}$$ (White and Critchley, 1999). F_v represents the variable fluorescence emission on the dark-adapted sample at a specific moment and F_m is the maximum fluorescence emitted when the PSII reactions centres are closed by a short saturation pulse.

Before the PAM experiments each flask was adapted to dark conditions for 20 min. Leaves were cut and placed individually on the magnetic support and an operational minimum fluorescence (F_0) above 0.150 was guaranteed. The maximum fluorescence (F_m) was registered following a short saturation pulse (800 ms,

3500 µmol m⁻² s⁻¹) and the F_v/F_m parameter was computed. Leaves were then exposed for 5 min to actinic light at 66 µmol m⁻² s⁻¹ for normal light (NL) samples and at 102 µmol m⁻² s⁻¹ for the high light (HL) samples, after which another SP was emitted to obtain maximum fluorescence under light adapted conditions (F'_m) and to calculate Φ_{II}.

5. Chlorophylls and carotenoids quantification

Leaves physiologically analogous to those used for PAM experiments were selected, placed in pre-weighted Eppendorf tubes and total fresh weight was determined using a high precision scale (Mettler H54AR). Photosynthetic pigments were extracted adding 1 mL acetone (80% v/v) per tube and incubating in the dark, at 4 °C, for 24 h. Absorbance was read at 663.2, 646.8 and 470 nm, and the concentration in chlorophyll a, b and carotenoids of the cetonic solutions was calculated according to Lichtenthaler (1987; Table II).

Table II: Equations for the determination of chlorophyll a and b and carotenoids concentrations in solutions (µg/mL), when using acetone 80% (v/v) as solvent (Lichtenthaler, 1987).

Pigments	Equations
Chlorophyll a	$= 12.25 \times A663.2 - 2.79 \times A646.8$
Chlorophyll b	$= 21.50 \times A646.8 - 5.10 \times A663.2$
Carotenoids	$= \dfrac{1000 \times A470 - 1.82 \times Chl.\,a - 85.02 \times Chl.\,b}{198}$

6. Yeast strain, culture media and growth conditions

The yeast *Saccharomyces cerevisiae*, haploid strain BY4741 (genotype: *MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*) (Brachmann *et al.*, 1998) was used in this work. Cell cultures were grown in liquid YPD medium [1% w/v yeast extract (Panreac), 1% w/v peptone (Becton, Dickinson and Company) and 2% w/v glucose], in Erlenmeyers with 1:5 ratio of culture to flask volume, in an orbital shaker at 30 °C and 200 rpm, or in YPethanol (1% w/v yeast extract, 2% w/v peptone and 1% ethanol with 1:10 ratio of culture volume and

flask volume. Spectrophotometric measurements at 600 nm were taken to monitor culture growth for all experiments performed.

7.   Yeast viability assays by drop test

In order to evaluate the antimicrobial activity of propolis extracts, a screening was developed to assess yeast viability when co-incubated with the extracts. The extracts tested were P10, P12, G11, G12, PV09, both ethanolic (EE) and *n*-hexane (HE), and C10.EE (Table I). A pre-inoculum of the selected yeast strain was inoculated in 5 mL YPD medium and incubated overnight at 30 °C, 200 rpm in an orbital shaker. The culture was then diluted in fresh medium to OD_{600} 0.1 and incubated again at 30 °C, 200 rpm, until exponential phase (OD_{600} 0.4 to 0.8). For each assay condition, a 5 mL volume of the exponential phase culture was transferred to glass test tubes, followed by the addition of the specific treatment and the mixture was incubated at 30 °C, 200 rpm. Aliquots were taken at incubation times of 0, 30, 60 and 90 min and sequentially diluted from 10^{-1} to 10^{-4}. A small volume (7.5 μL droplets) of each dilution was spotted on dried agar YPD plates (YPD with 2% w/v agar). The Petri dishes were left to dry at room temperature, incubated at 30 °C and photographed 48 h later.

The selected treatments –propolis extracts with concentrations ranging from 10 μg/mL to 500 μg/mL and controls (absolute ethanol or n-hexane) – were added to the glass test tubes and incubated as above described. The tested extract concentrations were: 50, 200 and 500 μg/mL for P10.EE; 200 and 500 μg/mL for P12.EE and P12.HE; 10, 50, 75, 125, 200 and 500 μg/mL for PV09.EE; 500 μg/mL for PV09.HE; 50 and 500 μg/mL for C10.EE; 50 and 500 μg/mL for G11.EE and G11.HE; and 200 and 500 μg/mL for G12.EE and G12.HE.

8.   Yeast intracellular oxidation state assessment by flow cytometry

Flow cytometry was performed to assess cell redox status by using dichlorofluorescein diacetate (H_2DCFDA; Sigma) as fluorochrome. An overnight-grown pre-inoculum (incubation at 30 °C, 200 rpm) of the selected yeast strain was diluted to obtain a 10 mL culture at OD_{600} 0.1. The culture was then incubated under the same conditions until the exponential phase. Cells were washed twice (with centrifugations at 17608 x*g*, 2

min) with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), diluted to OD_{600} 0.02 and 500 μL was taken for auto-fluorescence measurement. The fluorochrome was then added to the cells (0.05 mM final concentration) and incubation was performed in an orbital shaker for 1 h in the dark. Cells were washed in the same volume of PBS and the suspension distributed by 1 mL aliquots for the treatments. Treatments were made with propolis extracts (25, 50 and 100 μg/mL P10.EE and P12.EE; and 12.5, 25 and 50 μg/mL PV09) and controls were included (negative with absolute ethanol and positive with 0.01 M H_2O_2) before incubation for 20 min at 200 rpm, 30 °C. Each sample was analysed by flow cytometry in a Beckam Coulter Epics® XL cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW and the data was analysed with the "Flowing 2" software (Beckam Coulter, 2010).

9.   Yeast mitochondrial membrane potential assessment by flow cytometry

A pre-inoculum was prepared with a colony of the yeast strain in 5 mL YPethanol and was incubated at 30 °C, 200 rpm overnight. The suspension was diluted to OD_{600} 0.2 with fresh medium followed by incubation under the same conditions until the exponential phase. Cells were washed (with centrifugations at 17608 xg, 2 min) with deionized sterilized H_2O_2, diluted to OD_{600} 0.02 and distributed by aliquots for the treatments. Treatments were made with propolis extracts (50, 100 and 200 μg/mL P10.EE and P12.EE; and 50, 100 and 150 μg/mL PV09.EE) and a negative control (absolute ethanol) was included. Mixtures were incubated at 30 °C, 200 rpm for 60 min and rhodamine 123 (final concentration 50 mM) was added to each tube with subsequent incubation for 10 min at room temperature. Each sample was then analysed by flow cytometry in a Beckam Coulter Epics® XL cytometer as mentioned above in order to detect mitochondrial membrane potential variations.

10.   DNA damage assessment by comet assay

A 5 mL YPD pre-inoculum was prepared with a colony of the yeast strain and was incubated overnight at 3 °C, 200 rpm. The suspension was diluted to OD_{600} 0.1 with fresh medium and was incubated under the same conditions until the exponential growth phase (OD_{600} ~0.4).

Cells were harvested by centrifugation at 17608 x*g*, 2 min, and washed twice with the same volume of deionised water at 4 °C. Subsequently cells were incubated with lyticase buffer [200 U/mL lyticase, 500 μL S buffer 2x (2 M sorbitol, 50 mM KH_2PO_4, pH 6.5), 300 μL deionized H_2O and 50 mM β-mercaptoethanol (Sigma Aldrich) for 40 min at 30 °C, 200 rpm to obtain spheroplasts. Spheroplasts were washed twice with S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5), ressuspended and distributed by 100 μL aliquots. Propolis extracts were added (25, 50, 100 and 200 μg/mL P10.EE), controls were also included (absolute ethanol and 0.01 M H_2O_2) and the mixtures were incubated for 20 min at 30 °C, 200 rpm. The spheroplasts of each treatment were harvested by centrifugation, washed once with S buffer and the final pellet was ressuspended in 60 μL low-melting agarose (1.5% w/v in S buffer) at 35 °C. The mixture was spread onto glass slides previously coated with normal-melting agarose (0.5% w/v in deionized H_2O) and covered with cover slips. The slides were placed on ice in order to solidify the agarose and cover slips were removed. The slides were submerged for 20 min in lysing buffer [300 mM NaOH, 5 M NaCl, 0.5 M ethylenediamine tetraacetic acid (EDTA), 0.1 M Tris-HCl, 0.05% w/v lauroylsarcosine [Sigma Aldrich], pH 10], followed by 20 min in electrophoresis buffer (300 mM NaOH, 0.5 M EDTA, 0.1 M Tris-HCl, pH 10). Electrophoresis was performed with this buffer at 0.7 V/cm, 10 min at 4 °C. The microgels were neutralised with neutralisation buffer (10 mM Tris-HCl buffer, pH 7.4) for 10 min and fixed for 10 min with ethanol 76% (v/v) followed by 10 min with ethanol 96% (v/v). The glass slides were dried at room temperature and stored at 4 °C. The microgels were stained in GelRed (3x, Biotium) for visualization in a fluorescence microscope. Tail length was measured with the CometScore software.

11.   Statistical analyses

Data from the various experiments were analysed with the Prism v.5 (GraphPad, software, Inc.) statistical software. Data from flax plant growth measurements were analysed running one-way ANOVA followed by Tukey *post-hoc* test for multiple comparisons. The distribution's normality was assumed and homogeneity of variances tested with Bartlet's test. The data was then plotted as column or line graphs as mean values with 2 standard deviation (SD) bars. To also analyse the factor "year of collection", a two-way ANOVA with Bonferroni *post-hoc* test was performed with results obtained with both Pereiro extracts (P10 and P12). The pigment quantification experiments statistical data and difference between conditions can be found on the Annex section. For the maximum quantum efficiency of PSII (F_v/F_m) parameter, and to meet homogeneity of variances assumption, a data transformation was employed as follows:

$$x' = \arcsin\left(\sqrt{x}\right)$$

From the transformed data, a one-way ANOVA followed by a Tukey test was performed. In each comet assay experiment, at least 20 random comets were analysed to calculate the mean. Comet assay data are presented as the mean ± standard deviation of the means obtained in three independent experiments. Data were analysed with one-way ANOVA with Kruskal-Wallis test and Dunn's multiple comparison *post-hoc* tests.

Results

1. Effects of propolis extracts on yeast cell growth and viability

The biological activities of Portuguese propolis extracts, namely the antioxidant, anticancer and antimicrobial, have been studied and are well document in the literature (Burdock, 1998; Moreira *et al.*, 2008; Falcão *et al.*, 2010; Fokt *et al.*, 2010). Given the aforementioned biological activities, it was decided to measure the effect of the selected extracts on *S. cerevisiae* through an assay of viability in order to identify the most active extracts and concentrations.

To start an overall screening was performed in which all the extracts were tested at a considerably high concentration (500 µg/mL). As can be seen on Fig.1 the ethanolic extracts from Pereiro 2010 (P10.EE) and Póvoa de Varzim (PV09.EE) have shown a clear inhibition of growth after 90 minutes of incubation, with virtually no colonies visible at this point. The ethanolic extracts obtained from Pereiro 2012 (P12.EE) and Gerês 2012 (G12.EE) samples showed some inhibitory activity, though more modest. Côa (C10.EE) and Gerês 2011 (G11.EE) ethanolic extracts, as well as all of the n-hexane extracts did not appear to inhibit yeast cell growth at this concentration, and therefore most likely are devoid of toxicity.

Figure 1. Viability assays by drop dilution test method with 500 µg/mL of propolis extracts. Colonies are viewed after 48 hours of growth. **(A)** Cells were plated after 180 minutes of incubation with the ethanolic extracts. **(B)** Cells were plated after 90 minutes with the ethanolic extracts. **(C)** Cells were plated after 180 minutes of incubation with the *n*-hexane extracts. **(D)** Cells were plated after 90 minutes of incubation with the *n*-hexane extracts.

