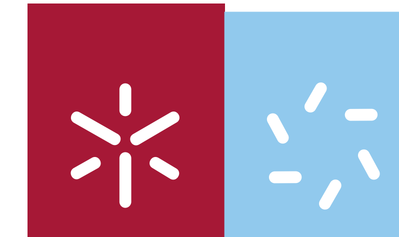


Universidade do Minho
Escola de Ciências

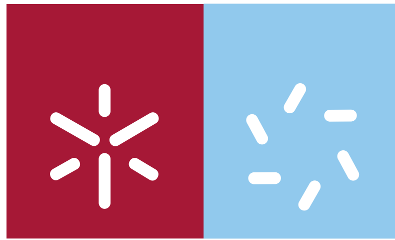
Márcia Andreia Oliveira Cruz

Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis

Outubro de 2011

Márcia Andreia Oliveira Cruz **Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis**

UMinho | 2011



Universidade do Minho
Escola de Ciências

Márcia Andreia Oliveira Cruz

Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis

Dissertação de Mestrado
Mestrado em Biotecnologia e Bio-empreendedorismo em Plantas Aromáticas e Medicinais

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

Outubro de 2011

Declaração

Nome: Márcia Andreia Oliveira Cruz

Endereço electrónico: marciaaocruz@gmail.com **Telefone**: 912060518

Número do Bilhete de Identidade: 13235377

Título da tese: Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis

Orientadores: Prof. Doutora Cristina Aguiar e Prof. Doutor Rui Oliveira

Ano conclusão: 2011

Designação do mestrado: Mestrado em Biotecnologia e Bio-empreendedorismo em Plantas Aromáticas e Medicinais

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

Universidade do Minho, 31 de Outubro de 2011

Assinatura:

Acknowledgments/Agradecimentos

My academic life was only possible with the help of my parents and my brother. The effort, care and love that they gave me through these years were essential to the success of my work. For all that support, I want to thank them a lot. I will love you forever!

Thanks to my boyfriend, always my company, in the best and worst moments, my friend and my love.

To my supervisors, Cristina Aguiar and Rui Oliveira, I thank all their availability, patience, and friendship.

I also want to thank professors Ana Cunha and Margarida Ferreira for helping me to prepare and to analyse propolis extracts. Thanks for sharing your knowledge with me.

I would like to thank Alberta, Ana Raquel and Isabel for their friendship, help and company in the laboratory.

Finally, I would like to show my gratitude to all the staff of the Department of Biology for all assistance in the realization of this work, especially to Cristina Ribeiro for the great help in cytometry and to Isabel.

A huge thank to Pedro Fernandes from "*Mel do Abel*", for the kindness in providing Propolis to this work.

Avaliação e caracterização das propriedades antioxidante e antigenotóxicas de propolis português

Resumo

O própolis é uma substância produzida pelas abelhas (*Apis mellifera* L.) após a colheita de brotos e cascas de plantas e pela mistura posterior com a enzima salivar β-glicosidase. As abelhas utilizam o própolis nos seus favos como proteção, para reparar danos, construir locais assépticos para os ovos da rainha, e também como isolante térmico. A composição química do própolis varia geograficamente, com a flora disponível, a época da colheita, e da raça das abelhas. Vários grupos de compostos podem ser encontrados nos extractos de própolis, tais como, polifenóis, terpenóides, esteróides e aminoácidos. Estes compostos têm estado associados a diversas actividades biológicas: antioxidante, antimicrobiana, *scanvenger* de radicais livres, antigenotóxico/genotóxico e antimutagénico. O própolis português tem sido pouco estudado o que abre a perspectiva da sua valorização económica através da validação cientifica das suas actividades biológicas normalmente atribuídas a amostras de outras origens. Assim, o nosso objectivo prende-se com a análise e estudo do própolis português, nomeadamente no que respeita à sua caracterização química e avaliação das suas actividades biológicas. Uma amostra colhida na Beira Interior (Côa) foi usada para preparar um extracto etanólico de própolis (PEE) para testar em diferentes ensaios utilizando *Saccharomyces cerevisiae* como modelo biológico. Para investigar o efeito protector do PEE em células de levedura, efectuaram-se ensaios de viabilidade com peróxido de hidrogénio (H_2O_2). Para avaliar o efeito antigenotóxico de PEE usou-se o ensaio cometa e para verificar a sua actividade antioxidante intracelular usou-se citometria de fluxo. A amostra de própolis foi analisada quimicamente para quantificar o teor em polifenóis totais e flavonóides, e os métodos de DPPH e ABTS foram usados para demonstrar a actividade antioxidante *in vitro*. Os nossos resultados sugerem que o própolis português tem capacidade antioxidante quando avaliada pelo ensaio DPPH (*in vitro*) e por citometria de fluxo (*in vivo*). Do mesmo modo, a viabilidade celular da levedura aumentou, tanto em condições de pré-incubação e co-incubação, na presença de um agente oxidante (H_2O_2). No entanto em incubações prolongadas de células com PEE observamos um decréscimo da viabilidade. O ensaio cometa sugere que o PEE tem efeito antigenotóxico, ao proteger o DNA contra stresse oxidativo, e genotóxico quando usado sozinho na incubação de células. Para além da acção antioxidante, estes resultados sugerem uma acção pró-oxidante do PEE.

Evaluation and characterization of antioxidant and antigenotoxic properties of Portuguese propolis

Resume

Propolis is a substance produced by bees (*Apis mellifera* L.) after harvest of buds and bark of plants and by subsequent mixing with the salivary enzyme β-glucosidase. Bees use propolis in their combs as protection, to repair damage, to build aseptic locals for the eggs of the queen, and also as a thermal insulator. The chemical composition of propolis varies geographically, with the available flora, the time of collection and the race of the bees. Different group of compounds can be found in propolis extracts, such as polyphenols, terpenoids, steroids and amino acids. These compounds have been associated with diverse biological activities: antimicrobial, antioxidant and scavenger of free radicals, antigenotoxic and genotoxic, antimutagenic. Portuguese propolis has been insufficiently studied, which possibilitates the opportunity for its economic valorization by scientifically support the biological activities commonly assigned to samples from other origins. Thus, our objective relates to the analysis and study of Portuguese propolis, particularly in what concerns chemical characterization and evaluation of biological activities. A sample collected in Beira Alta (Côa) was used to prepare an ethanol extract (PEE) and this extract was tested in different assays, using *Saccharomyces cerevisiae* as biological model. To investigate the protector effect of PEE in yeast cells, viability assay was made using hydrogen peroxide (H_2O_2). Comet assay was made to evaluate the antigenotoxic effect of PEE and flow cytometry to verify the antioxidant activity. The sample was analyzed chemically to quantify total polyphenolic and flavonoids content, and DPPH and ABTS to demonstrate the antioxidant activity *in vitro*. Our results suggest that Portuguese propolis has antioxidant capacity when assessed by the DPPH assay (*in vitro*) and flow cytometry (*in vivo*). Accordingly, viability has improved when propolis was assayed, either by pre-incubation or co-incubation with yeast cells shocked with an oxidant agent (H_2O_2). However, prolonged incubation of cells with high concentrations of PEE promoted decrease of viability. Results obtained by the comet assay suggest that PEE has antigenotoxic activity, protecting the genome against oxidative stress, and genotoxic effect when used alone in incubation of yeast cells. Besides the antioxidant activity, we provide results suggesting a prooxidant activity of PEE.

Index

1. Introduction 1
1.1. Oxidative stress and genome integrity 1
1.1.1. Oxidative stress....................1
1.1.2. DNA damage and repair 2
1.1.3. Methods for DNA damage assessment 3
1.1.4. Prevention of DNA damage/oxidative stress using phytochemicals 5
1.2. Propolis 6
1.2.1. Chemical composition of propolis and biological activities 7
1.2.2. Polyphenol and flavonoid compounds....................8
1.2.3. Portuguese propolis 8
1.3. Biological problem and objectives of this work 9
2. Material and Methods....................11
2.1. Yeast strain, media and growth conditions 11
2.3. Viability assay 11
2.4. Comet assay 12
2.5. Flow cytometry....................13
2.6. Chemical analysis of Propolis 14
2.6.1. Quantification of total polyphenols content 14
2.6.2. Quantification of flavonoids 14
2.6.3. DPPH assay 14
2.6.4. ABTS assay 15
2.7. Statistic analysis....................15
3. Results 16
3.1. Effects of PEE on cell viability under stress conditions....................16
3.2. PEE protects yeast cells from DNA damage by H_2O_2 19
3.3. Chemical characterization of propolis 22
3.4. PEE decreases intracellular oxidation....................23
4. Discussion 25
5. Future perspectives....................28
6. References 29

Abbreviation list:

8-oxoG – 8-oxo-7,8-dihydroguanine

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

A/P – apuric/apyrimidinic

BER – Base Excision Repair

CAT – catalase

DNA – deoxyribonucleic acid

DSB – double-strand breaks

DPPH – 1,1-diphenyl-2-picrylhydrazyl

PEE – propolis ethanol extract

EDTA – ethylenediamine tetraacetic acid

GSH-Px – glutathione peroxidase

GSH-Red – glutathione dehydrogenase

G6PD – glucose-6-phosphate dehydrogenase

HR – homologous recombination

H_2DCFDA – dichlorofluorescein diacetate

MMR – mismatch repair

NADPH – nicotinamide adenine dinucleotide phosphate (reduced form of $NADP^+$)

NER – Nucleotide Excision Repair

NMA – Normal Melting Agarose

NHEJ – non-homologous end joining pathways

N^7-megG – N^7-methylguanine

PBS – phosphate buffered saline

PCR – polymerase chain reaction

ROS – reactive oxygen species

SOD – superoxide dismutase

SSB – single-strand breaks

Tg – 5,6-dihydroxy-5,6-dihydrothyamine

TLS – translesion synthesis

UV – ultra-violet light

1. Introduction

1.1. Oxidative stress and genome integrity

1.1.1. Oxidative stress

Reactive oxygen species (ROS) are formed during the reduction of molecular oxygen to water. This is a reaction occurring in aerobic organisms and it is involved in the production of energy in the electron transport chain. ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are constantly produced by the mitochondrial respiratory chain, ionizing radiation, metabolism of exogenous compounds, as a response against infections and inflammation (Huang *et al.*, 2005) and by antioxidant defenses. They interfere with cell components including nucleic acids, proteins and lipids, causing several damages if not neutralized (Tran *et al.*, 1995; Collins, 2009).

In homeostasis, ROS are balanced by antioxidants but if this equilibrium is disrupted, either by an excess of free radicals production or by deficient antioxidant defenses, cells become under oxidative stress (Collins, 2009). This stage causes cellular damage, which may be involved in human diseases such as atherosclerosis, cancer, and neurodegenerative diseases (Parkinson and Alzheimer) (Good *et al.*, 1996; Gssen *et al.*, 1997; Halliwell *et al.*, 1999) and in processes such as aging and apoptosis (Laun *et al.*, 2001).

Aerobic organisms can counteract the effects of ROS by enzymatic antioxidant or nonenzimatic processes that attract and inactivate ROS and maintain the redox stability of cells (Huang *et al.*, 2005). Superoxide anion ($O_2^{\cdot-}$) produced by cellular respiration or by enzymatic reactions, such as NADPH oxidase and xanthine oxidase, is rapidly converted to H_2O_2, the principal cellular mediator of oxidative stress. Many mechanisms are intrinsically correlated with the formation of ROS and, among them the Fenton reaction has an important role (Figure 1). This reaction occurs in the presence of H_2O_2 that can be formed by endogenous metabolism or by an exogenous source. In the reduction of O_2 in the electron transport chain a small amount of superoxide ($O_2^{\cdot-}$) is formed and is converted to H_2O_2 by superoxide dismutase, which reacts with Fe^{2+}, formed by the release of protein-bound iron, resulting in the formation of $^{\cdot}OH$ radicals. The presence of NADH promotes Fe^{2+} replenishing by reduction of Fe^{3+}. H_2O_2 can be depleted by catalase, peroxidases and reduced glutathione (Henle *et al.*, 1997). The mechanisms of defense

from ROS in cells include low molecular weight scavengers, such as α-tocopherol, cysteine, β-carotene, reduced glutathione or ascorbic acid and enzymatic systems, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione dehydrogenase (GSH-Red) and glucose-6-phosphate dehydrogenase (G6PD) (Halliwell *et al.*, 1989; Izawa *et al.*, 1995).

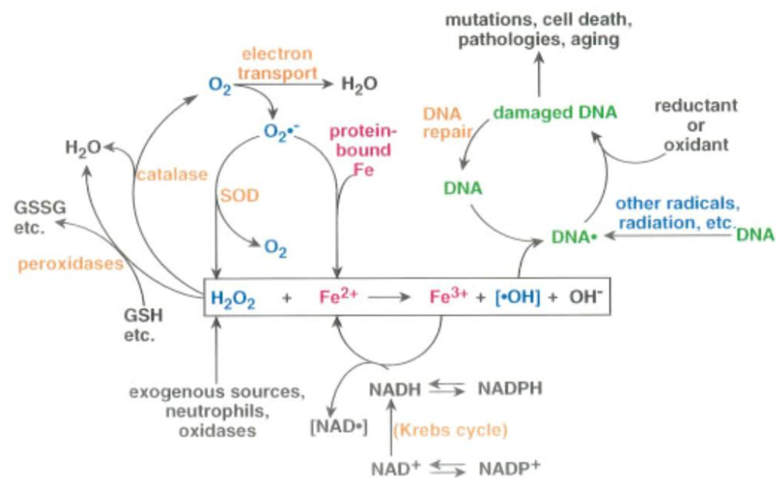


Figure 1 – Cellular reactions leading to oxidative damage in DNA via the Fenton reaction (Adapted from Henle *et al.*, 1997).

1.1.2. DNA damage and repair

The integrity and stability of DNA is essential for the survival and normal function of organisms. However, there are constantly damages by endogenous and exogenous genotoxic agents, including those that are produced by oxidative electron transport chain in mitochondria, ionizing radiation, metabolism of exogenous compounds, infection and inflammation. The DNA damage that can occur involves single- and double-stranded DNA breaks (SSB and DSB, respectively), base and sugar modifications, formation of apurinic/apyrimidinic (AP) lesions, and DNA-protein crosslinks (Newcomb *et al.*, 1998; Wang *et al.*, 1998; Friedberg, 2003; Boiteux *et al.*, 2004). One of the most frequent lesions in DNA - the AP sites - can be formed by spontaneous hydrolysis of the N-glycosidic bond or by elimination of damaged or inappropriate bases. These lesions can be mutagenic and can lead to cell death (Huang *et al.*, 2005). DNA bases are under several types of damage occurring by methylation, oxidation and deamination, that can cause lesions such as N^7-methylguanine (N^7-meG), 8-oxo-7,8-dihydroguanine (8-oxoG), 5,6-dihydroxy-5-6-dihydrothymine (Tg) and uracil (in DNA). 8-oxoG is the most abundant product

of DNA oxidative damage and can produce GC to TA transversions, pairing with adenine, and potentially increases the risk of mutation and cancers (Kelley *et al.*, 2003). AP sites block DNA replication and transcription (Lindahl *et al.*, 1974; Cadet *et al.*, 1997). The cleavage of AP sites by AP endonucleases or by DNA N-glycosylases/AP lyases forms SSBs with 3'- or 5'-blocked ends that cannot be used as substrates by DNA polymerases or DNA ligases (Krokan, 1997). Besides, 3'- or 5'-blocked SSBs can be converted into highly toxic DSBs after DNA replication (Caldecott, 2001).

Normally, organisms can respond to alterations in their genomic DNA by repairing the damage and restoring the genome to the normal physical and functional state, or they can support the lesions in a way that reduces their lethal effects (Friedberg and Wood, 1996). This DNA repair is possible due to cellular mechanisms such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), translesion synthesis (TLS), homologous recombination (HR) and non-homologous end joining (NHEJ) pathways (Prakash *et al.*, 2000; Sluppehaug *et al.*, 2003; Boiteux *et al.*, 2004).

The major DNA repair pathway for the removal of endogenous DNA damage in yeast and mammalian cells seems to be the BER pathway. This pathway is involved in the removal of DNA lesions such as 8-oxoG, uracil, thymine glycols and hydrates. Specific DNA glycosylases participate in BER and occur in two steps: these enzymes catalyze the excision of the base by cleavage of the glycosidic bond, leaving noncoding AP sites in DNA; then, the AP site is cleaved by AP endonucleases and the resulting gap is filled by action of DNA polymerase β (Boiteux *et al.*, 2004; Hanna *et al.*, 2004). The nucleotide excision repair (NER) pathway operates on a large spectrum of base damages, mainly lesions that destabilize the double helix and perturb the DNA structure. These are usually bulky lesions produced by environmental mutagenic and carcinogenic agents such as ultra-violet light (UV) or DNA intrastrand and interstrand crosslinks. This pathway consists in the incision of the DNA strand on both sides of the lesion, resulting in the removal of the damage in an oligonucleotide fragment (25-30 oligonucleotides), followed by repair synthesis and ligation steps (Friedberg *et al.*, 1996).

1.1.3. Methods for DNA damage assessment

DNA damage has been studied in a variety of organisms such as bacteria, cyanobacteria, phytoplankton, macroalgae, plants and animals like humans (Horio *et al.*, 2007). Detection of

DNA damage is crucial in the study of processes such as carcinogenesis and ageing (Kumari *et al.*, 2008). Several methods have been developed for DNA damage/repair assessment such as polymerase chain reaction (PCR), comet assay, halo, terminal deoxyribonucleotidyltrasnferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay, HPLC-electrospray tandem mass spectrometry, fluorescence *in situ* hybridization (FISH) and flow cytometry (FCM) (Kumari *et al.*, 2008). The correlation between the potentialities of each methodology, its advantages and disadvantages and the kind of specific results that can be obtained dictates the selection of the method.

The comet assay or single cell gel electrophoresis (SCGE) was initially developed by Ostling and Johanson (1984) to quantify DNA damage using a microgel electrophoresis technique in neutral conditions, allowing exclusively the detection of DNA double-strand breaks. In 1988, Singh *et al.* adapted this method to alkaline conditions, allowing assessment of both double- and single-strand DNA breaks, as well the alkali labile sites expressed as frank strand breaks in the DNA (Dhawan *et al.*, 2009). This method has been used to study the role of oxidative stress in human diseases, to detect effects of environmental exposure to genotoxins, and to better understand the importance of antioxidants in our diet (Collins, 2009). Nowadays the assay is well established in genotoxicity testing, it is simple, rapid and visual, and allows assessment of DNA damage and repair in individual cell populations. Other advantages include sensitivity (detection of low levels of DNA damage requires small number of cells - ~10,000 - per sample), flexibility (allows the use of proliferating as well as non-proliferating cells), low cost and speed (Dhawan *et al.*, 2009). Nevertheless, the comet assay has some limitations such as the requirement of viable, non-aggregated cell suspension and the impossibility of discrimination between strand breaks from base damage in sample containing necrotic and apoptotic cells. In addition, variation of results can occur due to sample variability (cells and cultures), image analysis systems or visual scoring, and the use of different DNA damage parameters (e.g. olive tail moment and percentage of DNA in the tail), which contribute to inter-laboratory variability (Dhawan *et al.*, 2009). Even though, the advantages of the comet assay outweigh these disadvantages and it is, nowadays, intensively used to assess DNA damage and repair both quantitatively and qualitatively in individual cells (Olive *et al.*, 2006).

1.1.4. Prevention of DNA damage/oxidative stress using phytochemicals

Currently, the interest in finding naturally occurring antioxidants has increased, because they can replace synthetic antioxidants, which are being restricted due to reports of suspected carcinogenicity. Herbs have been used for a large range of purposes including medicine, nutrition, fragrances, cosmetic and industrial uses mainly due to their polyphenol contents, that promote the antioxidant potential, flavor and fragrance (Zeng *et al.*, 2001).

Chronic diseases such as cardiovascular diseases, cancer, or Parkinson and Alzheimer diseases tend to be associated to a large production of free radicals, which lead to oxidative stress. However, balanced diets, rich in fruits and vegetables have been associated with lower risk of these diseases, mainly because of antioxidant properties displayed by some of their constituents such as the polyphenolic compounds flavonoids (Arts *et al.*, 2005). In plants, the role of polyphenols is associated with defense mechanisms. In stress conditions, such as temperature alterations, UV exposure and pathogenic attacks, plants increase polyphenol production (Dixon *et al.*, 1995).

One of the most important attributes of polyphenols is their capacity to protect against oxidative damage. They are beneficial in heart diseases to protect from peroxidation of low-density lipoprotein (LDL), and prevent against cancer and genomic instability by combating oxidative DNA damage (Fergunson, 2001). Polyphenols have been associated to antioxidant activity *in vitro*, being capable of scavenging a wide range of reactive oxygen and nitrogen species, such as superoxide anion, hydroxyl radical, peroxyl radicals, nitric oxide and peroxynitrous acid. Polyphenols can also chelate metal ions, such as iron and copper, preventing or minimizing their participation in Fenton reaction, and thus decreasing pro-oxidant activity of these reactive species (Manach *et al.*, 2004; Halliwell *et al.*, 2005).

Nevertheless, some studies have shown flavonoid pro-oxidant activity *in vitro*. Pro-oxidant activity appears to require the presence of Fe^{3+} and high flavonoid concentrations, increasing the formation of Fe^{2+} that reacts with H_2O_2 in the Fenton reaction (Laughton *et al.*, 1989). In addition, kaempferol can induce DNA degradation and concurrent lipid peroxidation in rat liver nuclei under aerobic conditions (Sahu *et al.*, 1994).

In the presence of copper and absence of H_2O_2, flavonoids may act as pro-oxidants rather than antioxidants, and this activity *in vitro* increases with concentration (Cao *et al.*, 1997). The generation of reactive species and the subsequent damage to macromolecules in flavonoids-Cu^{2+}-O_2 systems can be accounted for the following reaction sequences (Figure 2), where F represents flavonoids with the structure presented in Figure 3. However, this mechanism may occur in a different way in the intercellular medium because different cellular constituents can interfere with these reactions (Cao *et al.*,1997).

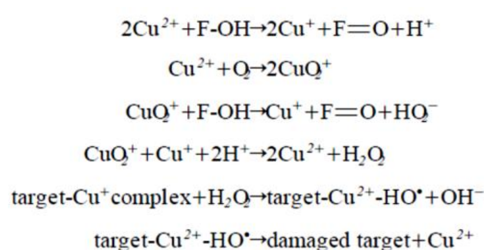


Figure 2 – Generation of reactive species using copper as catalyst of Fenton reaction. Adapted from Cao *et al.* (1997).