After this initial screening, four ethanolic extracts were selected – P10.EE, P12.EE, G12.EE and PV09.EE – and tested at lower concentrations (Fig.2). P10.EE and PV09.EE were tested at 200 μg/mL for the former and also 75 and 125 μg/mL for the latter. The results obtained suggest that Portuguese propolis extracts can be toxic to yeast cells, namely the polar (ethanolic) fraction of Pereiro and Póvoa de Varzim.
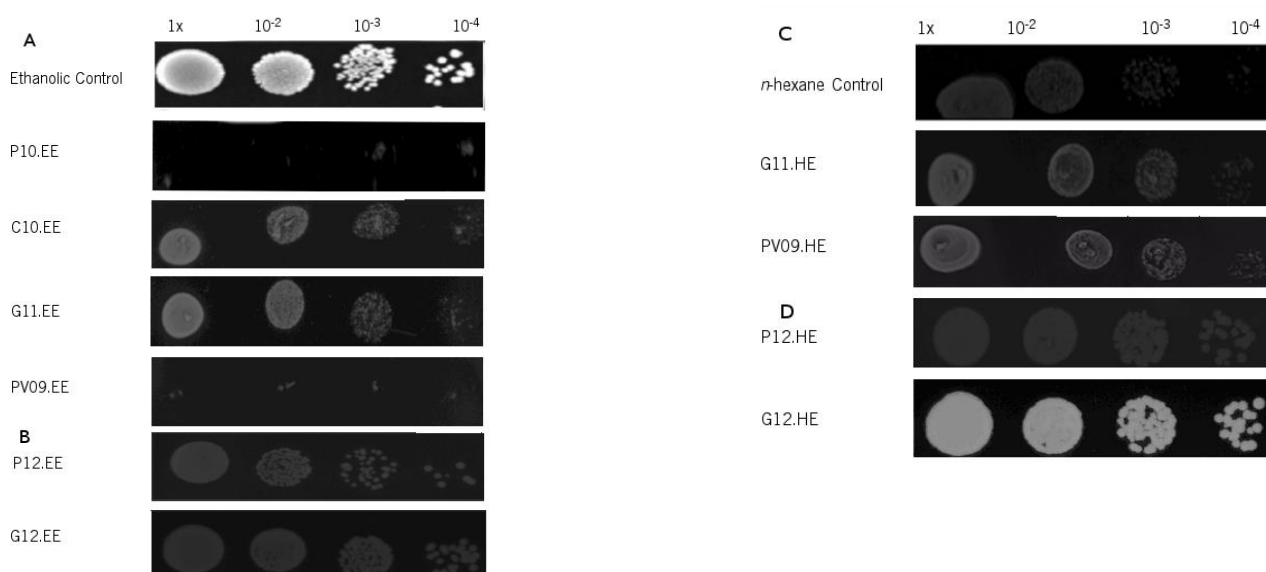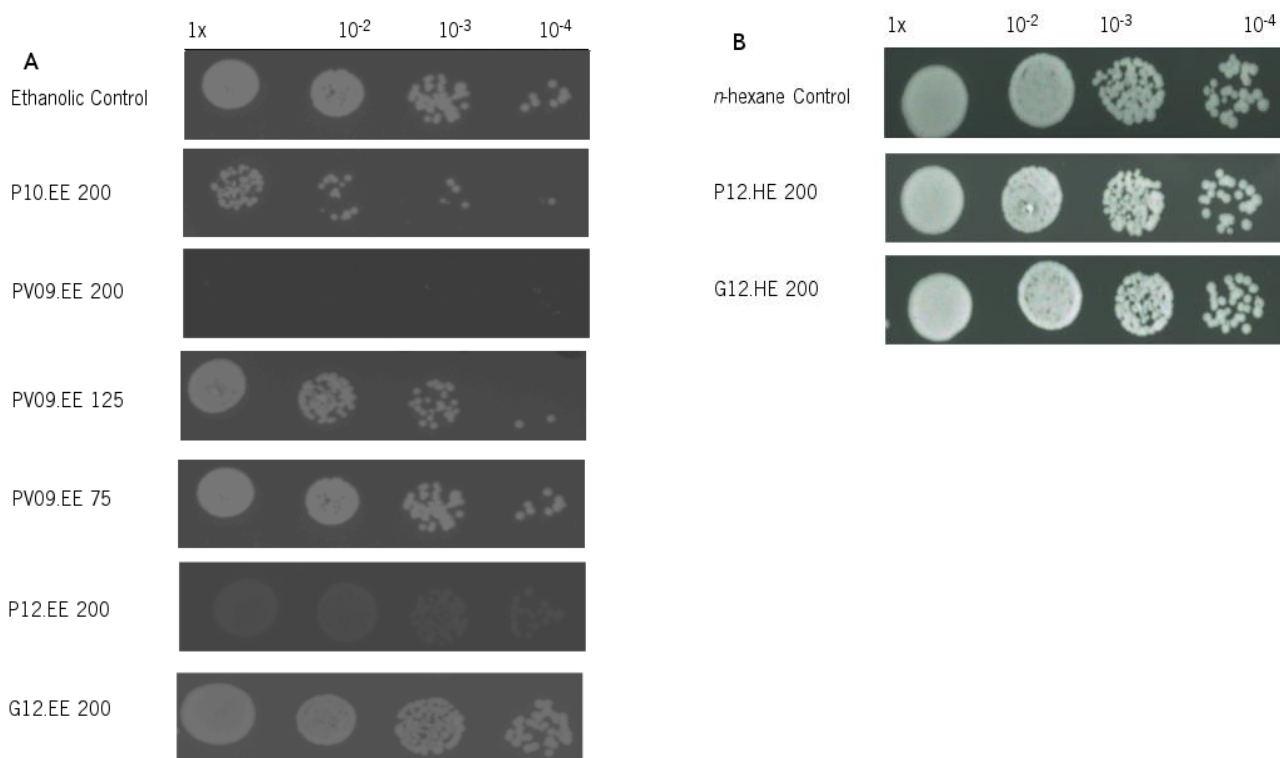

Figure 2. Viability assays by drop dilution test method with lower concentrations of propolis extracts. Colonies are viewed after 48 hours of growth. **(A)** Ethanolic extracts P10.EE, P12.EE and G12.EE at 200 μg/mL; PV09.EE at 200, 125 and 75 μg/mL **(B)** *n*-hexane extracts at 200 μg/mL. All were subjected to 90 minutes incubation with the propolis extracts.

2. Propolis extracts effects on yeast DNA integrity

Comet assay allows for the assessment of the toxicity of a given compound to DNA. Upon significant damage DNA becomes fragmented and migrates outwards. When an electric field is applied, the migration is augmented and unidirectional. A characteristic DNA trail then forms, the so called comet tail, whose length can be measured and correlated to DNA damage (Collins, 2009). After the initial viability screening results, indicating strong toxicity to yeast, the P10.EE extract was chosen to this assay, given also the few studies available for propolis genotoxicity. Yeast spheroplasts were incubated with ethanol and hydrogen peroxide as controls (Fig.3) (see Material and Methods) and three concentrations of P10.EE (50, 100 and 200 μg/mL)

were tested. At the lower concentration of 50 μg/mL there appears to be no significant increase in tail length compared to the negative control. At 100 μg/mL, tail length increases slightly in between the negative and positive control thresholds, and at the 200 μg/mL (where the extract has been previously found to inhibit yeast growth), a clear, significant increase occurs. At this concentration, genotoxicity of the extract is similar to the DNA damage imposed by H_2O_2.
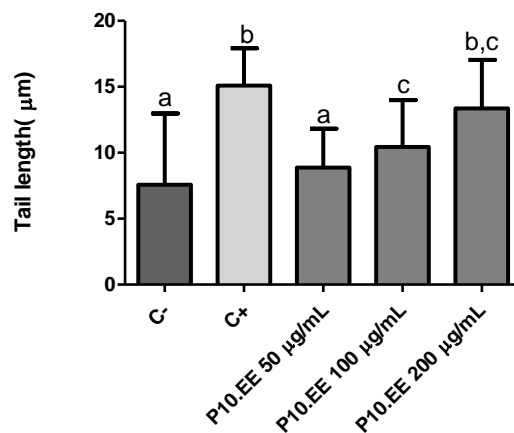

Figure 3. Comet assay tail length mean values for *S. cerevisiae* cells suspension with P10.EE. Columns represent mean values (n= 50) and the bars on top the respective SD.

3.   Effects of propolis extracts on intracellular oxidation status of yeast cells

A flow cytometry approach was chosen to assess the potential antioxidant activities of the Portuguese propolis extracts, particularly of those showing significant bioactivities earlier (Fig.1 and 2) P10.EE, P12.EE and PV09.EE. The fluorochrome employed is the key to obtain relevant data by flow cytometry. Many fluorochromes can be employed like methoxycoumarin, fluorescein, cascade blue, propidium iodide or rhodamine each binding to the cells through different mechanisms and allowing for the measurement of different phenomena. Fluorescein diacetate (H_2DCFDA) allows for the assessment of the oxidation state of the cells in vivo. Once the non-fluorescent membrane-permeant H_2DCFA molecule enters the cell, it is cleaved in its acetate moieties by esterases converting it in the impermeable H_2DCF compound. This compound can then be subsequently oxidised by several ROS producing the highly fluorescent 2',7'-dichlorofluorescein (DCF). The amount of fluorescence measured correlates with the oxidative state of the cell, with a higher fluorescence indicating higher oxidation and potentially more damage (Conour *et al.*, 2004).

To infer whether these propolis extracts could significantly alter intracellular oxidation status, experiments were performed by simple incubation of cells with propolis extracts and also by co-incubation (the extract was incubated at the same time as the stress inducer agent) and pre-incubation (cells are incubated

with the extracts, then washed and incubated with the positive control) with H_2O_2. In the below figures 4, 5 and 6, the fluorescence is plotted across the x axis for simple, co and pre-incubation conditions.

From figure 4 it is possible to observe that all the three extracts tested appear to reduce the intracellular oxidation levels of the yeast cells below that of the negative control, the incubation with ethanol, at both concentrations tested. At 200 μg/mL, the reduction of fluorescence and hence of oxidation, was remarkably more significant than on 100 μg/mL of P10.EE and P12.EE extracts.
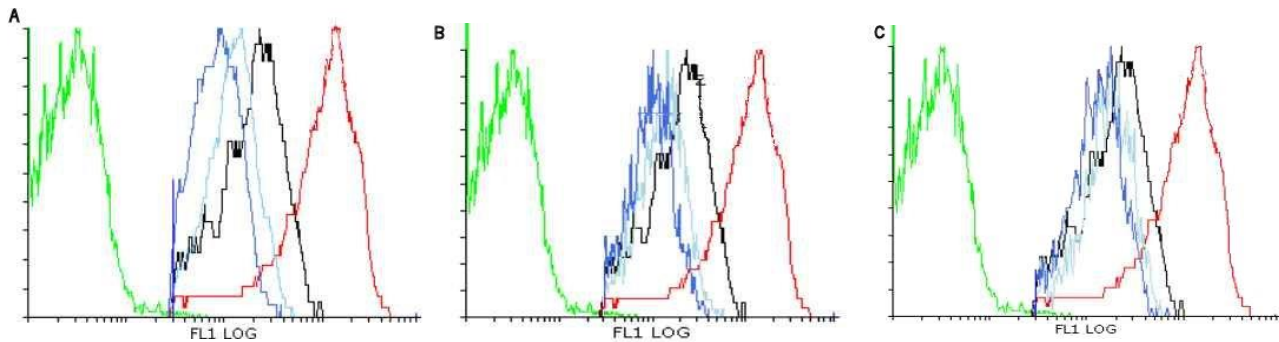

Figure 4. Intracellular oxidation of yeast cells incubated with propolis extracts and control treatments, measured by flow cytometry. Green – autofluorescence. Black – negative control (treated with the solvent of the extracts) Red – Cells treated with H_2O_2 at 0.01 M (positive control). (A) Cells with P10.EE extracts at 100 μg/mL – light blue and 200 μg/mL – dark blue. (B) Cells with P12.EE at 100 μg/mL – light blue and 200 μg/mL – dark blue. (C) Cells with PV09.EE at 25 μg/mL – light blue and 50 μg/mL – dark blue.

Given these significant results on a stand-alone incubation with the extracts, a second set of experiments were performed by co-incubating hydrogen peroxide along with the cell suspension and propolis extracts, as well as pre-incubating cells with extracts and H_2O_2. The extracts concentrations tested were lower than the stand-alone incubation experiment. When co-incubated with H_2O_2, the oxidation levels of the yeast cells when incubated with the three extracts were found to be between the negative and positive controls. It is clear that the propolis extracts provide a non-negligible amount of protection against the hazardous effects of H_2O_2. Once again, there appears to be a concentration effect, being the highest extract concentrations, like 50 μg/mL on PV09.EE or 100 μg/mL on P10 and P12.EE, closer to the negative control oxidative state than the lowest concentrations (Fig.5).
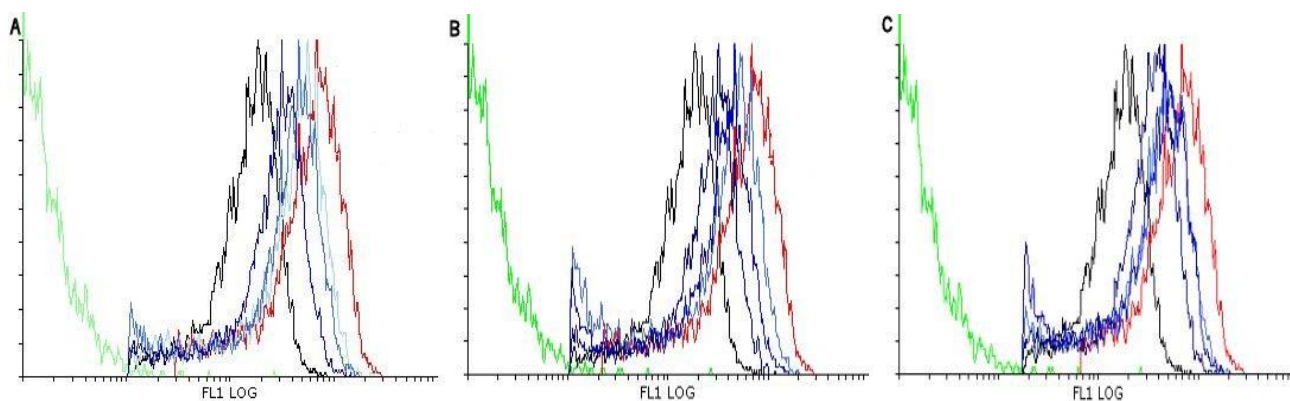
Figure 5. Intracellular oxidation of yeast cells co-incubated with propolis extracts and hydrogen peroxide and control treatments, measured by flow cytometry. Green – autofluorescence. Black – negative control (treated with the solvent of the extracts). Red – Cells treated with H_2O_2 at 0.01 M. **(A)** Cells with P10.EE extracts at 25 µg/mL – light blue; 50 µg/mL – medium blue and 100 µg/mL – dark blue. **(B)** Cells with P12.EE at 25 µg/mL – light blue; 50 µg/mL – medium blue and 100 µg/mL – dark blue. **(C)** Cells with PV09.EE at 12.5 µg/mL – light blue; 25 µg/mL – medium blue and 50 µg/mL   – dark blue.