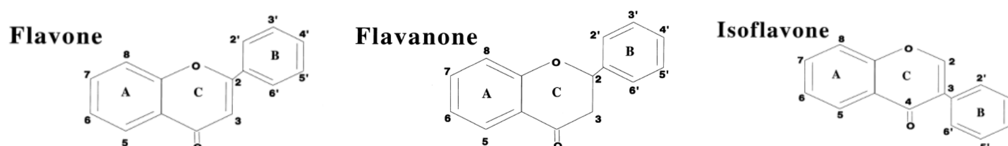


Figure 3 – Struture of the flavonoids Flavone, Flavanone and Isoflavone (Cao *et al.*, 1997).

1.2. Propolis

Propolis is a resinous substance collected by honey bees (*Apis mellifera* L.) from the buds and bark of plants such as poplar (*Populus spp.*), birch (*Betula alba*), beech (*Fagus sylvatica*), chestnut (*Aesculus hippocastanum*) and alder (*Alnus glutinosa*) (Silica *et al.*, 2005; Chen *et al.*, 2007) or from plants such as rosemary (*Baccharis dracunculifolia*), eucalyptus (*Eucalyptus* sp.) and pine (*Araucaria angustifolia*) (Sforcin *et al.*, 2005). Bees mix the resinous substance collected from plants with the salivary enzyme β-glucosidase causing the hydrolysis of glycosyl flavonoids into flavonoids aglucones (Pereira *et al.*, 2002).

Etymologically, the word propolis comes from the Greek words *pro* – for or in defense – and *polis* – city – meaning defense of the hive (Sforcin, 2007). Bees use propolis in their combs as protection, to repair damage (cracks and/or openings), to build aseptic locals for the eggs of

the queen, to embalm killed invaders - avoiding problems arising from the putrefaction of the corpses (Simões *et al.*, 2004) and preventing the proliferation of microbial infections (Laskar *et al.*, 2010) - and also as a thermal insulator (Moreira *et al.*, 2008; Fokt *et al.*, 2011).

1.2.1. Chemical composition of propolis and biological activities

The composition of propolis varies geographically, with the available flora and the season of collection as well as with the race of the producing bees (Miguel *et al.*, 2006; Sforcin *et al.*, 2011). In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% of other substances (Kalogeropoulos *et al.*, 2009). This product is a complex mixture where about 300 compounds have been identified so far. Chemical compounds present in propolis belong to different groups, such as polyphenols (flavonoids including flavones, flavonones, flavonols, dihydroflavonols and chalcones; phenolic acids and their esters), terpenoids, steroids and amino acids (Banskota *et al.* 2002; Usia *et al.*, 2002; Melliou *et al.*, 2004; Kalogeropoulos *et al.*, 2009). With appropriate solvents such as water, methanol, hexane, acetone and ethanol, extracts of propolis can be obtained containing these compounds. The most used and more efficient in extracting the majority of the main propolis bioactive compounds seems to be ethanol (Gómez-Caravaca *et al.*, 2006; Miguel *et al.* 2010), also used by beekeepers to make propolis “tincture”.

Propolis has a huge variability worldwide and as such the chemical composition among samples from different continents differs considerably. The American propolis is mainly composed of terpenoids and prenylated derivatives of *p*-coumaric acids while European (Miguel *et al.*, 2010) and Asian (Usia *et al.*, 2002) propolis contain several types of flavonoids and phenolic acid esters. These compounds, in particular polyphenolic components, caffeic acid derivatives and flavonoids, have been associated with biological activities of propolis, namely antimicrobial (Murad *et al.*, 2002; Silici *et al.*, 2005; Orsi *et al.*, 2005; Kalogeropoulos *et al.*, 2009; Nolkemper *et al.*, 2010) cytotoxic and hepatoprotective (Banskota *et al.*, 2000), radioprotective (Benkovic *et al.*, 2008), antimutagenic (Varanda *et al.*, 1999; Pereira *et al.*, 2008), antioxidant (Mohammadzadeh *et al.*, 2007; Moreira *et al.*, 2008; Miguel *et al.*, 2010; Valente *et al.*, 2010; Laskar *et al.*, 2010) and as a scavenger of free radicals (Banskota *et al.*, 2000; Cardile *et al.*, 2003). Tavares *et al.* (2006) showed that Brazilian green propolis acted as antigenotoxic at low concentration and as genotoxic substance at high concentration. Compounds that have “double face”, in other words, two different and opponent modes of action, are considered to

have a “Janus” effect (from the name of the roman god with two faces), and this type of compounds have been called Janus compounds (von Borstel *et al.*, 1998).

1.2.2. Polyphenol and flavonoid compounds

The human diet is made up of more than 8000 polyphenols. They are phytochemicals derived from phenylalanine containing an aromatic ring with a reactive hydroxyl group. They can be divided into different classes, being the following the most important: flavonoids and phenolic acids (eg. gallic acid), stilbenes (eg. resveratrol) and lignins (eg. secoisolariciresinol). The group of flavonoids comprises seven classes: flavones, flavonol, flavonones, anticianidines, flavan, isoflavones, and chalcones (Araújo *et al.*, 2011). Polyphenols functions in plants are related with the formation of flowers, fruits and seed pigmentation; the attraction of pollinators and dispersion of seeds; protection from UV radiation and the promotion of plant-microorganism interactions (Duthil *et al.*, 2000; Schijlen *et al.*, 2004). Polyphenols are fundamental in the human diet due to the antioxidant (Gladine *et al.*, 2007) and chemopreventive properties (Araújo *et al.*, 2011), protection of UV radiation (Liu *et al.*, 2008) and prevention of oral diseases (Petti *et al.*, 2009).

1.2.3. Portuguese propolis

In the Northern Hemisphere bees collect propolis in final spring, summer and beginning of autumn (Bankova *et al.*, 1998; Sforcin, 2007). Results obtained with Portuguese propolis indicate differences in polyphenols composition of samples collected in winter and in spring, being such content higher in spring. Miguel *et al.* (2010) took samples from different areas of Algarve (Portugal) and concluded that large distance between apiaries are not necessary to found significant differences in propolis phenol and flavonoids contents. The presence of polyphenols such as flavonoids may explain the antioxidant capacity of propolis, since they can act as scavengers of free radicals. Samples from Algarve display higher scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH) capacity in winter than in spring which is unexpected because propolis samples collected in spring usually have higher polyphenols and flavonoids content. So, this suggests that propolis phenol content and the antioxidant activity do not correlate, unlike what happens with propolis from the North and Centre of Portugal (Moreira *et al.* 2008); however, the studies available are still scarce to support this.

Chemical analysis of propolis from Northeast of Portugal demonstrated the presence of 37 compounds such as methylated and/or esterified or hydroxylated derivatives of common

polar flavonoids, peculiar derivatives of pinocembrin/pinobanksin containing a phenylpropanoic acid derivative moiety in their structure and a *p*-coumaric ester derivative dimmer (Falcão *et al.*, 2010). The chemical analysis of Portuguese propolis is very important in order to identify its compounds, to ascribe bioactive compounds to propolis activities, and to compare national propolis with other worldwide samples.

1.3. Biological problem and objectives of this work

Excessive agricultural and industrial activities promote increasing release of toxic substances in the environment, leading to deterioration of air, water and soil quality, which comprehend several risks to the survival of species (Dhawan *et al.*, 2009). Nowadays, the interest in natural products has been increasing since the suspected toxicity of some synthetic compounds used in food (Stone *et al.*, 2003), and because of that industries such as cosmetic and pharmaceutical have increased their efforts in obtaining bioactive compounds from natural products by extraction and purification (Halliwell, 1997).

Many natural products have been identified as containing several bioactivities capable to provide protection against disorders associated with cancer, cardiovascular diseases, aging and neurodegenerative diseases such as Parkinson and Alzheimer (Good *et al.*, 1996; Gassen *et al.*, 1997; Halliwell *et al.*, 1999; Russo *et al.*, 2003). These activities are correlated with the presence of polyphenols, mainly flavonoids, which have high antioxidant properties and also protect nuclear DNA from damage caused by hydrogen peroxide through the role of iron chelation (Melidou *et al.*, 2005). However, some studies showed that several flavonoids act, either, as prooxidant and genotoxic (Cao *et al.*, 1997).

Saccharomyces cerevisiae has been used as a model to understand the complex physiological, biochemical and molecular processes in metazoan cells. The advantages of this biological model are well known and relate to fast growth, cheap cultivation and tractability. The molecular mechanisms of fundamental cellular processes are very similar between higher eukaryotes and yeast, in transcription, replication, and DNA repair. The full genome sequence of *S. cerevisiae* is now available, and so this organism is one of the most studied model systems in cell biology, molecular biology and genetics (Grzelak *et al.*, 2006).

The aim of this work relates to the analysis and study of the antioxidant and antigenotoxic properties of Portuguese propolis. This work comprises a chemical characterization and the investigation of biological activities in a sample collected in Beira Alta (Côa), used to prepare a propolis ethanol extract (PEE), which was used in the following studies.

In order to investigate the antigenotoxic/genotoxic effect of PEE the comet assay was performed to measure DNA damage after pre-, and co-incubation of *S. cerevisiae* cells with propolis. Hydrogen peroxide, which causes base oxidation and single-strand breaks mediated by the highly reactive hydroxyl radicals (Miloshev *et al.*, 2002), was used as stressing agent. Cell viability under stress (5 mM H_2O_2) and non-stress conditions, was also evaluated in pre-, co- and post-incubation assays to understand propolis effects. To complete the study, flow cytometry was used to evaluate intracellular oxidation and antioxidant activity *in vivo*.

2. Material and Methods

2.1. Yeast strain, media and growth conditions

In all experiments the haploid *Saccharomyces cerevisiae* strain BY4741 (*MATahis3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann *et al.*, 1998) was used. Cells were grown on liquid YPD medium (1% w/v yeast extract, 1% w/v peptone and 2% w/v glucose), in an orbital shaker at 30 °C and 200 revolutions per minute (rpm). Growth of cultures was monitored by optical density measurement at 600nm (OD_{600}).

2.2. Propolis extract

Propolis was obtained in August 2010 from an apiary in Côa (Beira Interior), Portugal. For alcoholic extraction the raw propolis was incubated with 100 mL absolute ethanol in an orbital shaker at room temperature and in the dark. The solution was filtered (Whatman filter nr. 4) and the residue was re-dissolved in 100 mL absolute ethanol three times more. The filtrates were pooled and dried in a rotary evaporator, at 40 °C under stirring, yielding the propolis ethanolic extract (PEE), which was stored in the dark at 4 °C until further use.

2.3. Viability assay

Cultures of 5mL YPD media were incubated overnight at 30 °C and 200 rpm (pre-inoculum). Pre-inocula were diluted with fresh media to obtain 50 mL cultures with OD_{600} 0.1 and incubated under the same conditions until OD_{600} 0.4-0.8 (exponential phase), ensuring growth for 2 generations. For each assay, cells were harvested from 5 mL of the culture by centrifugation at 5869 x g, 2 min at 4 °C, washed twice with the same volume of sterilized deionized H_2O at 4 °C and suspended in the same volume of S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5) at 4 °C. Assays were performed with these cell suspensions using PEE in pre-incubation, co-incubation and post-incubation conditions.

A volume of 100 µL of cell suspension (in S buffer) was removed, serially diluted to 10^{-4} in sterilized deionized H_2O and spread on solid YPD medium (YPD with 2% w/v agar), in order to obtain a control situation.

In pre-incubation conditions, different stock solutions of PEE were added to the cell suspension for 300 µg/mL, 100 µg/mL or 25 µg/mL final concentration (final volume of 5 mL

was maintained in all samples). Alternatively, S buffer and ethanol were added to a similar cell suspension, to be used as control. The different suspensions were incubated for 20 min, at 30 °C, 200 rpm. At the end of this incubation time, 100 µL was taken, serially diluted to 10^{-4} in sterilized deionized H_2O and transferred to YPD plates as 7 drops of 40 µL each. Cells of the suspension were washed twice with the same volume of sterilized deionized H_2O at 4 °C and suspended in the same volume of S buffer. From a stock solution of 1M H_2O_2, 25 µL were added for 5 mM final concentration and the suspension was incubated for 90 min under the same conditions. Samples were harvested at 5, 10, 15, 30, 60 and 90 min, serially diluted to 10^{-4} in sterilized deionized H_2O and 7 drops of 40 µL each were transferred to YPD plates. Plates were incubated at 30 °C for 48 h and the colonies were counted. Survival rates were calculated as percentage of colony-forming units (CFU), assuming 100% survival for cells of the suspension before any treatment (H_2O_2, propolis or ethanol).

In co-incubation experiments, the procedure was the same, except for the simultaneous incubation with PEE or ethanol and H_2O_2. In post-incubation experiments the procedure was also similar except for the previous incubation with H_2O_2 (5 mM final concentration) with subsequent incubation with PEE or ethanol (control) for 20 min.

2.4. Comet assay

Cultures in 5 mL YPD medium were incubated overnight at 30 °C, 200 rpm (pre-*inoculum*). Pre-inocula were diluted to obtain 10 mL cultures with OD_{600} 0.1 and were incubated under the same conditions until OD_{600} 0.4-0.8 (exponential phase), ensuring 2 generations growth. Cells were harvested by centrifugation of 1 mL of the suspension at 17608 x g, 2 min at 4 °C, and washed twice with the same volume of deionized H_2O at 4 °C. The pellet was ressuspended in lyticase buffer (200U/mL lyticase, 500 µL S buffer 2x, 300 µL deionized H_2O and 50 mM β-mercaptoethanol) and incubated at 30 °C, 200 rpm for 40 min in order to obtain spheroplasts. Spheroplasts were washed twice with deionized H_2O, ressuspended in the same volume of S buffer and distributed by aliquots of 50 µL. Each aliquot was centrifuged at 17608 x g, 2 min at 4 °C and the pellet ressuspended in 500 µL S buffer. PEE (300 µg/mL, 100 µg/mL and 25 µg/mL), H_2O_2 (10 mM), and S buffer or ethanol (controls), were added according to the type of incubation described above: pre-, co- and post-incubation. In all cases, the incubation time was 20 min, followed by two washes with deionized H_2O. The resultant pellet was ressuspended in 500 µL of 1.5% (w/v in S buffer) low melting agarose (LMA) at 35 °C, spread onto glass slides

previously layered with 0.5% (w/v) normal melting agarose (NMA), covered with cover slips and incubated in ice in order to solidify the agarose. The cover slips were removed after 5min and the glass slides were submerged 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 Laurylsarcosine, pH 10) for 20 min. Glass slides were incubated in electrophoresis buffer (300 mM NaOH, 0.5 M EDTA, 0.1 M Tris-HCl, pH 10) for 20 min, placed in the electrophoresis chamber with electrophoresis buffer and electrophoresis was performed at 0.7 V/cm for 10 min at 4° C. The gels were neutralized with 10 mM Tris-HCl buffer, pH 7.4, for 10 min, and samples were fixed, firstly during 10 min in 76% v/v ethanol and then 10 min with 96% v/v ethanol. The slides were dried at room temperature or in a laminar flow chamber and visualized immediately or stored at 4 °C until observation. The comets were analyzed by fluorescence microscopy (Leica DMB 5000, black and with camera) after staining with 10 µL of GelRed™ (diluted 10,000 fold from the stock solution; Biotium). The tail length was measured with CometScore software.

2.5. Flow cytometry

Cultures in 5 mL YPD medium were incubated overnight at 30 °C, 200 rpm (pre-*inoculum*). Pre-inocula were diluted to obtain 10 mL cultures with OD_{600} 0.1 and were incubated under the same conditions until OD_{600} 0.4-0.8 (exponential phase), ensuring 2 generations growth. Cells were harvested by centrifugation of 1 mL of the suspension at 17608 x g, 2 min at 4 °C and washed twice with the same volume of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The suspension was diluted to OD_{600} 0.02 and 500 µL were removed for auto fluorescence measurement. Dichlorofluorescein diacetate (H_2DCFDA) of was added to the suspension (50 µM final concentration) before incubation at 30 °C, 200 rpm during 1 h in the dark. Cells were washed twice with the same volume of PBS and aliquots of 1 mL were mixed with the PEE and H_2O_2 in co-incubation conditions and incubated at 30 °C, 200 rpm, 20 min in dark. Treatments were as follows: PBS, 10 mM H_2O_2, 2% ethanol, 300 µg/mL PEE, 300 µg/mL PEE and 10mM H_2O_2, 100 µg/mL PEE, 100 µg/mL PEE and 10mM H_2O_2, 25 µg/mL PEE, and 25 µg/mL PEE and 10mM H_2O_2. Twenty thousand cells of each sample were analyzed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green flurescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. Data were analyzed and histograms were made with the WinMDI 2.8 software.

2.6. Chemical analysis of Propolis

Spectrophotometric methods were used to perform PEE chemical analysis: the determination of total polyphenols and flavonoids contents and the evaluation of the antioxidant capacity of the sample.

2.6.1. Quantification of total polyphenols content

To quantify total polyphenol content in PEE, 300 mg ethanol solution of propolis, diluted 4-fold (0.0499 mg/g final concentration), mixed with 2.0 g deionized H_2O, 200 mg Folin-Ciocalteu reagent, 2,0 g of 10% $NaCO_3$, and H_2O to complete 10,000 g final mass of the mixture. The reducing power of phenols in the mixture was observed by optical density at 760 nm (OD_{760}) after 1 h incubation. Polyphenol concentration in propolis sample was calculated taking the standard gallic acid as reference and results were expressed as gallic acid equivalents.

2.6.2. Quantification of flavonoids

This method allows quantification of substances (flavonoids) capable to inhibit the chelation of aluminum of the ethanolic solution of $AlCl_3 \cdot 6H_2O$. Five hundred mg of the solution $AlCl_3 \cdot 6H_2O$ 2% were added to 300 mg of ethanolic solution of propolis (10,887 mg/g) and ethanol was used to complete the mixture final mass of 10,000 g. After 30 min, chelation of aluminum was followed by optical density at 420 nm (OD_{420}). Flavonoids concentration in PEE was calculated comparing with quercetin (1,2mg/g), used as standard, and results are expressed as quercetin equivalents.

2.6.3. DPPH assay

One of the main referred propolis activities is antioxidant capacity. This capacity can be tested by chemical assays such as DPPH or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods. DPPH assay quantifies the existence of antioxidant activity of a solution that can reduce the DPPH molecule, by the capture of the free electron of nitrogen atom present in DPPH. xThis reduction is visible by the loss of violet colour and is followed by OD_{517}, after 20 min in dark. Two hundred mg of PEE 4x diluted (0.0498 mg/g final concentration) were added to 500 mg ethanol solution of DPPH (0.5 mM) and the final mass of the mixture (3,000 g) was completed with absolute ethanol. After 20 min incubation OD_{517} was measured and the amount of

antioxidant substances present in PEE was calculated taking the standard gallic acid as reference (5 mg/g 5x diluted) and results were expressed as gallic acid equivalents.

2.6.4. ABTS assay

The antioxidant capacity of propolis extract was also determined by the ABTS assay, which quantifies the substances capable to inhibit oxidation of ABTS radical. ABTS work-solution (2.5 g) was added to 100 mg of PEE (10,887 mg/g) and ethanol was added to complete a final mass of the mixture of 3,000 g. After 30 min incubation in the dark, the optical density of the samples was measured at 734 nm and the amount of antioxidant substances present in propolis sample was calculated taking the standard gallic acid as reference (5mg/g 5x diluted) and results were expressed as gallic acid equivalents.

2.7. Statistic analysis

The experiments were done in triplicate and results are presented as a mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison of more than two means and Tukey's test was used to perform multiple comparisons. All asterisks indicate statistically significant differences when compared to the respective control: * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$.

3. Results

Several reports in the literature have recently provided evidence of the anticancer and antioxidant properties of ethanolic extracts of Portuguese propolis (Moreira *et al.*, 2008; Miguel *et al.*, 2010; Valente *et al.*, 2011). Based on the documented antioxidant properties and flavonoids presence in propolis, we decided to investigate the effect of a Portuguese propolis ethanol extract (PEE) against oxidative stress in *S. cerevisiae* cells measured as viability and antigenotoxicity.

3.1. Effects of PEE on cell viability under stress conditions

In viability assays, three methodologies were applied: pre-incubation, co-incubation and post-incubation. In all cases, cultures of yeast cells were diluted 50 fold in S buffer when incubated with PEE. In pre-incubation experiments, cultures were incubated with PEE at 30 °C, 200 rpm for 20 min, washed twice with deionized H_2O and subsequently incubated with 5 mM H_2O_2 under the same conditions. Aliquots of the culture were harvested at different time-points, diluted (10^{-4} dilution) and plated solid YPD medium in order to count colonies after 48 h incubation at 30 °C. Cell death was considered as loss of viability expressed as percentage of colonies of test plates when compared to the reference plate, without toxic treatment. A control experiment without PEE and H_2O_2 was included so that viability of untreated and non-stressed cells could be determined. Results obtained (Figure 4) show that yeast cells when exposed to 5 mM H_2O_2 had a significant decrease in survival rate. However, when cells were pre-treated with 300 µg/mL (Figure 4A) and 100 µg/mL (Figure 4B) of PEE, loss of viability was slower during the 90 min of exposure to 5 mM H_2O_2. As expected, cells without any treatment and treated only with PEE or ethanol 2% showed a nearly constant survival rate for 90 min incubation. These results suggest that PEE in pre-incubation protect yeast cells against oxidative stress, by promoting cell adaptation to oxidative stress.

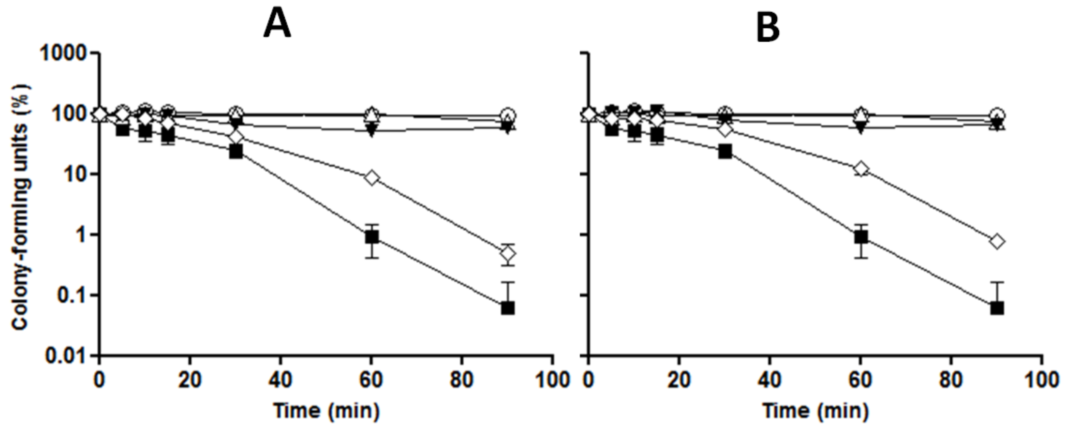


Figure 4 – Pre-incubation with PEE increases viability of *S. cerevisiae* cells under oxidative stress. Yeast cells were incubated with PEE (A: 300 µg/mL; B: 100 µg/mL) for 20 min, washed and suspended in S buffer, and subsequently, incubated with 5 mM H_2O_2 for different time-points (0, 5, 10, 20, 30, 60 and 90 min). At each time-point, an aliquot was collected, diluted to 10^{-4} and spread on YPD plates. Colonies were counted after 48 h incubation at 30 °C. The same procedure was applied to all treatments: cells only with S buffer (-○-), treated with H_2O_2 (-■-), treated with 2% ethanol (-△-), treated only with PEE (-▼-) and treated with PEE and H_2O_2 (-◇-). Data are the mean±SD of three independent experiments.