In the pre-incubation procedure, the effect on the decrease of intracellular oxidation, while undoubtedly still occurs, does not seem to be as significant as on the co-incubation assay (Fig.6).
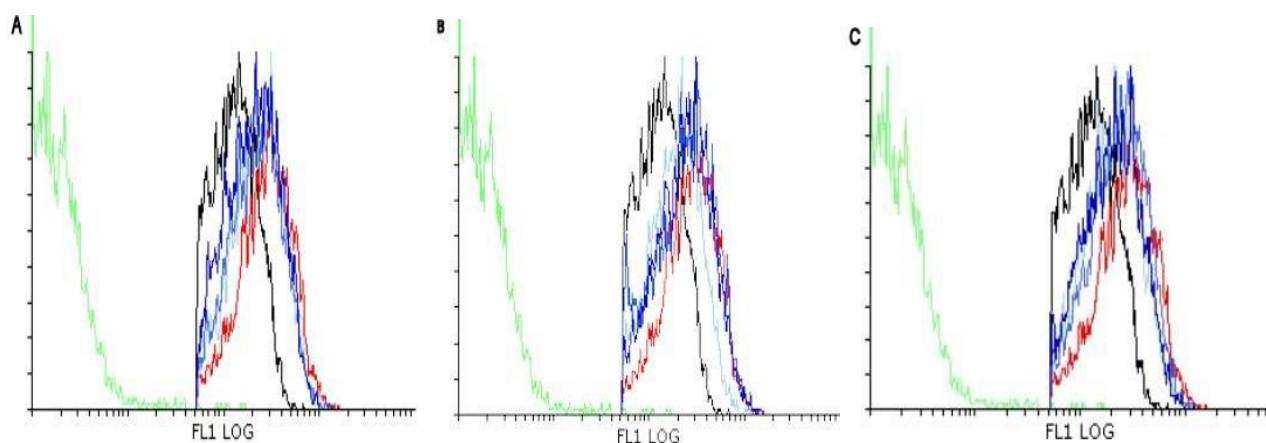
Figure 6. Intracellular oxidation of yeast cells with propolis extracts and control treatments pre-incubated with H_2O_2, measured by flow cytometry. Green – autofluorescence. Black – negative control (treated with the solvent of the extracts). Red – Cells treated with H_2O_2 at 0.01 M. **(A)** Cells with P10.EE extracts at 25 µg/mL – light blue; 50 µg/mL – medium blue and 100 µg/mL – dark blue. **(B)** Cells with P12.EE at 25 µg/mL – light blue; 50 µg/mL – medium blue – and 100 µg/mL – dark blue. **(C)** Cells with PV09.EE at 12.5 µg/mL – light blue; 25 µg/mL – medium blue and 50 µg/mL   – dark blue.

4. Effects of propolis extracts on mitochondrial function and membrane potential

In order to assess the effect of the selected Portuguese propolis extracts in eukaryotic cells and its potential targeting to mitochondria, the mitochondrial membrane potential variation was measured by flow cytometry coupled to rodamine 123 staining. Rhodamine 123 is a fluorochrome that allows for the probing of mitochondrial function, since its accumulation in the mitochondria is a measure of mitochondria membrane potential ($\Delta\Psi$m) (Ludovico *et al.*, 2001). Mitochondria are the only organelles known to have a significant membrane potential, with a negative charge inside. ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, membranes or DNA and can also increase the tendency of mitochondria to release proteins such as cytochrome c (cyt c) to the cytosol by mitochondrial outer membrane permeabilization, which results in the activation of the cell's apoptotic machinery. Being this organelle a primary source of some reactive oxygen species such as superoxide anions that permeate the mitochondrial membrane and a potential target for propolis compounds, this technique seems adequate to evaluate propolis toxicity and disclose its mode of action, making it a relevant situation to measure via a fluorescence activated cell sorting (FACS) approach (Ludovico *et al.*, 2001; Drakulic *et al.*, 2005; Murphy, 2008).

As depicted in figure 7, cells incubated with P10.EE show a remarkable decrease of fluorescence, hence of membrane potential, particularly at 100 and 200 µg/mL. Interestingly, P12.EE displayed a smaller effect with the same concentrations. PV09.EE appears to be the most toxic of the tested extracts, as the membrane potential decrease was more acute even at the 50, 100 and 150 µg/mL.
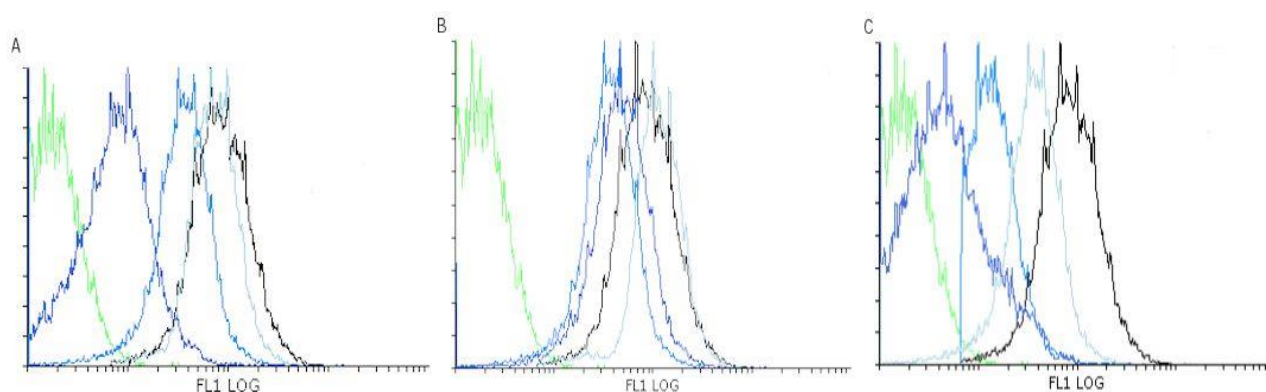
Figure 7. Mitochondrial membrane potential variation (**Ψ∆m**) of yeast cells with propolis extracts and ethanolic control treatments measured by flow cytometry with rhodamine 123 labeling. Green – autofluorescence Black – negative control (treated with the solvent of the extracts) **(A)** Cells with P10.EE extracts at 50 µg/mL – light blue;100 µg/mL – medium blue and 200 µg/mL – dark blue; **(B)** Cells with P12.EE at 50 µg/mL – light blue ;100 µg/mL – medium blue – and 200 µg/mL – dark blue; **(C)** Cells with PV09.EE at 50 µg/mL – light blue ; 100 µg/mL – medium blue and 150 µg/mL – dark blue.

5.   Propolis extracts effects on plant growth and photosynthetic activity

To test propolis extracts phytotoxicity, and similarly to the approach used with the yeast model, a first screening experiment was run in which flax plants were grown on culture media where the extracts were incorporated at a final concentration of 500 µg/mL. Methanol was used instead of ethanol for the controls, due to its lower phytotoxicity. Two weeks after germination roots, hypocotyl and epicotyls lengths were measured (Fig. 8). All the extracts tested, with the exception of G11.HE, have demonstrated the ability to inhibit significantly root development (Fig. 8 A). A minor impact was observed in the aerial parts (Fig. 8 B and C). The ethanolic extracts of Póvoa de Varzim 2009 (PV09.EE) and Pereiro 2010 (P10.EE) strongly inhibit root growth to values below 25% of the control, followed by P12.EE that reduced root length to 40% (Fig. 8 A). Also, PV09.EE and P10.EE were the only extracts that significantly to reduced hypocotyl development (Fig. 8 B and C). Flax plants grown in the presence of these two extracts are remarkably shorter than those grown in the control (Fig. 8), and exhibited a profuse formation of lateral roots (Fig. 9). Shorter roots and some ramification can also be observed with P12.EE, although not as pronouncedly as with PV09.EE and P10.EE (Fig. 9 D, F, H, respectively). PV09.EE caused the strongest effects on roots of all the extracts tested (Fig. 9 F).
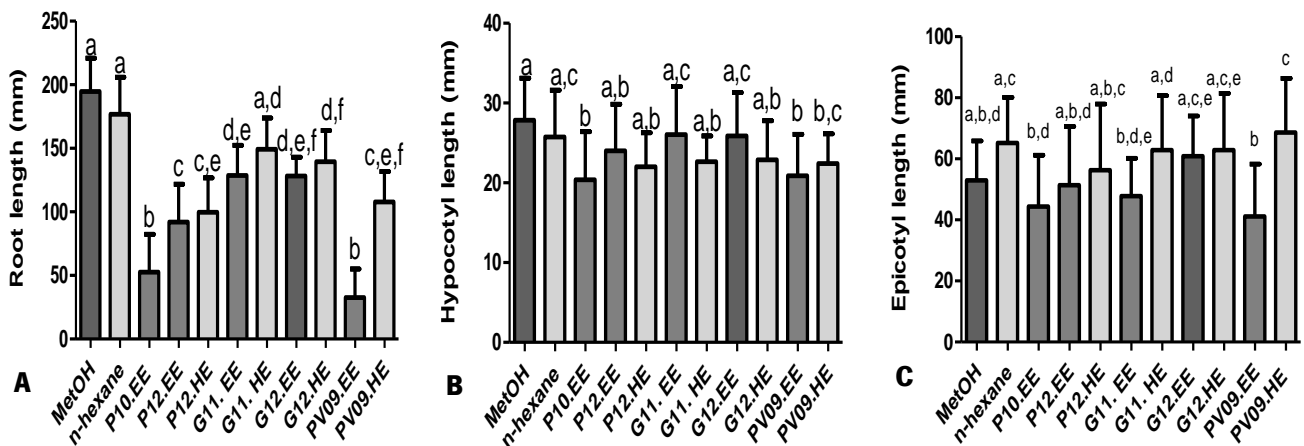
Figure 8. Average length of three main plant organs - root, hypocotyl and epicotyl - of *in vitro* flax plants grown in MS medium with different propolis extracts for two weeks. Columns represent mean values (n= 6) and the bars on top the respective SD.
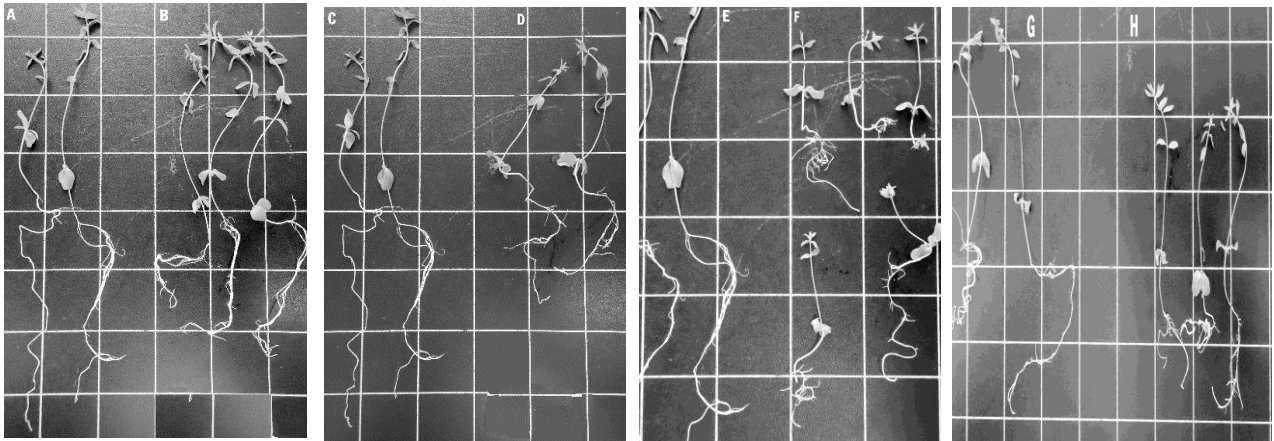
Figure 9. Several specimens of flax photographed after two weeks growth in culture media supplemented with propolis extracts or the respective solvents (controls). **(A)** *n*-hexane control plants. **(B)** PV09.HE plants. **(C)** Methanol control plants, **(D)** P12.EE plants, **(E)** Methanol control, **(F)** PV09.EE, **(G)** Methanol control and **(H)** P10.EE. Each square of the grid has 3 cm of width.

The P10.EE, P12.EE and PV09.EE extracts induced lateral root growth as can be clearly seen on figure 10 (A, E and F). The n-hexane extract PV09.HE appears to have little effects on root development when compared to its ethanolic counterpart.
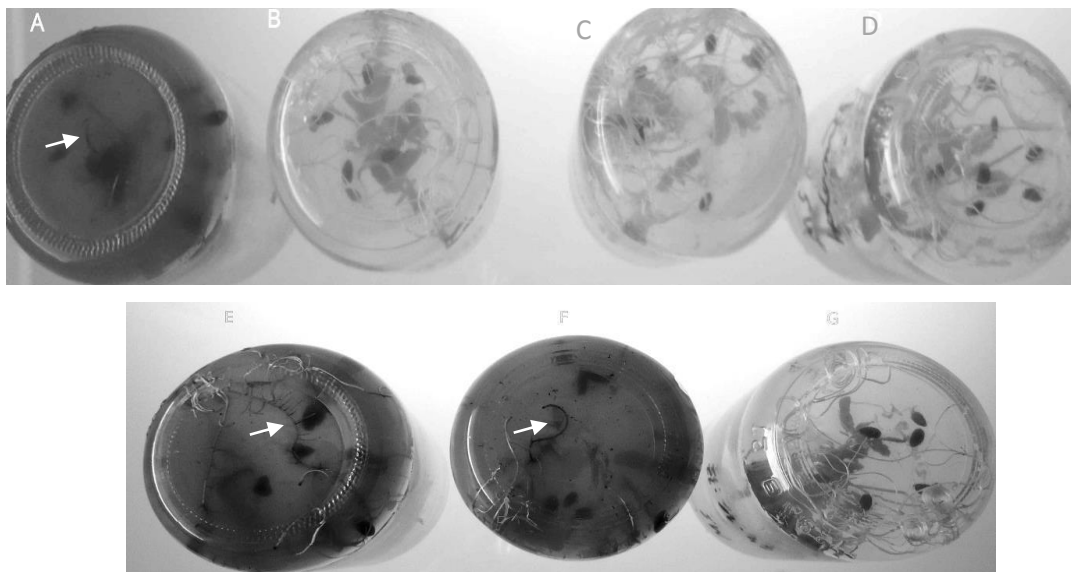
Figure 10. Bottom view of culture flasks for some flax plants cultivated in medium with propolis extracts. Different responses to the extract can be observed in root development. **(A)** PV09.EE, **(B)** Methanol control, **(C)** PV09.HE, **(D)** *n*-Hexane control, **(E)** P12.EE, **(F)** P10.EE and **(G)** Methanol control.

A particular morphological feature was observed in roots of some plants grown on extracts such as PV09.EE, PV09.HE, P10.EE and P12.EE. The apical tip of the root has several layers of cells growing outwards in scrambled, and in a non-organised way (Fig.11).
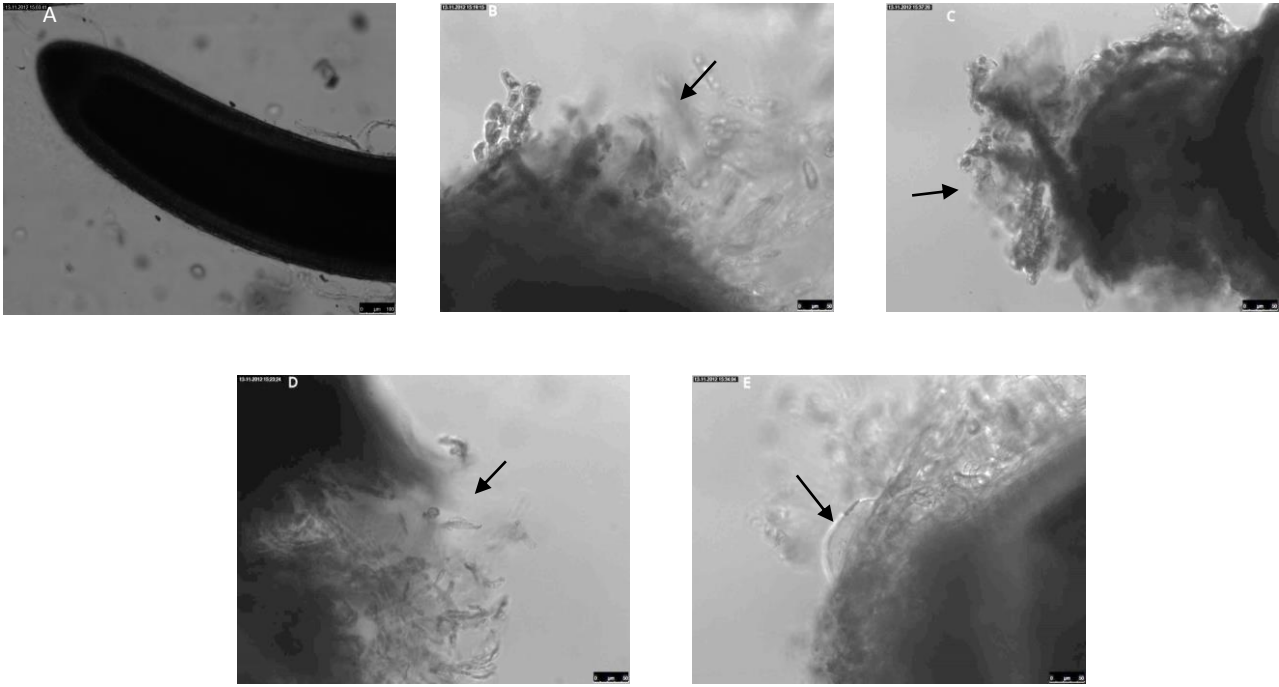
Figure 11. Bright field photomicrographs of selected flax plant roots at 80x **(A)** and 160x. A particular morphological trait on the apical portion of the root is evident (see the arrows). **(A)** *n*-hexane control, **(B)** P10.EE, **(C)** P12.EE, **(D)** PV09.EE and **(E)** PV09.HE.

To evaluate effects on the photosynthetic function, the photochemical maximum quantum efficiency of PSII (F_v/F_m) was determined by the PAM fluorometry technique (Fig.12). Although many extracts at the concentration tested reduced F_v/F_m, only with P10.EE the values were significantly lower than in the control. It is important to point out that the variability has increased with the treatments (observable by the SD increase), what may mask any potential effect.
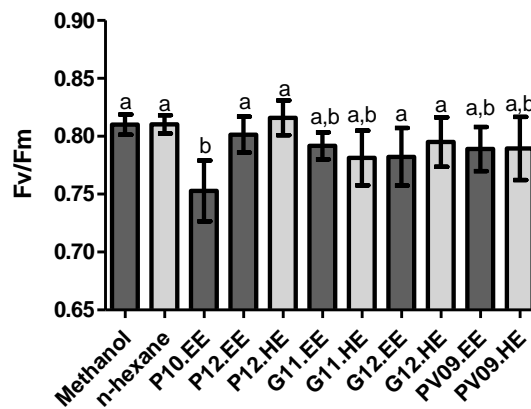
Figure 12. Maximum quantum efficiency (F_v/F_m) several flax plants cultivated in medium with propolis extracts. Columns represent mean values (n= 6) and bars the respective standard deviation (SD).

6. Effects of propolis extracts in plants exposed to light induced photooxidative damage

To evaluate potential protective effects of propolis extracts against oxidative stress induced by exposure to excess light, an experiment was set up where flax plants were grown at a high light (HL) intensity (102 μmol m^{-2} s^{-1}; the normal light (NL) regime is of about 30 μmol m^{-2} s^{-1}) and three n-hexane selected Portuguese propolis extracts (G11.HE, P12.HE and PV09.HE) and four ethanolic extracts of G11.EE, P10.EE, P12.EE and PV09.EE were tested at the following final concentrations: 0.125, 0.25, 0.5 and 1 mg/mL (50, 100, 200 and 400 mg/mL extract concentration) for the n-hexane extracts and 0.0625, 0.125, 0.25 and 0.5 mg/mL (25, 50, 100 and 200 mg/mL extract concentration) for the ethanolic ones. The criteria for this selection were related to significant activity inhibiting plant growth demonstrated previously on the ethanolic extracts and the potential for protective and growth stimulation activity on n-hexane based extracts.

After one week in culture for the n-hexane samples and two weeks for the ethanolic ones, the culture flasks were visually inspected and photographed (Fig.13 and 14). It was also possible to distinguish a dose-dependent inhibition of early plant growth, something that the previous experiment of plant culture was not designed for. The most active extracts were once again the ones with ethanol as a solvent, namely, P10.EE, PVO9.EE and to a lesser degree P12.EE.
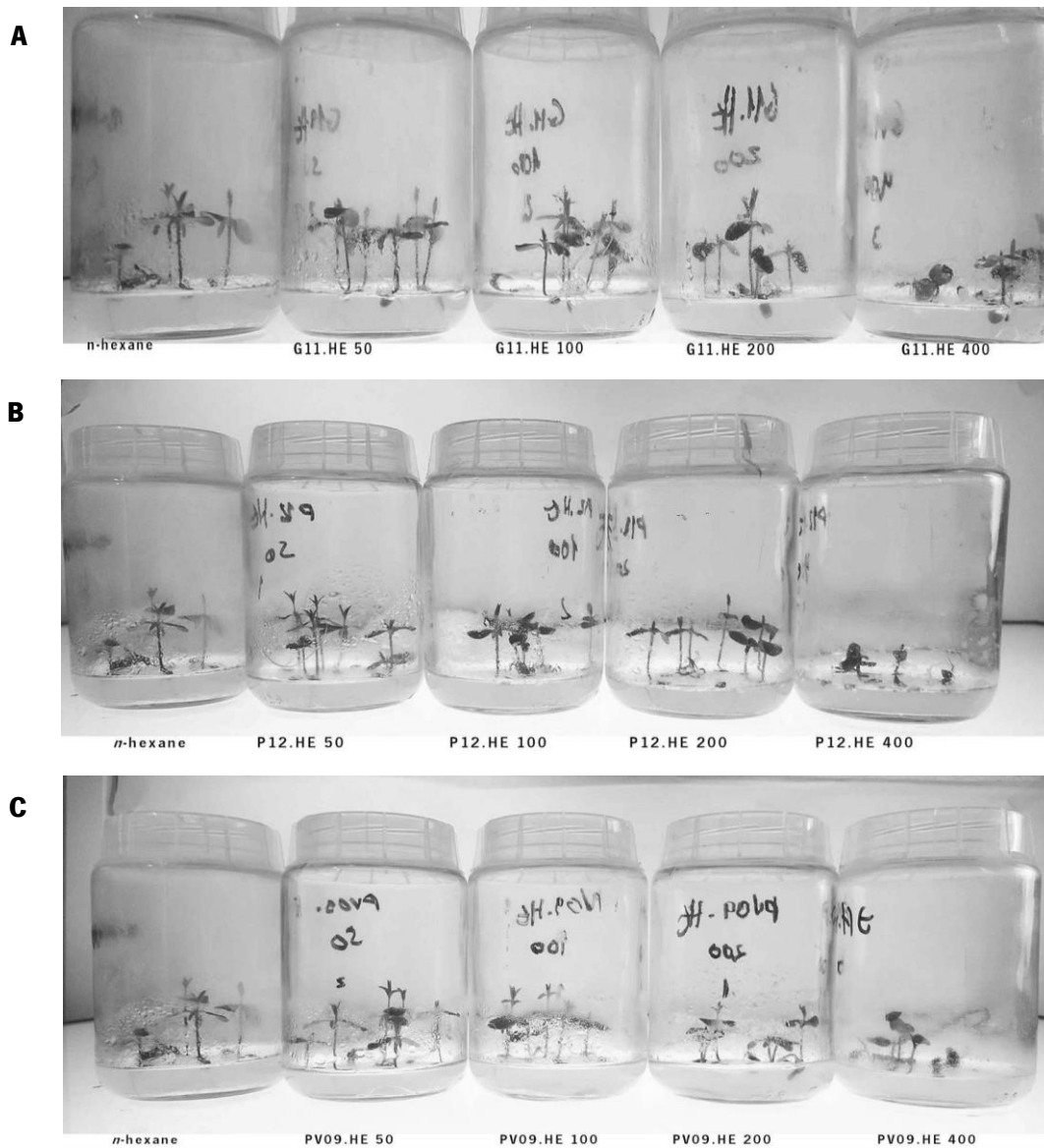
Figure 13. Plant culture flasks with MS medium and the *n*-hexane extracts, G11.HE **(A)**, P12.HE **(B)** and PV09.HE **(C)**, at concentrations from 50 to 400 mg/mL.
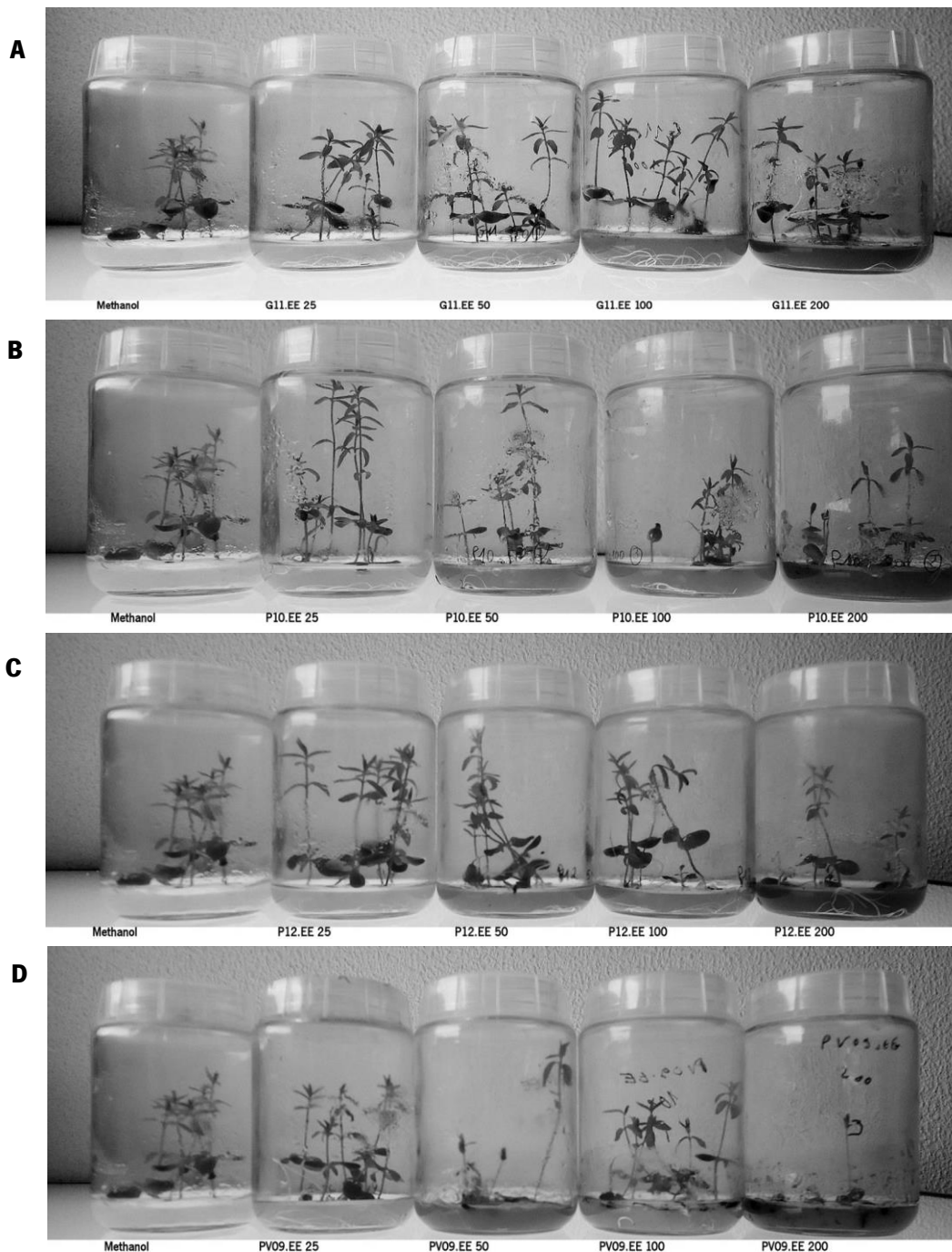
Figure 14. Plant culture flasks with MS medium and G11.EE **(A)**, P10.EE **(B)**, P12.EE **(C)** and PV09.EE **(D)** propolis extracts at concentrations from 25 to 200 mg/mL.