To investigate a direct antioxidant activity of PEE on the toxicant we have performed co-incubation experiments. Cells were incubated simultaneously with PEE (300 µg/mL, 100 µg/mL and 25 µg/mL) and 5 mM H_2O_2 under the same conditions. Aliquots of the culture were harvested at different time-points, diluted (10^{-4} dilution) and plated on YPD plates in order to count colonies after 48 h incubation at 30 °C. Cell death was considered as loss of viability expressed as percentage of colonies of test plates when compared to the reference plate, without toxic treatment. A control experiment without PEE and H_2O_2 was included so that viability of untreated and non-stressed cells could be determined. Results obtained (Figure 5) show that yeast cells when exposed to 5 mM H_2O_2 had a significant decrease in survival rate. However, when cells were incubated with of PEE, the loss of viability was slower during the 90 min of exposure to 5 mM H_2O_2. As expected, cells without any treatment and treated only with PEE or ethanol 2% displayed a nearly constant survival rate during all the experiment. At 100 µg/mL (Figure 5B) PEE provided more protection when compared with the other concentrations (figs. 5A and 5C). These results suggest that PEE protects yeast cells against oxidative damage also in co-incubation with H_2O_2.

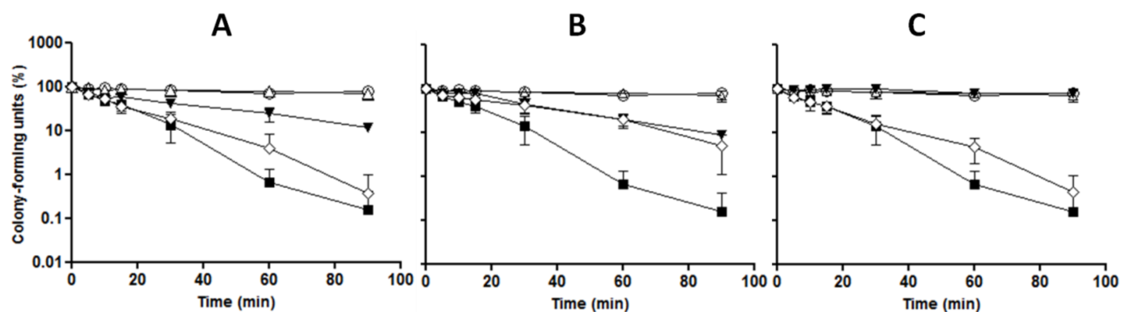


Figure 5 – PEE increases viability of *S. cerevisiae* cells when co-incubated with H_2O_2. Yeast cells were co-incubated with PEE (A: 300 μg/mL; B: 100 μg/mL; C: 25 μg/mL) and 5 mM H_2O_2 for different time-points (0, 5, 10, 20, 30, 60 and 90 min). At each time-point, an aliquot was collected, diluted to 10^{-4} and spread on YPD plates. Colonies were counted after 48 h incubation at 30 °C. The same procedure was applied to all treatments: cells only with S buffer (-○-), treated with H_2O_2 (-■-), treated with 2% ethanol (-△-), treated only with PEE (-▼-) and treated with PEE and H_2O_2 (-◇-). Data are the mean±SD of three independent experiments.

After investigating protection in pre- and co-incubation, we have performed post-incubation experiments to study if PEE promotes recovery from damage caused by H_2O_2 in yeast cells. Cells were incubated with 5 mM H_2O_2 during 20 min, at 30 °C, 200 rpm, washed twice and subsequently incubated with PEE (300 μg/mL, 100 μg/mL and 25 μg/mL) for 20 min. Aliquots of the culture were harvested at different time-points, diluted to 10^{-4} and spread on YPD plates in order to count colonies after 48 h incubation at 30 °C. Cell death was considered as loss of viability expressed as percentage of colonies of test plates when compared to the reference plate, without toxic treatment. A control experiment without PEE and H_2O_2 was included so that viability of untreated and non-stressed cells could be determined. Results obtained (Figure 6) show that post-treatments with PEE with yeast cells exposed to 5 mM H_2O_2 did not change significantly survival rate, except for 300 μg/mL (Figure 6A) PEE, which promoted faster loss of viability than with H_2O_2 alone. These results suggest that PEE at 100 μg/mL and 25 μg/mL did not improve oxidative damage recovery after damage in cells promoted by H_2O_2,. However, at 300 μg/mL evidence suggests that oxidative damage is more pronounced. As expected, cells without any treatment and treated only with PEE or 2% ethanol showed a nearly constant survival rate throughout the experiment.

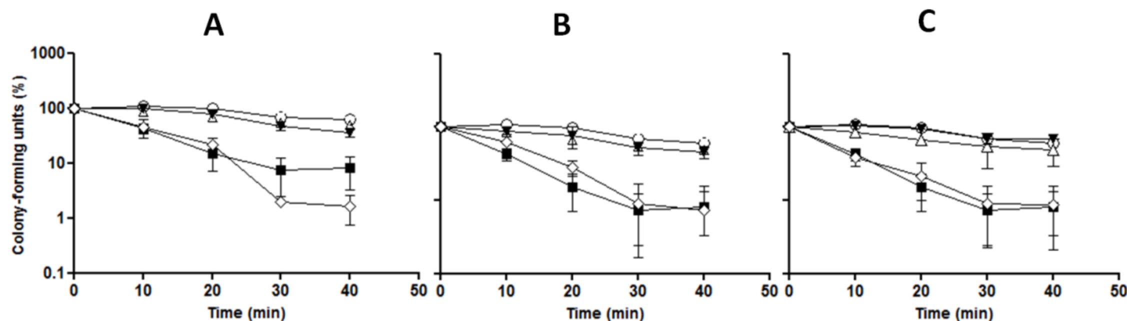


Figure 6 – Post-incubation with PEE did not affect the viability of *S. cerevisiae* cells exposed to oxidative stress. Yeast cells were incubated 10 min with 5 mM H_2O_2, washed and incubated with PEE (A: 300 μg/mL; B: 100 μg/mL; C: 25 μg/mL) for 10 min. At each time-point 0, 10, 20, 30, 40 min, an aliquot was collected, diluted to 10^{-4} and spread on YPD plates. Colonies were counted after 48 h incubation at 30 °C. The same procedure was applied to all treatments: cells only with S buffer (-○-), treated with H_2O_2 (-■-), treated with 2% ethanol (-△-), treated only with PEE (-▼-) and treated with PEE and H_2O_2 (-◇-). Data are the mean±SD of three independent experiments.

3.2. PEE protects yeast cells from DNA damage by H_2O_2

The capacity of propolis decrease DNA damage promoted by H_2O_2 has been scarcely reported before (Russo *et al.*, 2006). In addition, to our knowledge, Portuguese propolis has not been studied for antigenotoxicity. Therefore, we decided to investigate antigenotoxicity of Portuguese propolis, using the yeast comet assay in cells, under pre- and co-incubation conditions.

Yeast spheroplasts were pre-treated with 300 μg/mL, 100 μg/mL and 25 μg/mL in S buffer to maintain osmotic protection of spheroplasts, and then, exposed to 10 mM H_2O_2. Several controls were included: incubation only with S buffer; incubation with ethanol as control of the dilutions of PEE used; incubation with H_2O_2; and incubation only with PEE. Control experiments indicate that S buffer (untreated cells) and ethanol (PEE solvent) did not cause damage to DNA (Figure 7). As expected, H_2O_2 increased dramatically comet tail length and when yeast spheroplasts were treated with PEE before exposure to H_2O_2, a statistically significant decrease in comet tail length was observed when compared with the control situation (H_2O_2) (Figures 7 and 8).

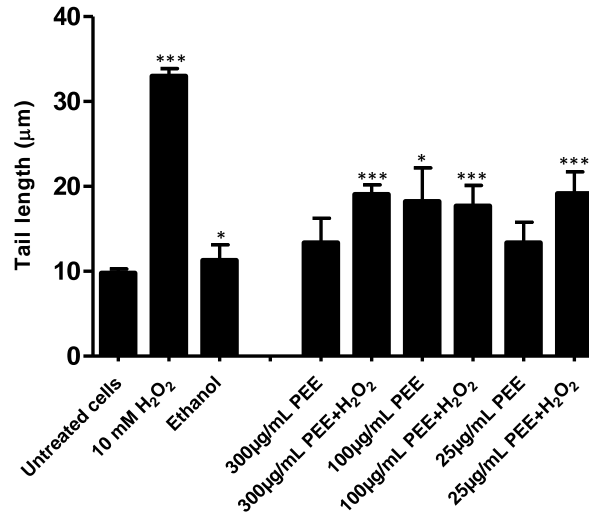


Figure 7 - Pre-treatment of *S.cerevisiae* cells with PEE protects DNA against oxidative damage by H_2O_2. Spheroplasts were incubated with PEE (300 µg/mL, 100 µg/mL and 25 µg/mL) for 20 min, washed, and subsequently incubated with 10mM H_2O_2 for 20min. In samples with exclusive PEE treatment H_2O_2 was replaced by S buffer. DNA damage was analyzed with the yeast comet assay (see Materials and Methods). Cells without any treatment were included in the experiment (untreated cells) as well as cells treated with ethanol and with H_2O_2 (10 mM H_2O_2) and treated only with PEE solvent (ethanol). Mean±SD values are from three independent experiments (* represent $p < 0.05$ and *** $p < 0.001$).

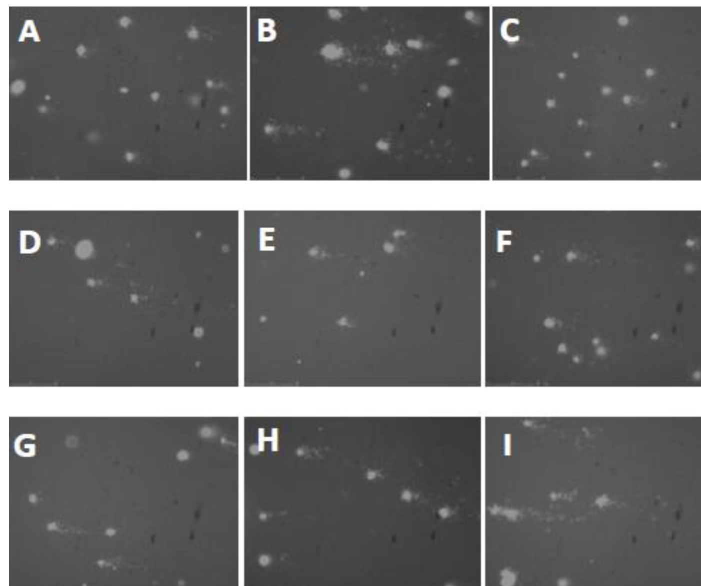


Figure 8 – Photomicrographss of yeast comets after DNA staining with GelRed. **A**: control experience only with S buffer; **B**: cells treated with ethanol before incubation with 10 mM H_2O_2; **C**: incubation with ethanol before incubation with S buffer; **D**: incubation with 300 µg/mL PEE before incubation only with S buffer; **E**: incubation with 300 µg/mL PEE before incubation with 10 mM H_2O_2; **F**: incubation with 100 µg/mL PEE before incubation only with S buffer; **G**: incubation with 100 µg/mL PEE before incubation with 10 mM H_2O_2; **H**: incubation with 25 µg/mL PEE before incubation only with S buffer; **I**: incubation with 25 µg/mL PEE before incubation with 10 mM H_2O_2. All images were obtained at 400x magnification.