Like what was done in the first screening experiment the growth flax of two-week-old plantlets was quantitatively assessed and the average lengths of main root, hypocotyl and longer epicotyl can be seen respectively in figures 15, 16 and 17. No significant effect was observed due high light exposure with respect to early plant development because all organs had similar dimensions when comparing the controls (Figs. 15,

16 and 17). Also, no conspicuous interaction was observed between light and extracts because growth responses are in line with those observed in the first experiment where these extracts were tested at a final concentration of 500 µg/mL (Fig. 8). Comparing global effects on growth it is possible to conclude that roots were the most affected organs followed by the hypocotyl and that, with very few exceptions, a dose-dependent inhibitory effect was observed (Figs. 15, 16, 17).

In relation to root growth, plants grown in P10.EE and P12.EE exhibited a concentration-dependent inhibition but it is clear the stronger effect exerted by P10.EE, corroborating previous results. At the highest concentration tested P10.EE had a reduction of root length of about 28.7% (comparing to control plants) while P12.EE only 60.5% (Fig. 15 A). Regarding PV09.EE, which is the most active extract (Fig. 15 B), the differences in root growth start with the 50 mg/mL extract concentration being significantly lower than the controls (78%), with some variations in the in between 100 mg/mL and another decrease at the maximum 200 mg/mL (21.7%). G11.EE only showed significant differences at the highest extract concentration (75.9%) (Fig. 15 C).
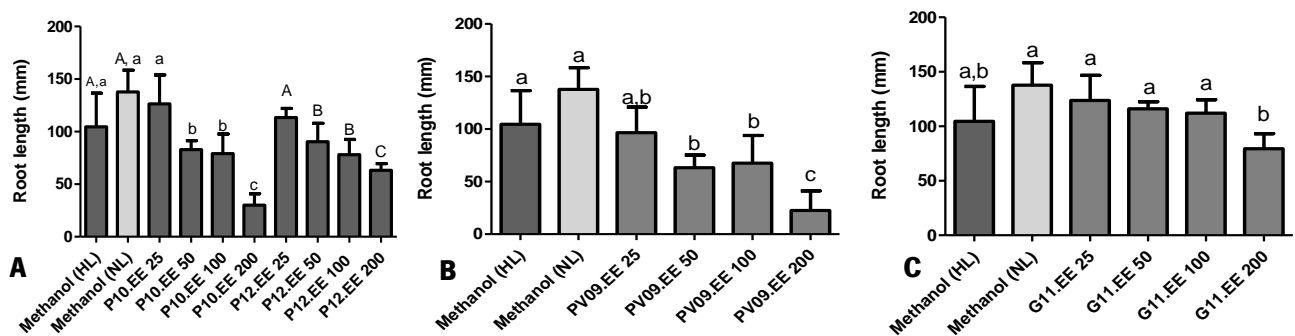
Figure 15. Average values of root length for several flax plants cultivated in medium with propolis extracts of Pereiro (both P10.EE and P12.EE), PV09.EE and G11.EE at 25, 50, 100 and 200 mg/mL concentrations. Plants were exposed to photoinhibitory light intensities. NL stands for the normal light control condition and HL for the photoinhibitory high light condition. Columns represent mean values (n= 6) and the bars on top the respective SD. The P10.EE and P12.EE samples were analysed together with a two-way analysis of variance with Bonferroni test. The remaining samples were processed with one-way ANOVAs and Tukey test.

Regarding the hypocotyl, extracts P10.EE and P12.EE reduced growth to 75% and 72% of the controls for the higher concentrations (Fig. 16 A), but PV09.EE being effective only at the highest concentration tested was the most effective extract by reducing growth to 34.7% of control (Fig. 16 B). Again, no significant differences on hypocotyl growth were registered with G11.EE (root length reduced to just 97% of the control) (Fig. 16 C).

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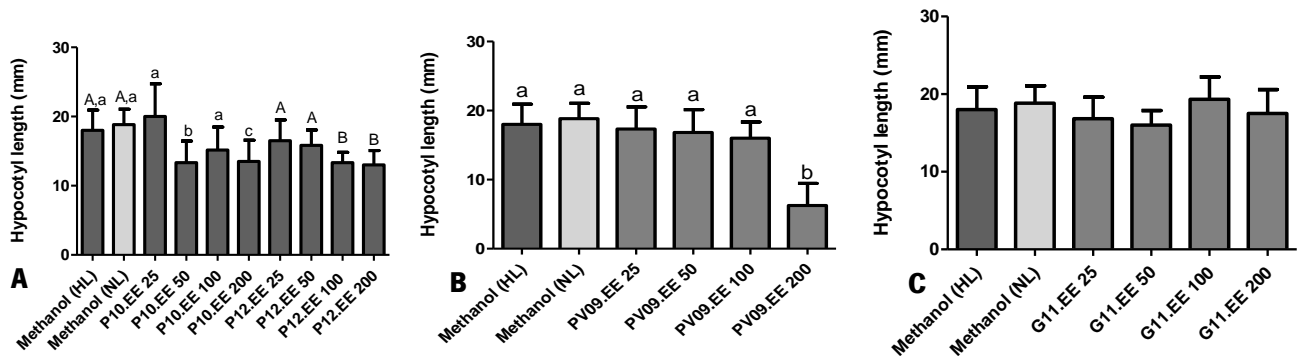
Figure 16. Average values of hypocotyl length for several flax plants cultivated in medium with propolis extracts of Pereiro (both P10.EE and P12.EE), PV09.EE and G11.EE at 25, 50, 100 and 200 mg/mL concentrations. Plants were exposed to photoinhibitory light intensities. NL stands for the normal light control condition and HL for the photoinhibitory high light condition. Columns represent mean values (n= 8) and the bars on top the respective SD. The P10.EE and P12.EE samples were analysed together with a two-way analysis of variance with Bonferroni test. The remaining samples were processed with one-way ANOVAs and Tukey test.

In what concerns the epicotyl growth (Fig. 17) a very different picture was observed when comparing to the results obtained for the other two organs.
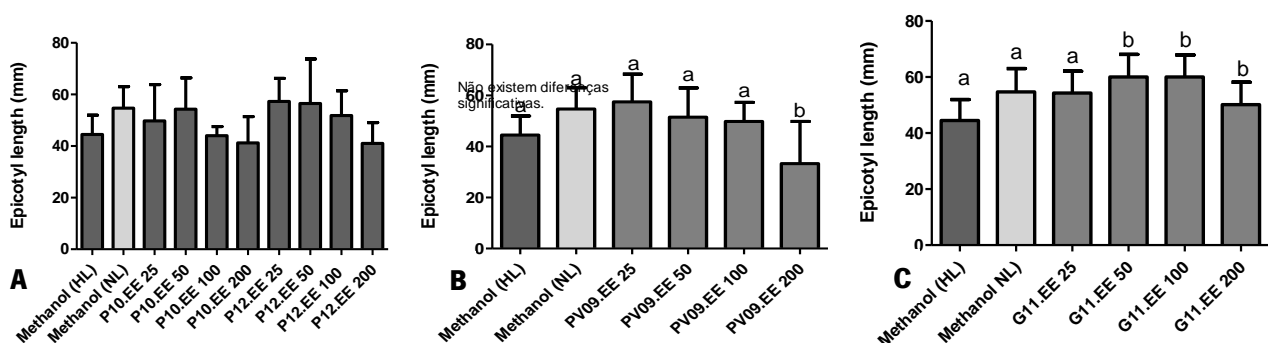
Figure 17. Average values of epicotyl length for several flax plants cultivated in medium with propolis extracts of Pereiro **(A)** (both P10.EE and P12.EE), PV09.EE **(B)** and G11.EE **(C)** at 25, 50, 100 and 200 mg/mL concentrations. Plants were exposed to photoinhibitory light intensities. NL stands for the normal light control condition and HL for the photoinhibitory high light condition. Columns represent mean values (n= 8) and the bars on top the respective SD. The P10.EE and P12.EE samples were analysed together with a two-way analysis of variance with Bonferroni test. The remaining samples were processed with one-way ANOVAs and Tukey test.

The ethanolic extracts from Pereiro (P10.EE and P12.EE) were not effective at any concentration (Fig. 17 A). PV09.EE was again inhibitory for the highest concentration but only moderately (74% of the HL control) (Fig. 17 B). Interestingly, G11.EE, the least phytotoxic extract so far tested, with no effects on root or hypocotyl growth, induced a significant increase in epicotyl length for the 3 higher concentrations (up to 34% more) (Fig. 17 C).

Overall the extracts seemed to affect hypocotyl growth to a lesser degree than they do in root growth, albeit still more on epicotyl growth.

To access the potential of the Portuguese propolis to protect against higher than normal light intensity with the *in vitro* cultures of flax with *n*-hexane and ethanolic extracts that were grown for two weeks exposed to a photoinhibitory light intensity, leaves were collected per sample and its contents in photosynthetic pigments measured spectrophotometrically (Fig. 18 and 20). All the statistical analyses were made by 1-way ANOVA's with Tukey post-test and the differences between all conditions can be consulted on Table I to V (Annex). From the same cultures chlorophyll fluorescence photosynthetic parameters (F_v/F_m, Φ_{II} and NPQ) were measured on leaves by PAM fluorometry.

Photoprotective effects of the Portuguese propolis extracts, both *n*-hexane and ethanolic, against light stress were evaluated in *in vitro* grown flax plantlets measuring photosynthetic pigments contents and *in vivo* photosynthesis by PAM fluorometry. The cultures were exposed to higher than normal light intensities (about 102 µmol m^{-2} s^{-1}) to simulate the photoinhibition situation. A clear evidence that plants were subjected to photoinhibitory light conditions was the highly significant decrease in total chlorophyll content of plants grown at HL when compared to those grown under NL conditions (Fig. 18 and 20). The high light and normal light controls of total chlorophylls are significantly different on both experiments. In relation to the *n*-hexane extracts selected, G11.HE, P12.HE and PV09.HE, total chlorophyll content and chlorophyll *a* over *b* ratio were

Figure 18. Photosynthetic pigments quantifications in leaves of flax plants grown on MS medium with ethanolic propolis extracts at 50,100, 200 and 400 mg/mL. **(A)**Total chlorophyll content of leaves of flax plants grown on MS medium with the selected n-hexane propolis extracts at 50, 100, 200 and 400 mg/mL. **(B)** Chlorophyll a over chlorophyll b ratio. The single dot at the concentration zero is the normal light control condition.

measured in flax plantlets grown with extract concentrations from 50 to 400 mg/mL (0.125mg/mL to 1mg/mL final concentration) (Fig 18).

Fig. 18 A displays the variation of total chlorophyll content in response to increasing concentrations of the *n*-hexane extracts tested. At the higher concentration, all the three extract show a significant rise, though the values are still way below the NL control. All extracts had reduced this light stress inhibitory effect on chlorophyll content in a concentration dependent-manner, however, the pattern of variation as a function of extract concentration is not linear. P12.HE cultures increased over the photoinibitory high light control until the 100 mg/mL extract concentration, and started decreasing at that point. PV09.HE shows a very similar behaviour to P12.HE, with a significant rise until that same concentration, moreover at the very same 100 mg/mL G11.HE has the sharpest increase of chlorophyll content of all three.

The ratio of chlorophyll *a* over chlorophyll *b* (Fig. 18 B) drop at 50 mg/mL on all three extracts and rises again from 100 to 400 mg/mL extract concentrations to ratios very close to the normal light control's, though overall the chlorophylls ratio differences are not very significant.

The maximum quantum efficiency (F_v/F_m) of dark-adapted leaves from plant cultures exposed to photoinhibitory high light intensity was measured by the PAM method and results are shown in figure 19 for the *n*-hexane extracts. No high light-induced effect was observed for this parameter because differences between HL and NL controls were not significant, but comparing *n*-hexane with ethanol controls of another independent experiment suggests that probably *n*-hexane has an inhibitory effect on F_v/F_m (Fig. 19 and 21).

Figure 19. Maximum quantum efficiency (F_v/F_m) values for several flax plants cultivated in medium with propolis extracts (G11.HE, P12.HE and PV09.HE) and exposed to photoinhibitory light intensities. Columns represent mean values (n= 8) and the bars on top the respective SD. NL stands for the normal light control condition and HL for the photoinhibitory high light condition.

Interestingly, plants growing in medium with *n*-hexane extracts reveal a concentration-dependent increase in the F_v/F_m parameter up to the considered normal values for healthy plants (around 0.8 to 0.83). P12.HE at the highest concentration exerted an inhibitory effect (Fig. 19 B), while G11.EE (Fig. 19 B) and PV09.HE (Fig. 19 C) increased the parameter.

Photoinhibition in flax plantlets was also studied in an independent experiment (Fig.20) with a selection of ethanolic extracts, namely G11.EE, P10.EE, P12.EE and PV09.EE with extract concentration from 25 to 200 mg/mL (0.625 mg/mL to 0.5 mg/mL of final concentrations). In addition to chlorophyll pigment quantification and chlorophyll a/b ratio, the total carotenoid pigments were also measured (Fig. 20).
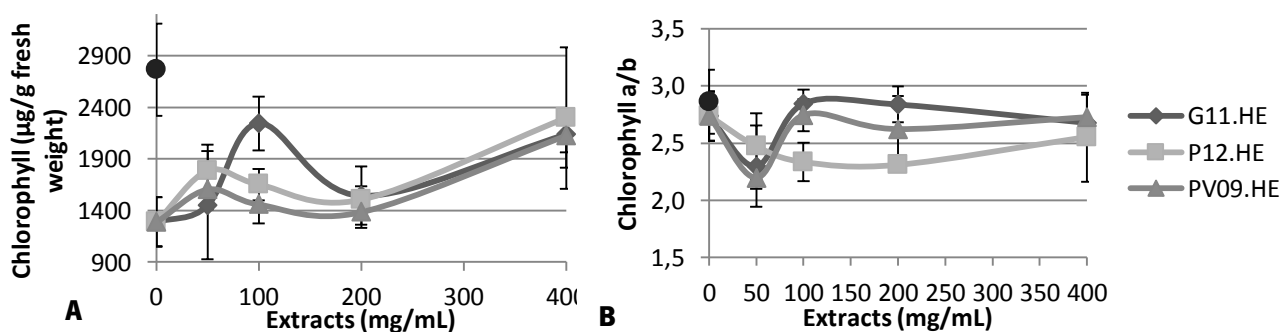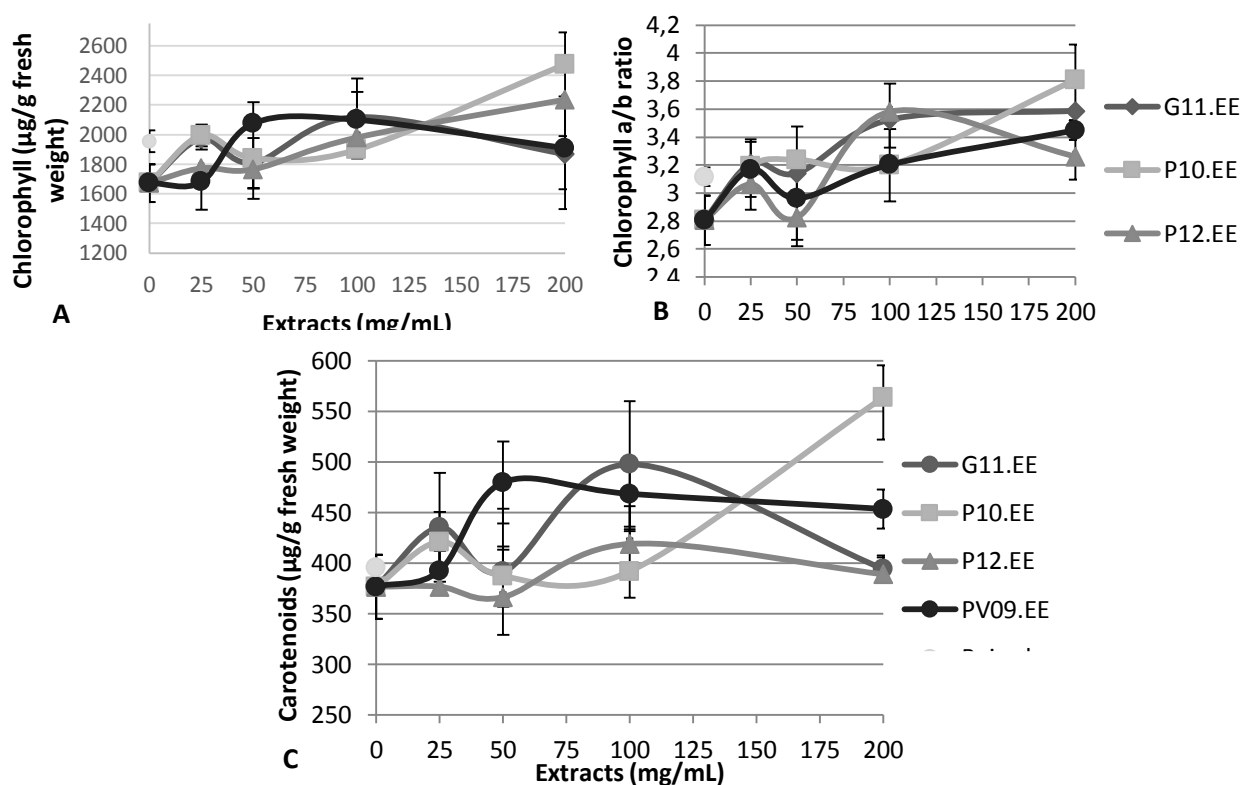

Figure 20. Photosynthetic pigments quantifications in leaves of flax plants grown on MS medium with ethanolic propolis extracts at 25, 50, 100 and 200 mg/mL. **A)** Total chlorophyll a and b on leaves of flax plants grown on MS medium with the selected ethanolic propolis extracts at 25, 50, 100 and 200 mg/mL. **B)** Ratio of chlorophyll a over chlorophyll b with the same propolis extracts and concentrations. The single dot at the concentration zero is the normal light control condition. **C)** Total carotenoids on leaves of flax plant grown as described above.

In PAM measurements, along F_v/F_m, the Φ_{II} and NPQ parameters were determined (Fig. 21, 22 and 23).

While *n*-hexane extract obtained from the Gerês sample (G11.HE) was one of the most active extracts increasing chlorophyll contents (Fig. 18 A), the ethanolic one (G11.EE) was the least (Fig. 20 A). On the other hand, ethanolic extracts from Pereiro samples (P10.EE and P12.EE) have promoted a significant increase in chlorophyll content, well above the control, at the highest concentration. PV09.EE increased slightly the chlorophyll content at lower concentration, but at the higher 200 mg/mL concentration the effect is reversed.

Regarding the chlorophyll *a* / *b* ratio, the variation according to the extracts concentration is somewhat erratic, however, the overall tendency to promote a net recovery from the light stress-induced decrease is clear for ethanolic extracts (Fig. 20 B). G11.EE extracts induced a significant increase of the ratio starting with the 25 mg/mL concentration and raised further in 100 and 200 mg/mL. In P10.EE the ratio suffers no significant variation until the highest concentration where it increases significantly, while on P12.EE only the two upper concentrations display a significant and the sharpest decrease of these set of extracts. PV09.EE promoted a significant increase only at the highest concentration (Fig. 20 B). Globally these results suggest that the increase in total chlorophylls is specially explained by an increase in chlorophyll *a*.

The carotenoids total content variation with extracts increasing concentration was also difficult to interpret and is generally affected by larger standard deviations, however the tendency to increase at lower or higher EE concentrations is similar to what was noticed for the previous parameters (Fig. 20 C). G11.EE did not decrease the carotenoid levels significantly for the most part. While the other extracts have peaked the plant content in carotenoids at low to medium concentrations maintaining or decreasing thereafter, P10.EE increased the carotenoid content very significantly only at 200 mg/mL, similarly to what was observed regarding chlorophyll content (Fig. 20 A). P12.EE variations were not statistically significant.

Regarding the ethanolic extracts G11.EE (Fig. 21 A), P10.EE (Fig. 21 B) and P12.EE (Fig. 21 C), all have promoted a significant decrease in F_v/F_m (Fig. 21). Comparing to the controls, G11.EE (Fig. 20 A) exert significant inhibition at lower concentrations (from 25 to 100 mg/mL), P10.EE (Fig. 20 B) displayed a significant reduction only at the highest concentrations (100 and 200 mg/mL) and P12.EE (Fig. 20 C) had no inhibitory effect. However, at the highest concentration (200 mg/mL), all extracts had increased relatively to 100 mg/mL (Fig. 21).

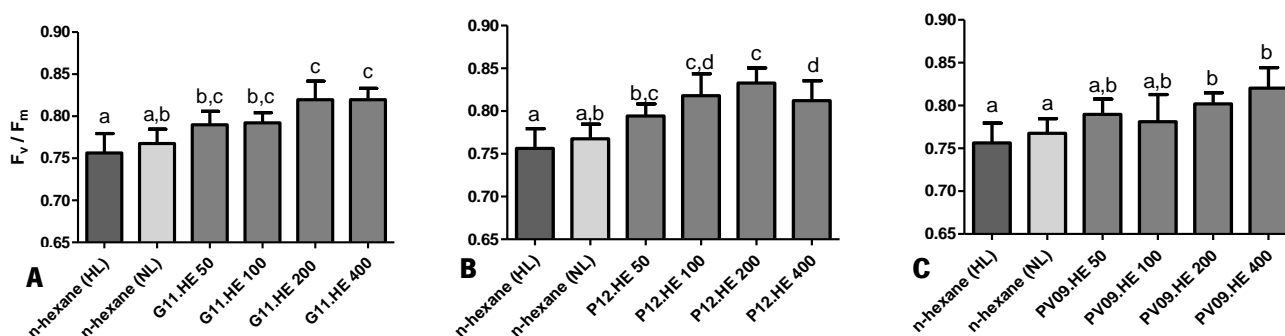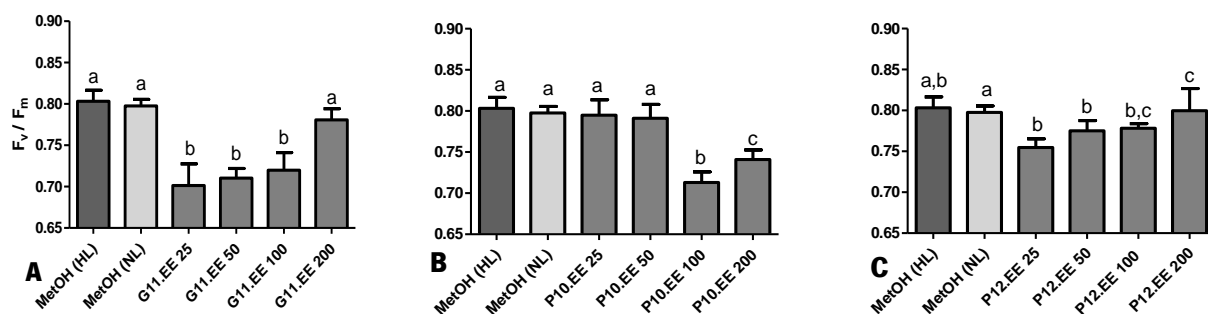**Figure 21.** Maximum quantum efficiency (F_v/F_m) values for several flax plants cultivated in medium with propolis extracts (G11.EE, P10.EE and P12.EE) at 25, 50, 100 and 200 mg/mL concentrations and exposed to photoinhibitory light intensities. Columns represent mean values (n= 8) and the bars on top the respective SD. NL stands for the normal light control condition and HL for the photoinhibitory high light condition.

Concerning the effective quantum yield (Φ_{II}), a measure of photosynthetic efficiency, the results were more dramatic when comparing with those of maximum quantum yield (Fig. 22).

This parameter has responded very differently when comparing Gerês (Fig. 22 A) with Pereiro ethanolic extracts. While G11.EE significantly reduced Φ_{II} at the lowest concentration tested (25 mg/mL) and steadily increase as a function of concentration up to control values, extracts from Pereiro samples were not effective at lower concentrations but reduced this parameter for higher ones. In particular P10.EE, one of the most active extracts for the parameters tested in this work, revealed a very significant decrease from 50 mg/mL to 200 mg/mL, where it reached around half of the control value (Fig. 22 B). P12.EE started to decrease from 25 to 100 mg/mL, but at the highest concentration (200 mg/mL) it produced an increase to values not statistically different from the HL control (Fig. 22 C).
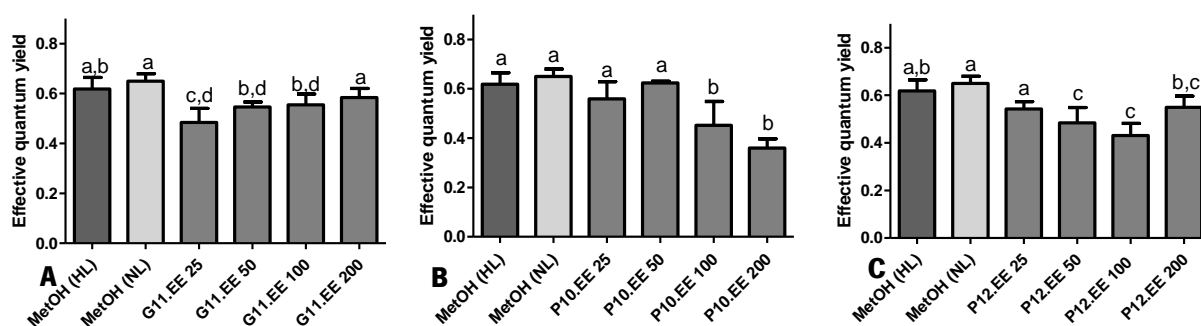
Figure 22. Effective quantum yield (Φ_{II}) values for several flax plants cultivated in medium with propolis extracts several flax plants cultivated in medium with propolis extracts (G11.EE, P10.EE and P12.EE) at 25, 50, 100 and 200 mg/mL concentrations and exposed to photoinhibitory light intensities. Columns represent mean values (n= 8) and the bars on top the respective SD. NL stands for the normal light control condition and HL for the photoinhibitory high light condition.