When cells were incubated only with 100 μg/mL of PEE, a statistically significant increase in comet tail length compared with cells treated with ethanol was observed, suggesting that PEE has also genotoxic activity. As depicted in Figure 7, genotoxicity did not correlate directly with PEE, however, this activity is in accordance with a previous report by Tavares *et al.* (2006) who concluded that PEE is both, genotoxic and antigenotoxic.

Subsequently, we decided to assay co-incubation of yeast cells with PEE and H_2O_2 to investigate if antioxidant activity of PEE protects DNA cells from damage under oxidative stress conditions. In this experiment we incubated spheroplasts with PEE (300 μg/mL, 100 μg/mL and 25 μg/mL) and 10 mM of H_2O_2 for 20 min and then we analyzed DNA damage. Several controls were included: incubation only with S buffer; incubation with ethanol as control of the dilutions of PEE used; incubation only with H_2O_2; and incubation only with PEE. Control experiments indicate that S buffer did not cause damage of DNA, but ethanol caused a significant increased in the tail length of the comets (Figure 9). In addition, H_2O_2 increased dramatically comet tail length and when yeast spheroplasts were treated only with PEE, we observed a statistically significant increase in tail length comparing with the control (ethanol). These results suggest that PEE acted as a genotoxic agent to *S. cerevisiae* cells. However, in the presence of a toxic agent, H_2O_2, 300 μg/mL PEE displayed antigenotoxic activity, since the tail length of the comets had a statistically significant decrease relatively to the control (H_2O_2) (Figures 9 and 10). So, in co-incubation conditions PEE can act either, as an antigenotoxic and as genotoxic agent. Interestingly, while genotoxicity was observed for all assayed concentrations, antigenotoxicity was only observed for the higher concentration (300 μg/mL).

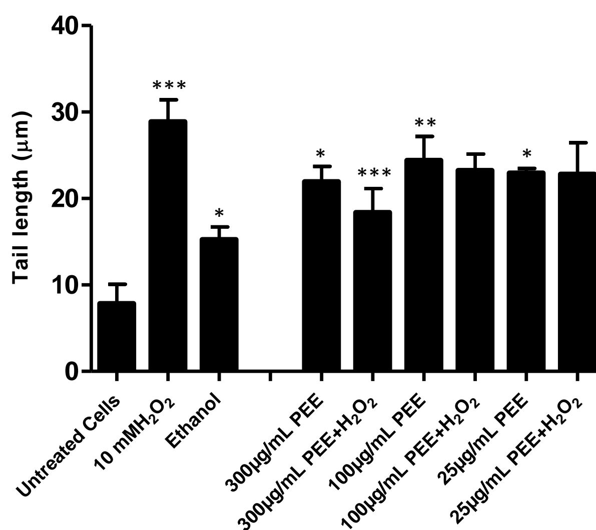


Figure 9 – Co-incubation of *S. cerevisiae* cells with 300 μg/mL PEE protects cells from DNA damage under oxidative stress with 10 mM of H_2O_2. In samples with exclusive PEE treatment H_2O_2 was replaced by S buffer. DNA damage was analyzed with the yeast comet assay (see Materials and Methods). Cells without any treatment were included in the experiment (untreated cells; suspended in S buffer) as well as cells treated with ethanol and with H_2O_2 (10 mMH_2O_2) and treated only with PEE solvent (ethanol). Mean±SD values are from three independent experiments (* represent $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

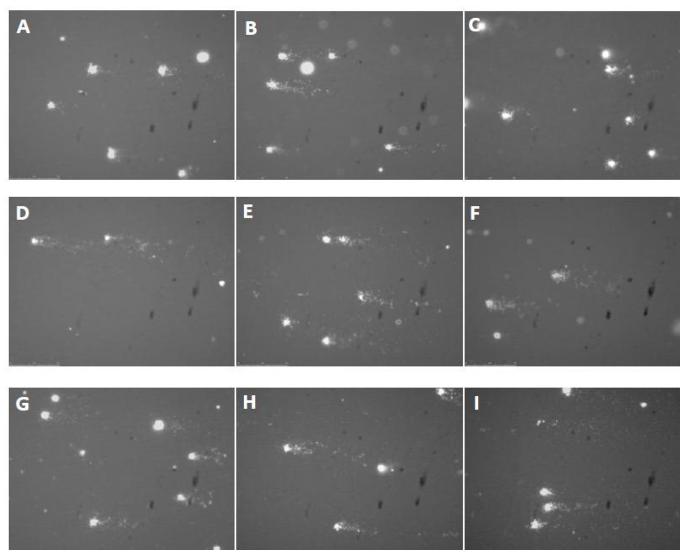


Figure 10 – Photomicrographs of yeast comets after DNA staining with GelRed. **A**: control experience only with S buffer; **B**: cells treated with 10 mM H_2O_2; **C**: incubation with ethanol; **D**: incubation with 300 µg/mL PEE; **E**: incubation with 300 µg/mL PEE and 10 mM H_2O_2; **F**: incubation with 100 µg/mL PEE; **G**: incubation with 100 µg/mL PEE and 10 mM H_2O_2; **H**: incubation with 25 µg/mL PEE; **I**: incubation with 25 µg/mL PEE and 10 mM H_2O_2. All images were obtained at 400x magnification.

3.3. Chemical characterization of propolis

To characterize chemically propolis used in this work we analyzed the total content of polyphenols and flavonoids, the main potentially bioactive compounds found in propolis. We analyzed total polyphenols with the Folin-Ciocaulteu method by measuring OD_{760} after reaction PEE, Folin-Ciocaulteu reagent and Na_2CO_3, the result was expressed in mg galic acid equivalent/g of sample. To analyze the flavonoids content, we used $AlCl_3{\cdot}H_2O_2$, which is quelated by flavonoids, resulting a yellow color proportional to the concentration flavonoids and the result was expressed in mg quercetine equivalent /g of sample. In addition, we used DPPH (violet color) and ABTS (blue-green color) radicals to quantify the *in vitro* antioxidant capacity of PEE, both in mg galic acid equivalent/g of sample. The results are express in table 1. We can't comparing ours results of DPPH and ABTS with others reports, because no one did the methods in mg/g of sample like us.

Table 1 – Chemical analysis of PEE. Total polyphenols and flavonoids content, and *in vitro* antioxidant activity of PEE by DPPH and ABTS methods.

Total Polyphenols (mg GAE/g)	Flavonoids (mg QE/g)	DPPH (mg GAE/g)	ABTS (mgGAE/g)
160,40±16,56	30,21±0,52	50,46±3,00	46,29±12,41

3.4. PEE decreases intracellular oxidation

Chemical characterization of PEE suggests antioxidant capacity of PEE, which correlates with results obtained in viability assays with cells stressed with H_2O_2. To investigate if PEE influences the intercellular oxidation in the presence of H_2O_2, we have performed experiments in co-incubation conditions using flow cytometry with H_2DCFDA as probe. This substance enters the cell and is deacetylated to dichlorofluorescein ($DCFH_2$) by intracellular esterases. The deacetylated form $DCFH_2$ is hydrophilic and becomes trapped inside the cells due to impermeability of plasma membranes. In the presence of oxidants, $DCFH_2$ oxidizes, forming DCF, which is fluorescent with excitation at 530nm and emission at 485nm. With this approach, we expected to observe a decrease in fluorescence in cells treated with PEE and H_2O_2 10mM comparing with controls (H_2O_2). As depicted in Figure 11, treatment with PEE decreased intracellular fluorescence in a dose-dependent manner when compared with cells treated only with 10 mM H_2O_2. To verify that fluorescence measured in the cytometer was from intracellular fluorochrome, we analyzed an aliquot of cells from each sample in fluorescence microscopy (Figure 12). As shown in Figure 12, a significant proportion of cells displayed intracellular fluorescence upon incubation with H_2DCFDA.

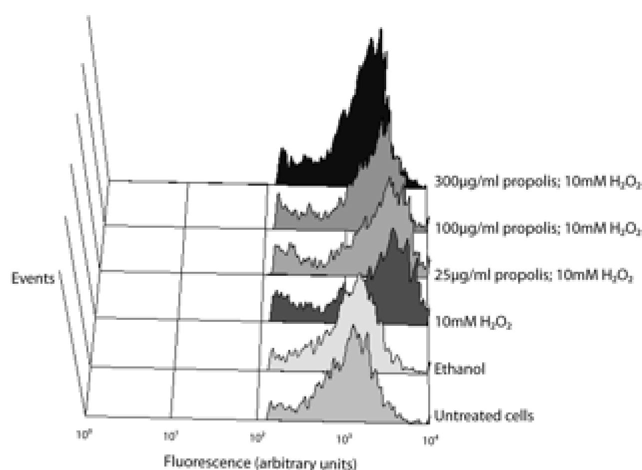


Figure 11 – Intercellular oxidation of *S. cerevisiae* cells co-incubated with PEE and 10 mM H_2O_2, by flow cytometry. Cells were loaded with H_2DCFDA, treated with PEE and 10 mM H_2O_2 for 20 min and analyzed for fluorescence by flow cytometry. Data are from a representative experiment from three independent experiments.

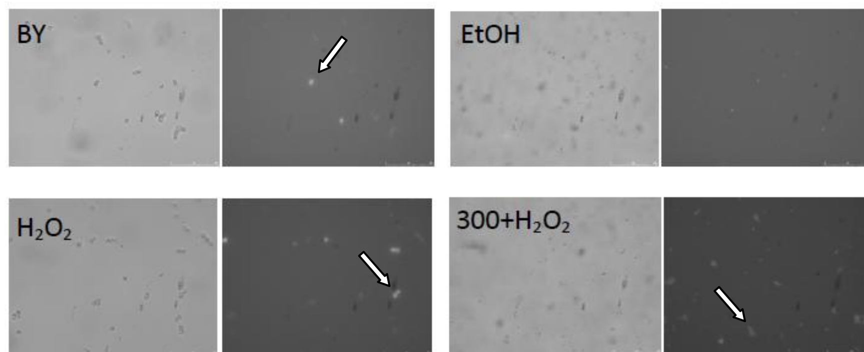


Figure 12 – Photomicrographs of *S. cerevisiae* cells with fluorescence (H_2DCFDA) after co-incubation with S buffer (BY; 200x magnification), ethanol (EtOH; 400x magnification), H_2O_2 10 mM (H_2O_2; 200x magnification), and PEE and H_2O_2 ($300+H_2O_2$; 400x magnification). For each sample bright field (left image) and fluorescence microscopy (right image) are presented. Arrows point to cells displaying fluorescence.