The non-photochemical quenching (NPQ) - the amount of radiation energy that is absorbed by the PSII but that is not conveyed to photosynthetic processes, like the xanthophyll cycle - was determined following the determination of the previous photochemical parameters (Fig. 23). Although control plants under NL and HL were not significantly different it seems that high light treatment have induced higher NPQ levels. Similarly to what was found for effective quantum yield, Gerês and Pereiro extracts promoted different effects: G11.EE were ineffective at all the concentrations tested (Fig. 23 A) while Pereiro extracts induced a dose-dependent increase in NPQ. P10.EE increased significantly the amount of energy dissipated non-photochemically (Fig. 23 B) at concentrations higher than 50 mg/mL more than doubling the control level at 200 mg/mL; P12.EE was effective above 25 mg/mL (Fig. 23 C) but at the highest concentration caused a reduction to values not statistically different from the HL control.
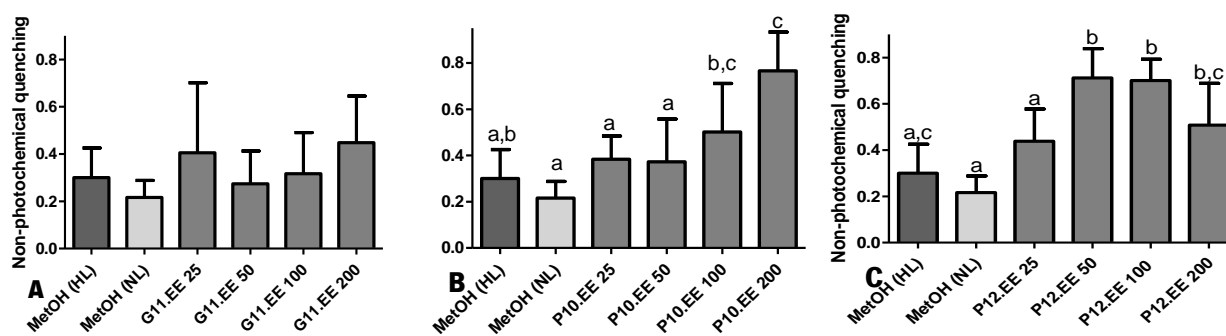
Figure 23. Non-photochemical quenching (NPQ) for several flax plants cultivated in medium with propolis extracts (G11.EE, P10.EE and P12.EE) at 25, 50, 100 and 200 mg/mL concentrations and exposed to photoinhibitory light intensities. Columns represent mean values (n= 8) and the bars on top the respective SD. NL stands for the normal light control condition and HL for the photoinhibitory high light condition.

Discussion

Propolis is a well-known by-product of the beehive with many reported bioactivities (Marcucci, 1995; Burdock, 1998; Lofty, 2006; Fokt *et al.*, 2010). Portuguese propolis has only been a subject of few studies in the last decade, remaining a topic of interest in many aspects. Not only the already reported genotoxic and antioxidant Portuguese propolis properties (Tavares *et al.*, 2006; Moreira *et al.*, 2008; Miguel *et al.*, 2010; Cruz, 2011) need deepening insights, but also new bioactive properties and the elucidation of propolis constituents' biological activities and targets are to be unveiled. A renewed scientific interest for propolis has risen in recent years for its potential uses in pharmaceutical industry but also as a food preservative. Propolis extracts have well-reported antioxidant activities, free radical scavenging or antimicrobial activities (Grange and Davey, 1990; Mirzoeva *et al.*, 1997; Menezes *et al.*, 1997; Lofty, 2006; Sforcin *et al.*, 2007; Falcão *et al.*, 2010; Fokt *et al.*, 2010). Regarding Portuguese propolis, only few bioactivities like the antioxidant activity (Moreira *et al.*, 2008; Miguel *et al.*, 2010) has been reported so far.

In the present work, a combined approach was followed using the yeast *Saccharomyces cerevisiae* and *in vitro* cultures of flax (*Linum usitatissimum*) as biological models. Regarding *Saccharomyces*, there are very few studies of propolis using this yeast model (Alves de Castro *et al.*, 2011), particularly with Portuguese samples (Cruz, 2011). In what concerns propolis effects against plants, and although allelopathy is a common rule in species communication, only very few investigations have reported phytotoxic activity (Gusman *et al.*, 2008) and no references were found in the literature so far for Portuguese propolis. Therefore the need for this study was evident. The main goals of this work were to investigate Portuguese propolis dual properties, toxic and protective, using the yeast model to evaluate cytotoxic, genotoxic/genoprotective activities and the plant model to assess its phytotoxicity/photoprotection capacity against light-induced stress. These properties would open new avenues in antimicrobial and bioherbicidal research and also in UV-induced cell damage therapeutics.

1. **Propolis ethanolic extracts are cytotoxic decreasing yeast cell growth and viability**

After the preparation of *n*-hexane or ethanolic extracts, these were tested on viability assays at a high concentration (500 µg/mL). The results of this preliminary screening allowed the selection of extracts of particular interest for further experiments. None of the *n*-hexane extracts caused significant growth inhibition (Fig. 1) and were therefore discarded from the extracts panel for microbial studies. The ethanolic extracts obtained from propolis samples of Póvoa de Varzim (PV09.EE) and Pereiro (P10.EE and P12.EE) displayed the strongest effects with the presence of only a few yeast colonies (Fig.1 and 2), and thus were further tested using a range of lower concentrations, in an attempt to characterise its dose-dependent inhibitory profile. Other ethanolic extracts such as G12.EE showed more modest growth inhibition and C10.EE and G11.EE displayed no significant activity whatsoever on yeast and were also excluded from further experiments.

2. **Propolis extracts from Pereiro samples are genotoxic inducing DNA damage on yeast**

In order to investigate genotoxic effects of Portuguese propolis, comet assay was performed in yeast with P10.EE. The antigenotoxicity and genotoxicity of propolis extracts has been described before as a Janus type effect (Tavares *et al.*, 2006; Cruz, 2011). In other words, at lower doses there is a described chemoprotective activity on DNA, but unequivocal signs of genotoxicity appear at higher extract concentration. In this work however, neither antigenotoxicity nor the existence of a dual effect were seen. Nonetheless, our results show clear genotoxic activity at the higher tested concentration (200 µg/mL) and absence at the lowest (50 µg/mL; Fig. 3). This supports the view of a dose-dependent effect, and also is coherent with the results obtained by viability assays (Fig. 2), where yeast growth was inhibited by 200 µg/mL P10.EE. The bioactive compounds of propolis described in many samples from different origins, including Portuguese propolis (Bankova *et al.*, 1998; Salomão *et al.*, 2004; Falcão *et al.*, 2010), such as flavonoids, may be responsible for these activities, as they can act either as free radical scavengers or prooxidants, depending on the concentration (Tavares *et al.*, 2006).

3. Propolis extracts promote mitochondrial damage in yeasts induced by reactive oxygen species

One of the main cellular targets of the cytotoxicity exhibited by the Portuguese propolis extracts tested in the wide batch of viability assays performed, could be the cell mitochondria. It has been described in the literature that propolis extracts induce programmed cell death and target the mitochondrial membrane protein cytochrome *c* (Alves de Castro *et al.*, 2011). To prove the hypothesis, flow cytometry with a fluorochrome whose fluorescence is linked to mitochondrial membrane potential (Ludovico *et al.*, 2001), such as rhodamine 123, was performed and the membrane potential variation according to the incubations with the propolis extracts was measured (Fig. 7). Yeast cell were grown in a non-fermentable carbon source, so that the mitochondria represents the only possible path to cell's survival, through respiration. Cells incubated with P10.EE showed a remarkable reduction of membrane potential, P12.EE caused a slighter effect at the same concentrations and PV09.EE was the most toxic, reducing far more the membrane potential even at lower concentrations. This reduction on membrane potential leads to a permeabilization of the outer membrane and release of mitochondrial proteins such as cytochrome *c* that are known to trigger programmed cell death (Murphy, 2009). These results support the hypothesis that one possible mechanism of the extracts cytotoxicity is by mitochondrial damage, which could probably be a mechanism shared with other eukaryots.

4. Propolis ethanolic extracts have antioxidant activity in yeast cells decreasing the basal intracellular oxidation status but also the high oxidation levels induced by hydrogen peroxide

To assess the antioxidant activity of selected Portuguese propolis extracts, a flow cytometry technique was chosen with a redox-sensitive fluorochrome, dichlorofluorescein diacetate on yeast cells incubated with the extracts (PV09.EE, P10.EE and P12.EE) and co-incubated or pre-incubated with H_2O_2. Cells showed a dose-dependent decrease in intracellular oxidation in all three extracts, but more significantly with the PV09.EE and P10.EE extracts and in simple and co-incubation procedures, whereas in pre-incubation experiments, the antioxidant effect is less significant. To our knowledge, there are a few previous studies of Portuguese propolis samples that also reports antioxidant activity (Moreira *et al.*, 2008; Miguel *et al.*, 2010; Cruz, 2011). From these different actions in co- and pre-incubations (Fig. 4, 5 and 6), it can be hypothesised that the main mechanism of the antioxidant compounds of the extracts is by direct scavenging of free radicals and hazardous intracellular produced molecules, rather than an upstream regulatory chain of action by cell defence induced pathways, assuming that those pathways once triggered would still be effective regardless of

the continued presence of the antioxidant compounds. In short, the evidence sustain that the Portuguese propolis extracts are also rich in antioxidant compounds such as those like polyphenols and flavonoids that are commonly reported among the main constituents of the extracts and are of great antioxidant activity (Bankova *et al.*, 2000; Moreira *et al.*, 2008; Miguel *et al.*, 2010).

5.   Propolis extracts can inhibit plant growth and photosynthesis

Propolis extracts effects were also evaluated on two-weeks-old plantlets of flax germinated *in vitro* on MS medium with propolis extracts at 200 mg/mL (500 µg/mL final concentration). Phytotoxicity was assessed measuring plant growth (Fig. 8, 9 and 10), specifically the root, hypocotyl and epicotyl growth, and *in vivo* photosynthetic activity (Fig. 12).

Concerning the impact on early plant growth, the most toxic extracts were again P10.EE and PV09.EE, inhibiting root development quite pronouncedly and with milder inhibitory effects on hypocotyl and epicotyl development. This strong effect on the seminal root development may be explained by a cytotoxic effect, as observed in the yeast model. An increased root ramification could also be observed with these extracts as well as with some of the *n*-hexane ones, like PV09.HE. This may reflect an impairment in auxin polar transport to the root apex inducing lateral or adventitious root differentiation (Teale *et al.*, 2005; Cheng *et al.*, 2013). A particular morphological trait (Fig. 11) was observed in the root apex, with some outer layers of cells growing outwards in a scrambled, non-organized manner, in plantlets grown in the presence of some of the most active extracts, like ethanolic and *n*-hexane extracts obtained from Póvoa de Varzim samples (PV09.HE, PV09.EE) and the ethanolic extracts from Pereiro (P10.EE and P12.EE).

It is known that many plant exudates and compounds of the secondary metabolism, some of which are common in propolis composition, such as flavonoids, terpenes or alkaloids (Macías *et al.*, 2007; Gusman *et al.*, 2008; Falcão *et al.*, 2010), have natural allelopathic properties causing developmental changes on many plant species. Allelopathic interactions have already been described in Brazilian propolis, where propolis extracts inhibited radicular development on several botanical species with roots growing abnormally thicker (Gusman *et al.*, 2008). Our results go in line with this effect of propolis in root growth inhibition, but although increased root thickness was not observed for the Portuguese extracts tested, other conspicuous phenotypes were identified, suggesting that different propolis compounds with different cellular targets may play a role in this root development impairment syndrome. Further studies would be required for

a more accurate characterization of this broad effect on root development, namely for the identification of bioactive compounds and mechanisms of action.

In this first screening the impact of propolis extracts on the photosynthetic function of *in vitro* grown flax plantlets was assessed by PAM fluorometry, measuring the photochemical parameter maximum quantum efficiency (F_v/F_m) (Fig. 12). Only P10.EE showed a significant inhibitory effect, but knowing that this parameter is quite resilient to stress conditions, this suggests that at least this particular extract could severely hamper the photosynthetic apparatus of plants. In fact, this extract was also the most toxic in yeast, inducing the highest levels of oxidative stress in mitochondria and genotoxicity, suggesting that this impact on photochemical efficiency of PSII may be mediated by ROS production. It is noteworthy that many allelochemicals found in plants, like phenolic acids, have been suggested as likely repressors of photosynthesis (Leather and Heinhellig, 1988). Moreover, given that some commercial herbicides mode of action is by inhibiting photosynthesis (Duke, 2011), these findings could be of pivotal importance in possible applications of these extract's compounds in commercial herbicides.

6. Propolis ethanolic extracts have strong impacts on photochemical and non-photochemical quenching pathways but also protect flax plantlets from high light-induced photooxidative stress

A second experiment was deployed to study propolis effects in plantlets grown under high light (HL) stress conditions. Strong UV radiation but also excessive white light intensities may damage plants and hinder their photosynthetic capacity. This excessive light energy absorbed by the antenna that could not be efficiently processed by the photochemical pathway, generally causes damage through the formation of ROS species. The oxidative damage tackles certain key enzymatic processes related to photosynthesis such as those related with the biosynthesis of chlorophyll (Aarti *et al.*, 2007), but also cause direct damage to macromolecules of the photosynthetic apparatus such as proteins and thylakoid membrane lipids (Pospíšil, 2012). Portuguese propolis extracts, given its complex and rich chemical composition and its known antioxidant activity could conceivably counter the high light-induced damage on plants. As in the first screening experiment, also here plant growth (root, hypocotyl and epicotyl) and maximum quantum efficiency (F_v/F_m) was assessed, but additionally a thorough evaluation of propolis effects on overall photosynthetic performance

and non-photochemical dissipative capacity was performed by measuring the effective quantum yield (Φ_{II}), non-photochemical quenching (NPQ), and the total content in photosynthetic pigments.