4. Discussion

Our interest in Portuguese propolis relates to the existence of only few reports on biological activities, namely antioxidant and antigenotoxic and genotoxic. In the last decades propolis has been widely study to complement its use in medicine, cosmetic and food. Over the last decades, research has been engaged to support scientifically characteristics that confer propolis the traditionally known healing properties and subsequently increase its economical valorization. The principal significance of the study of propolis relates to the importance of standardization for its acceptance as a medicine (Sforcin *et al.*, 2011). One of the most documented characteristics of propolis is antioxidant activity (Silici *et al.*, 2005), free radical scavenging (Banskova *et al.*, 2000; Russo *et al.*, 2003; Simões *et al.*, 2004), antigenotoxicity and genotoxicity (Tavares *et al.*, 2006), antimutagenicity (Varanda *et al.*, 1999), anti-cancer activity (Valente *et al.*, 2011) and antimicrobial activity (Kalogeropoulos, *et al.*, 2009). In the literature on Portuguese propolis several reports mention mainly antioxidant potential (Moreira *et al.*, 2008; Miguel *et al.*, 2010; Moreira *et al.*, 2011) and the potencial to inhibit human renal cancer cell growth (Valente *et al.*, 2011).

In our work, we aimed to further support the antioxidant activity and the antigenotoxic/genotoxic activities of Portuguese propolis ethanolic extract. When we incubated cells firstly with PEE and then with H_2O_2, we observed an increase in cell viability relatively with the control (H_2O_2) (Figure 4). These results suggest that PEE activates the antioxidant mechanisms in cells, promoting an alert state in intercellular medium allowing cells to cope more efficiently with oxidative stress. Some reports suggest that polyphenols can act either, as anti-oxidant and as pro-oxidant, mainly by promoting the Fenton reaction with cooper ions (Cao *et al.*, 1997). In co-incubation with PEE and H_2O_2, we observed a more pronounced increase in cell viability when 100 μg/mL was used relatively with the control situation (H_2O_2) (Figure 5B). This suggests that a direct ROS scavenging effect (co-incubation) is more efficient than an activation of stress responses (pre-incubation) in promoting cell survival under oxidative stress. However, when cells were incubated only with PEE, in the concentration of 300 μg/mL and 100 μg/mL, we observed decreased cell viability in co-incubation than in pre-incubation experiments. In the former case cells were in contact with PEE and H_2O_2 for 90 min, far more than the 20 min incubation with PEE in pre-incubation experiments. Therefore, the pro-oxidant activity would have more impact in cell viability as is further supported by the dose-dependent decrease in cell

viability caused by PEE in co-incubation (Figure 5). To complete the study of the effect of PEE in cell viability, we did a post-incubation experience to verify if PEE can improve recovery from exposure to an oxidative agent. Our results suggest that PEE does not improve recovery upon oxidative shock and may even contribute to decrease cell viability, especially at high concentrations. This further support the prooxidant activity of PEE discussed above, which is especially patent at high concentrations.

We have performed the yeast comet assay to investigate the antigenotoxic/genotoxic effects of PEE. When cells were pre-treated with PEE, before H_2O_2 exposure, we detected a statistically significant decrease of comet tail length in all concentrations used (Figure 7). On the other hand, in incubation only with PEE, we observed an increase of comet tail length relatively of the control (ethanol) in the concentration of 100 µg/mL (Figure 7). These results, indicating that PEE has antigenotoxic and genotoxic activities, correlate with viability assays, in which protection against oxidative stress and toxicity were detected. We did a complementary experience, using co-incubation, to investigate if PEE protects DNA under oxidative stress with H_2O_2. Here, again, PEE displayed an antigenotoxic and genotoxic effect on yeast cells (Figure 9). Tavares *et al.* (2006) have reported before antigenotoxicity and genotoxicity of hydro-alcoholic propolis extracts from Brazilian propolis in mammalian cells. Our results are in accordance with this study and suggest that Portuguese propolis can have similar biological activities than propolis from South America.

Interestingly, genotoxicity of PEE, in experiments without H_2O_2 treatment, was higher in co-incubation than in pre-incubation experiments (Figures 7 and 9). This difference can be explained by the experimental design of these experiments, since in pre-incubation, cells were exposed to PEE, washed and incubated with S buffer, while in co-incubation, cells were analyzed with the comet assay immediately after incubation with PEE. Therefore, pre-incubation experiments allow cells to repair DNA damage before application of the comet assay. This argues in favor of an induction of an alert state of cells discussed above for viability assays. In fact here we show that PEE has mild genotoxic activity, which can be a consequence of the prooxidant properties of PEE that promote activation of cellular stress responses. Under these conditions, cells become more prepared to cope with stress leading to improved viability.

Our sample of propolis has a total polyphenolic and flavonoids content relatively close to propolis from Portuguese propolis from Fundão (Moreira *et al.*, 2008), Indian propolis (Laskar *et*

al., 2010) and to Brazilian propolis (Alencar *et al.*, 2007; Mello *et al.*, 2010). However, comparisons should be cautious due to differences between the solvent used for the extracts (different percentages of ethanol) and in the procedure to obtain the extracts. To our knowledge antioxidant activity of PEE (extraction only with ethanol), measured by the DPPH and ABTS assays, has not been reported before. Therefore, comparison, in terms of *in vitro* antioxidant capacity between Portuguese propolis and propolis from different origins is not possible. Nevertheless, we show evidence suggesting that Portuguese propolis has similar composition in polyphenolols and flavonoids, the phytochemicals with more potential to have biological activity found in propolis. This correlates with the *in vitro* antioxidant activity and protection against oxidative stress measured in terms of viability and antigenotoxicity.

To detect the intracellular antioxidant activity of PEE in living cells, we used flow cytometry with a redox-sensitive fluorochrome. As expected, cells displayed decrease in intracellular oxidation by incubation with PEE upon oxidative shock (Figure 11). This dose-dependent intracellular antioxidant activity is in accordance with the *in vitro* antioxidant activity and the protection effect in viability and antigenotoxicity upon oxidative shock.

In this work we present evidence strongly suggesting that Portuguese propolis has antioxidant and prooxidant activity and antigenotoxic and genotoxic activity, which fits in the so-called "Janus" effect. According to this model, substances that has dual effect, one positive and one negative, are "Janus" compounds (von Brostel *et al.*, 1998).

Portuguese propolis had been demonstrate an important significance in medicine, namely in Hereditary spherocytosis (HS) (Moreira *et al.*, 2011), to protect erythrocytes from damage promoted by free radicals and inhibition of human renal cancer cell growth (Valente *et al.*, 2011) and its antioxidant properties could be extensible used mainly in food industry (Moreira *et al.*,2008; Miguel *et al.*, 2010). However, additional studies are required to complete the evaluation of Portuguese propolis potentials.

5. Future perspectives

To better understand biological activities of propolis it would be important to complete the analysis of the sample used in this study with powerful analytical methodologies such as chromatography (HPLC, GC and MS). This would give detailed information on chemical composition, allowing to study year by year variations of samples from the same origin and to compare samples from different regions. In addition, the identification of biologically active compounds would allow to predicted medical applications and to guide investigation in the search of pharmacological effects.

The antigenotoxic/genotoxic effect of propolis can be investigated by taking advantage of the amenability of the genetic system of *S. cerevisiae*. Mutants affected in stress responses, in antioxidant proteins and in DNA repair pathways can be used in assays to help to identify the pathways involved in the protective activity of propolis. In addition, elegant screening systems with reporter gene-based genetic constructs can easily be adapted to the search of new biological activities of propolis at cellular level.

These two types of approach could be extended to Portuguese propolis from as many different origins as possible so that a map of properties could be created, assigning medical properties of propolis to the region of production. This would contribute to improve valorization of this natural product, enabling the creation of a Portuguese propolis-specific business activity.

6. References

Alencar, S.M., Oldoni, T.L.C., Castro, M.L., Cabral, I.S.R., Costa-Neto, C.M., Cury, J.A., Rosalen, P.L., Ikegaki, M. "Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis". *Journal of Ethnopharmacology*. **113**: 278-283 (2007)

Araújo, J.R.; Gonçalves, P.; Martel, F. "Chemopreventive effects of dietary polyphenols in colorectal cancer cell lines". *Nutrition Research*. **31**: 77-87 (2011)

Arts, I.C.W.; Hollman, C.H. "Polyphenols and diseases risk in epidemiologic studies". *American Journal of Clinical Nutrition.* **81**: 317-325 (2005)

Bankova, V.; Boudourova-Krasteva, G.; Popov, S.; Sforcin, J.M.; Funari, S.R.C. "Seasonal variations of the chemical composition of Brazilian propolis". *Apidologie*. **29**: 361-367 (1998)

Banskota, A.H., Tezuka, Y., Adnyana, I.K., Midorikawa, K., Matsushige, K., Message, D., Huertas, A.A.G., Kadota, S., "Cytotoxic, hepatoprotective and free radical scanvenging effects of propolis from Brazil, Peru, the Netherlands and China". *Journal of Ethnopharmacology*. **72**: 239-246 (2000)

Banskota, A.H.; Nagaoka, T.; Sumioka, L.Y. "Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines". *J Ethnopharm.* **80**: 67–73 (2002)

Benkovic, V., Knezevic, A.H., Dikic, D., Lisicic, D., Orsolic, N., Basic, I., Kosalec, I., Kopjar, N., "Radioprotective effects of propolis and quercetin in γ-irradiated mice evaluated by the alkaline comet assay". *Phytomedicine*. **15**: 851-858 (2008)

Boiteux, S.; Guillet, M., "Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*". *DNA Repair*. **3**: 1-12 (2004)

Cadet, J., Berger, M., Douki, T., Ravanat, J.L., " Oxidative damage to DNA: formation, measurement, and biological significance". *Rev. Physiol. Biochem. Pharmacol*. **131**: 1-87 (1997)

Cao, G., Sofic, E., Prior, R.L., "Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships". *Free Radical Biology & Medicine*. **22**: 749-760 (1997)

Cardile, V.; Panico, A.; Gentile, B.; Borrelli, F.; Russo, A., "Effect of propolis on human cartilage and chondrocytes". *Life Sciences*. **73**: 1027-1035 (2003)

Collins, A.R., "Review - The comet assay for DNA damage and repair – principles, applications, and limitations". *Molecular Biotechnology*. **26**: 249-261 (2004)

Collins, A.R., "Investigating oxidative DNA damage and its repair using the comet assay". *Mutation Research*. **681**: 24-32 (2009)

Chen, C. N.; Wu, C.-L.; Lin, J.-K., "Apoptosis of human melanoma cells induced by the novel compounds propolin A and propolin B from Taiwenense propolis". *Cancer letters*. **245**: 218-231 (2007)

Chen, C.-R.; Lee, Y.-N.; Chang C.-M. J.; Lee, M.-R.; Wei, I.-C., "Hot-pressurized fluid extraction on flavonoids and phenolic acids from Brazilian propolis and their cytotoxic assay *in vitro*". *Journal of the Chinese Institute of Chemical Engineers*. **38**: 191-196 (2007)

Dhawan, A.; Bajpayee, M.; Parmar, D., "Comet assay: a reliable tool for the assessment of DNA damage in different models". *Cell Biol.Toxicol.* **25**: 5-32 (2009)

Dixon, R.A., Paiva, N.L., Stress-induced phenylpropanoid metabolism. *Plant Cell*. **7**: 1085-1097 (1995)