High-light stress effects on photosynthesis and potential photoprotective role of propolis extracts were studied by growing flax plant cultures under high light intensities (102 µmol m^{-2} s^{-1}, which is approximately 3 times the normal level used in the *in vitro* cultures growth chamber) in the presence of increasing extracts concentrations. In this experiment three *n*-hexane extracts (G11.HE, P12.HE and PV09.HE) and four ethanolic extracts (PV09.EE, G11.EE, P10.EE and P12.EE) were tested (Fig. 13 and 14). Corroborating results obtained with the screening experiment, P10.EE, P12.EE and PV09.EE showed their toxicity by a clear dose-dependent inhibition of root development, but also at a lesser scale on hypocotyl growth (Fig. 15, 16 and 17). Pereiro extracts showed no significant effect on epicotyl whereas PV09.EE and curiously G11.EE display some significant effect on epicotyl growth, though its effects on root and hypocotyl were only marginal. Overall, the most toxic extracts displayed its effects mostly at the radicular development level. As discussed earlier, as the myriad of compounds that are present in propolis, ranging from flavonoids to terpenes or quinones, can target root development, it is likely that this effect on plant growth is explained by the action of some of these compounds. As referred above, one possible mechanism may be by these compounds acting as prooxidants causing cytotoxicity. As plant interactions frequently occur in nature through allelochemicals, released as root exudates or volatile blends (Delachiave *et al.*, 1999; Macías *et al.*, 2007), not only between plants species but also with other organisms (Macías *et al.*, 2007), it is likely to be the case with these propolis extracts (Gusman *et al.*, 2008; Badri and Vivanco, 2009).

The total amount of chlorophyll pigments (Fig. 18 and 20) in leaves may give an indication of the overall plant photosynthetic capacity and whether it has been hampered or enhanced by propolis extracts when subject to high light stress (Aarti *et al.*, 2007; Baker, 2008). The ratio of chlorophyll *a* over chlorophyll *b* translate the light environmental conditions during plant growth, with lower ratios indicating an adaptation to higher light intensity regimes, but can also indicate specific effects at the reaction centers of photosystems because only chlorophyll *a* is present in their core center (Papageorgiou and Govindjee, 2004). The significant reduction in total chlorophyll content of plantlets grown under high light suggests that these light conditions tested had effectively caused chlorophyll damage, probably by photooxidation and most likely through the formation of ROS in the photosystems (Aarti *et al.*, 2007; Kumar and Kasturi Bai, 2009). All the *n*-hexane extracts tested (G11.EE, P12.HE and PV09.HE) had caused a recovery of the chlorophyll contents (Fig. 18 A) at the highest concentration tested (400 mg/mL), but still below the NL levels, suggesting an alleviating effect from the photooxidative-induced damage to the chlorophylls. The first rise at lower extracts concentrations can be explained by a specific protective effect over chlorophyll *a* that is supported by the parallel rise in

chl.*a*/chl.*b* ratio (Fig. 18 B). P10.EE and PV09.EE caused the most significant increases in total chlorophylls and chl.*a*/chl.*b* ratio of the tested extracts (Fig. 20), which can be interpreted as an alleviation of ROS pressure especially over chlorophyll *a* and eventually over the photosystems.

Also interesting to note is the variation on carotenoid content (Fig. 20 C). PV09.EE and P10.EE increase this class of pigments significantly well above NL and HL control levels, which could be associated with the increase in non-photochemical processes (Fig. 23) stimulated by Pereiro ethanolic extracts. In fact, higher light intensities stimulate the acidification of thylakoid lumen and the regulation of the xanthophyll cycle (XC) to convert violaxanthin into zeaxanthin, which effectively quenches excess excitation energy by the non-photochemical pathway (Baker, 2008), and under high light regimes a higher concentration in XC carotenoids is generally observed.

Through chlorophyll fluorescence analysis by PAM fluorometry, the maximum quantum efficiency, effective quantum yield and non-photochemical quenching parameters were also determined on the plantlets cultures exposed to HL conditions, as referred above. The first two correlate, respectively, with the amount of excitation energy received by the plant that is potentially or effectively put to use in photosynthesis, while the non-photochemical quenching is the portion lost on processes unrelated to photosynthesis (Papageorgiou and Govindjee, 2004; Baker, 2008). The xanthophyll cycle also plays a role in protecting the chloroplast from oxidative damage, as the carotenoids actively scavenge ROS molecules like singlet oxygen and the excessive electron energy is dissipated as heat through NPQ (Munée-Bosch and Alegre, 2003).

The *n*-hexane extracts tested (G11.HE, P12.HE and PV09.HE) appear to have increased the F_v/F_m parameter (Fig. 19), while the ethanolic extracts of G11, P10 and P12 have decreased (Fig. 21) but independently from the light conditions, since no significant effects were induced by high light on this parameter. Curiously, *n*-hexane extracts by recovering the low F_v/F_m observed in control plants to the considered normal values, suggest that the solvent itself causes damage to PSII and that it is reverted by HE compounds in a dose-dependent manner. On the other hand, the ethanolic could cause F_v/F_m reduction at least in part by oxidative-induced damage.

The ethanolic extracts caused also significant reductions in effective quantum yield (Fig. 22) and increases in non-photochemical quenching (Fig. 23), which indicates divert of the energy received by light absorption away from the photochemical to non-photochemical pathways. It seems that a certain pattern is observed: the *n*-hexane extracts that have throughout the experiments shown little to no toxic effect on plant and yeast, appear to improve plant overall photosynthetic capacity under light stress conditions by protecting chlorophyll from photooxidative-induced damage and the ethanolic extracts that have exhibited the most toxic biological activities such as PV09.EE, P10.EE and P12.EE are also among the group that shifts the

photosynthetic function from photochemically productive to non-photochemically dissipative, reducing photosynthetic capacity of the plants. In fact, P10.EE was the extract that promoted the highest decrease in photosynthesis, increase in total carotenoids and in NPQ simultaneously, corroborating the idea that the increase in total carotenoids induced by EE were related with non-photochemical pathways. This reduction in the overall photosynthetic capacity may also explain the strong impact on plant development.

The combined experimental approaches of the present work allow for the characterisation of diverse biological activities in the set of Portuguese propolis extracts chosen. Ethanolic extracts such as PV09.EE, P10.EE and P12.EE exhibited significantly inhibitory effects in several cellular functions both in yeast and plant growth, appearing to constitute a toxic blend of compounds with a more general inhibitory action, such as prooxidative. The two samples from the Pereiro apiary show differences in their activity, being the sample collected in 2012 less bioactive than the one collected in 2010, highlighting the importance of data of collection in determining propolis composition and biological properties. This could be explained as propolis composition varies substantially with the time of the collect, bee behaviour patterns or changes to the surrounding flora (Bankova *et al.*, 1998; Falcão *et al.*, 2010). Together, the effects visible on plant chlorophylls and carotenoids levels and PAM parameters (F_v/F_m, Φ_{II} and NPQ) and on yeast mitochondria membrane potential seems to corroborate the bioactivities we found for the P10.EE, P12.EE and PV09.EE throughout this work, as well as pointing some basic targeting of the biological activities endured by these organisms. That is, primarily root and hypocotyl growth and photosynthetic apparatus on plants, and the mitochondria on yeast. These extracts show also an antioxidant effect on yeast, presumably connected to a direct scavenge of free radicals, which on non-lethal doses might contribute to up the cellular fitness, and might be of particular interest to pharmaceutical applications. Many compounds known to be common in propolis such as flavonoids are frequently reported to have antioxidant and free radical scavenging activity (Tavares *et al.*, 2006; Hernandez *et al.*, 2008; Moreira *et al.*, 2008).

The *n*-hexane propolis extracts appear to have little toxicity, let aside some misgravitropic responses, and therefore the bulk of their study was directed to the hypothesis of photoprotective activity with the experiment performed under photoinhibitory conditions. The most striking result obtained was the recovery of chlorophyll contents to normal NL levels in a dose-dependent manner and maintenance of chlorophyll a/b ratio. As F_v/F_m was not significant affected by the HL regime, photoprotective effects from photoinhibition could not be evaluated. However, the intrinsic toxicity of *n*-hexane observed for the dilutions used in F_v/F_m, if do not let to rule out possible changes in aspects of plant's physiology by recovering the low F_v/F_m observed in NL or HL control plants to considered normal values, suggest that some constituents of *n*-hexane extracts could avoid or repair *n*-hexane induced PSII damage, by a mechanism still unknown.

From the overall results obtained with these Portuguese propolis extracts, it is possible to forecast several commercial applications, ranging from its antioxidant activities that would make an interesting source of compounds for food or pharmaceutical industries, to its described phytotoxic activities that could eventually lead to the development of new herbicides.

Final Remarks and Future Perspectives

To better understand all the aforementioned biological activities of Portuguese propolis, a complete chemical characterization of the studied propolis extracts would be pivotal. This information would be invaluable to relate specific compounds or combinations of compounds to certain biological activities found in our work and reported in the literature, as well as to identify compounds or mixtures responsible for the biological activities not yet reported in the literature, such as the phytotoxic ones. The evaluation of isolated compounds and synergistic mixtures allow to elucidate its modes of action and also to further devise other commercial applications. Also the differences found on biological activities between the same apiary samples collected in different years (namely P10.EE and P12.EE), could be explained by the different chemical composition and even point towards the environmental changes on the flora or the bee's behavioral patterns of collection. Propolis from different regions, would likely reflect differences in the surrounding flora in its chemical composition.

In this work, we were able to highlight the genotoxicity of the P10.EE extract. Future studies to complement our findings would explore the genoprotective effects of P10.EE along with the remaining extracts. As reported before (Bankova *et al.*, 2000; Moreira *et al.*, 2008; Cruz, 2011) propolis extracts display concentration-dependent antioxidant and prooxidant activities. The nature of *S. cerevisiae* genetic system makes also easy to use mutant strains affected in stress responses, DNA repair and antioxidant defence pathways, which coupled with the chemical composition of the extract would elucidate specific cellular targets and modes of action of specific compounds.

The results obtained with flax plant *in vitro* cultures, revealed in the first place that this was an adequate plant model to evaluate phytotoxicity with great sensitivity at both physiological and developmental level. Additionally, PAM fluorometry was a powerful tool to analyse different aspects of photosynthetic function and proved to be very useful in the evaluation of both inhibitory effects of extracts on photochemical and non-photochemical parameters but also of its photoprotective potential. This tool would be further explored to study propolis effects against UV-induced damage in eukaryotic cells.

With respect to the broad inhibitory pattern of the ethanolic extracts PV09.EE and P10.EE, that caused strong effects both on root and yeast growth, flow cytometry approaches will be entailed to investigate if those effects were associated with any impairment in the cell cycle that would be of most relevance in drug development.

What is more, we believe that propolis is not biologically active as a mere consequence of being a mixture of phytochemicals, but more that propolis is produced with certain chemical and bioactive profiles to play ecologically important functions, like germination avoidance or antimicrobial activity (Marcucci, 1995; Sforcin, 2007), protecting the bees and the integrity of the beehive.

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Annex

The following tables are supplementary statistical data of the pigments extraction and quantification on flax plants. Data was analysed with one-way ANOVA followed by Tukey *post-hoc* test for multiple comparisons, except when the two Pereiro extracts were paired, on which case a two-way ANOVA with Bonferroni *post-hoc* test was performed. The statistically significant differences between conditions are listed with the appropriate letters.

Table I: Total amount of chlorophyll for flax cultures with n-hexane extracts.

Chlorophyll (µg/g fresh weight)			
	Extracts		
Control/Concentrations (µg/mL)	G11.HE	P12.HE	PV09.HE
n-hexane (HL)	a	a	a
n-hexane (NL)	b	b	b
50	a	a	a
100	b	a	a
200	a	a	a
400	a,b	b	b

Table II: Total amount of chlorophyll for flax cultures with ethanolic extracts.

Chlorophyll (µg/g fresh weight)				
	Extracts			
Control /Concentration (µg/mL)	G11.EE	P10.EE	P12.EE	PV09.EE
Methanol (HL)	a	a	a	a,b,c
Methanol (NL)	a	a	a	a
25	a	a	a	b
50	a	a	a	c
100	a	a	a	a
200	a	b	a	a

Table III: Ratio of chlorophyll a over chlorophyll for flax cultures with n-hexane extracts.

Chlorophyll a/b ratio

Control/Concentration (µg/mL)	Extracts		
	G11.HE	P12.HE	PV09.HE
n-hexane (HL)	a	a	a
n-hexane (NL)	a,b	a	a
50	a,c	a	a,b
100	a	a	a
200	a	a	a
400	a	a	a

Table IV: Ratio of a over chlorophyll for flax cultures with ethanolic extracts.

Chlorophyll a/b ratio

Control/Concentration (µg/mL)	Extracts			
	G11.EE	P10.EE	P12.EE	PV09.EE
Methanol (HL)	a,b	a	a	a
Methanol (NL)	a,c	a	a,b	a,b
25	b	a	a	a
50	a	a	a	a
100	c	a	b	a
200	d	b	b	b

Table V: Total amount of carotenoids for flax cultures with ethanolic extracts.

Carotenoids (µg/g fresh weight)

Control/Concentration (µg/mL)	Extracts			
	G11.EE	P10.EE	P12.EE	PV09.EE
Methanol (HL)	a	a	a	a
Methanol (NL)	a,b	a	a	a,c
25	a	a	a	a
50	b	a	a	b
100	a	a	a	c
200	a	b	a	a