Duthie, G.G.; Duthie, S.J., Kyle, J.A.M. "Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants". Nutrition Research Reviews. 13: 79-106 (2000)

Falcão, S.I.; Vilas-Boas, M.; Estevinho, L.M.; Barros, C.; Domingues, M.R.M.; Cardoso, S.M.; "Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds". *Anal Bioanal Chem* **396**: 887-897 (2010)

Ferguson, L.R. "Role of plant polyphenols in genomic stability". Mutation Research. 475: 89-111 (2001)

Folin, O.; Denis, W. “A colorimetric method for the determination of phenols (and phenol derivatieves) in urine.” J. Biol. Chem. 22:305-308 (1915)

Fokt, H., Pereira, A., Ferreira, A.M., Cunha, A., Aguiar, C. “How do bees prevent hive infections? The antimicrobial properties of propolis”. *In Méndez-Villas A (Eds) Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex Research Center*, **1**: 481-493 (2010)

Friedberg, E.C., Wood, R.P., “DNA excision repair pathways”. *DNA replication in Eucariotic Cells*. **8**: 249-269 (1996)

Friedberg, E.C., “DNA damage and repair”. *Nature*. **421**: 436-440 (2003)

Friedberg, E.C., McDaniel, L.D., Schultz, R.A., “The role of endogenous and exogenous DNA damage and mutagenesis”. *Current Opinion in Genetics & Development*. **14**: 5-10 (2004)

Gassen, M.; Yudim, M.B. “The potential role of iron chelators in the treatment of Parkinson’s disease and related neurological disorders”. *Pharmacol. Toxicol*. **80**: 159-166 (1997)

Gladine, C.; Morad, C.; Rock, E.; Bauchart, D.; Durand, D. “Plant extract rich in polyphenols (PERP) are efficient antioxidants to prevent lipoperoxidation in plasma lipids from animals fed *n*-3 PUFA supplemented diets”. *Animals Feed Science and Technology*. **136**: 281-296 (2007)

Gómez-Caravaca, A.M.; Gómez-Romero, M.; Arráez-Román, D.; Segura-Carretero, A., Fernádez-Gutérrez, A. “Advances in the analysis of phenolic compounds in products derived from bees”. Journal of Pharmaceutical and Biomedical Analysis. 41: 1220-1234 (2006)

Good, P.F.; Werner, P.; Hsu, A.; Olanow, C.W.; Perl, D.P. “Evidence of neuronal oxidative damage in Alzheimer’s disease”. *Am. J. Pathol.* **149**: 21-28 (1996)

Grzelak, A., Macierzyńska, E., Bartosz, G., “Accumulation of oxidative damage during replicative aging of the yeast *Saccharomyces cerevisae*”. *Experimental Gerontology*. **41**: 813-818 (2006)

Hanna, M.; Chow, B.L., Morey, N.J.; Jinks-Robertson, S.; Doetsch, P.W.; Xiao, W., "Involvement of two endonuclease III homologs in the base excision repair pathway for the processing of DNA alkylation damage in *Saccharomyces cerevisiae*". *DNA Repair*. **3**: 51-59 (2004)

Halliwell, B., Gutteridge, J.M.C. "Free radicals in biology and medicine". *Oxford: Clarandon Press*. (1989)

Halliwell, B. "Antioxidants and Human disease: a general introduction". Nutrition Reviews. 55: S44-S49 (1997)

Halliwell, B., "Oxygen and nitrogen are procarcinogens. Damage to DNA by reactive oxygen, chlorine and nitrogen sp.: measurement, mechanism and effects of nutrition". *Mutat. Res*. **443**: 37-52 (1999)

Halliwell, B.; Raftre, J.; Jenner, A. "Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not?" American Journal of Clinical nutrition. 81: 268S-276S (2005)

Henle, E.S., Linn, S., "Formation, prevention and repair of DNA damage by Iron/hydrogen peroxide". *The Journal of Biological Chemistry*. **272** (31): 19095-19098 (1997)

Horio, T., Miyauchi-Hashimoto, H., Kuwamoto, K., Yamazaki, F., Okamoto, H., "Photobiological information obtained from XPA gene-deficient mice". *Photochem. Photobiol*. **83**: 218-224 (2007)

Huang, M.-E.; Kolodner, R.D., "A biological Network in *Saccharomyces cerevisiae* prevents the deleterious effects of endogenous oxidative DNA damage". *Molecular Cell*. **17**: 709-720 (2005)

Izawa, S., Inoue, Y., Kimura, A., "Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae* ". *FEBS Letters*. **368**: 73-76 (1995)

Kalogeropoulos, N., Konteles, S.J., Troullidou, E., Mourtzinos, I., Karathanos, V.T., "Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus". *Food Chemistry*. **116**: 452-461 (2009)

Kelley, M.R., Kow, Y.W., Wilson III, D.M., "Disparity between DNA base excision repair in yeast and mammalian: translational implications". *Cancer Research*. **63**: 549-554 (2003)

Krokan, H.E., Standal, R., Slupphaug, G., "DNA glycosilases in the base excision repair of DNA". *Biochem. J.* **325**: 1-16 (1997)

Kumari, S., Rastogi, R.P., Singh, K.L., Singh, S.P., Sinha, R.P., "DNA damage: detection strategies". *EXCLI Journal*. **7**: 44-62 (2008)

Laskar, R.A.; Sk, I.; Roy, N.; Begum, N.A., "Antioxidant activity of Indian propolis and its chemical constituents". *Food Chemistry*. **122**: 233-237 (2010)

Laun, P., Pichova, A., Madeo, F., Funchs, J., Ellinger, A., Lohlwein, S., Dawes, I., Fröhlich, K.U., Breitenbach, M., "Aged mother cells of Saccharomyces cerevisiae show markers of oxidative stress and apoptosis". *Mol. Microbiol*. **39**: 1166-1173 (2001)

Lindahl, T., Nyberg, B., "Heat-induced deamination of cytosine residues in deoxyribonucleic acid". *Biochemistry*. **13**: 3405-3410 (1974)

Liu, M.-L.; Yu, L.-C. "Potential protection of green tea polyphenols against ultraviolet irradiation-induced injury on rat cortical neurons". Neuroscience Letters. 444: 236-239 (2008)

Laughton, M.J.; Halliwell, B.; Evans, P.J.; Hoult, J.R.S. "Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effexts on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA". *Biochem. Pharmacol.* **38**: 2859-2865 (1989)

Madeo, F., Herker, E., Wissing, S., Jungwirth, H., Eisenberg, T., Fröhlich, K.-U. "Apoptosis in yeast". Current Opinion in Microbiology. **7**: 655-660 (2004)

Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., "Polyphenols: food sources and bioavailability". American Journal of Clinical Nutrition. 79: 727-47 (2004)

Melidou, M., Riganakos, K., Galaris, D., "Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: The role of iron chelation". *Free Radical Biology & Medicine*. **39**: 1591-1600 (2005)

Melliou, E.; Chinou, I. “Chemical analysis and antimicrobial activity of Greek propolis”. *Planta Med.* **70**: 515–9 (2004)

Mello, B.C.B.S., Petrus, J.C.C., Hubinger, M.D., “Concentration of flavonoids and phenolic compounds in aqueous and ethanolic propolis extracts trough nanofiltration”. *Journal of food engineering*. **96**: 533-539 (2010)

Miloshev, G.; Mihaylov, I.; Anachkova, B., “Application of the single cell gel electrophoresis on yeast cells”. *Mutation research*. **513**: 69-74 (2002)

Miguel, M.G., Nunes, S., Dandlen, S.A., Cavaco, A.M.,Antunes, M.D.,”Phenols and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve, South of Portugal”. *Food and Chemical Toxicology*. **48**: 3418-3423 (2010)

Mohammadzadeh, S.; Sharriatpanahi, M.; Hamedi, M.; Amanzadeh, Y.; Ebrahimi, S.E.S.; Ostad, S.N., “Antioxidant power of Iranian propolis extract”. *Food Chemistry*. **103**: 729-733 (2007)

Moreira, L.L.; Dias, L.G.; Pereira, J.A.; Estevinho, L., “Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal”. *Food and Chemical Toxicology*. **46**: 3482-3485 (2008)

Moreira, L.L., Dias, T., Dias, L.G., Rogão, M., da Silva, J.P., Estevinho, L.M., “Propolis influence on erythrocyte membrane disorder (hereditary spherocytosis): A first approach”. *Food and Chemical Toxicology*. **49**: 520-526 (2011)

Murad, J.M., Calvi, S.A., Soares, A.M.V.C., Bankova, V., Sforcin, J.M., “Effects of propolis from Brazil and Bulgaria on fungicidal activity of macrophages against *Paracoccidioides brasiliensis*”. *Journal of Ethnopharmacology*. **79**: 331-334 (2002)

Nagaoka, T., Banskota, A.H.; Tezuka, Ya; Midorikawa, K.; Matsushige, K.; Kadota, Sh. “Caffeic acid phenethyl ester (CAPE): potent nitric oxide inhibitor from the Netherlands propolis”. *Biol Pharm Bull.* **26**: 487–91 (2003)

Newcomb, T.G.; Loeb, L.A. “Oxidative DNA damage and mutagenesis. In DNA repair in prokaryotes and lower eukaryotes”. J*.A. Nickoloff and M.F. Hoekstra, eds. (Totawa, NJ: Humana Press)*, 65-84 (1998)

Nolkemper, S., Reichling, J., Sensch, K.H., Schnitzler, P., "Mechanism of herpes simplex virus type 2 suppression by propolis extracts". Phytomedicine. 17: 132-138 (2010)

Olive, P.L.; Banáth, J.P., "The comet assay: a method to measure DNA damage in individual cells". *Nature Protocols*. **1**: 23-27 (2006)

Orsi, R.O., Sforcin, J.M., Funari, S.R.C., Bankova, V., "Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against Salmonella Typhimurium". International Immunopharmacology. 5: 359-368 (2005)

Ostling, O. & Johanson, K.J. "Microelectrophoretic study of radiation-induced DNA damage in individual mammalian cells".*Biochem. Biophys. Res. Commun*. **123**: 291-298 (1984)

Prakash, S., Prakash, L., "Nucleotide excision repair in yeast". *Mutation Research*. **451**: 13-24 (2000)

Pereira, A.D.S.; Seixas, F.R.M.S., Aquino Neto, F.R. "Própolis: 100 years of research and future perspectives". *Química Nova*. **25**: 321-326 (2002)

Pereira, A.D.; de Andrade, S.F.; Swerts, M.S.O.; Maistro, E.L., "First *in vivo* evaluation of the mutagenic effect of Brazilian green propolis by comet assay and micronucleus test". *Food and Chemical Toxicology*. **46**: 2580-2584 (2008)

Petti, S.; Scully, C. "Polyphenols, oral health and diseases: A review". *Journal of Dentistry*. **37**: 413-423 (2009)

Russo, A., Izzo, A.A., Borrelli, F., Renis, M., Vanella, A., "Free radical scavenging capacity and protective effect of Bacopa monniera L. on DNA damage". *Phytotherapy research*. **17**: 870-875 (2003)

Russo, A.; Troncoso, N.; Sanchez, F.; Garbarino, J.A.; Vanella, A., "Propolis projects human spermatozoa from DNA damage caused by benzo[α]pyrene and exogenous reactive oxygen species". *Life Sciences*. **78**: 1401-1406 (2006)

Sahu, S.C., Gray, G.C., "Lipid peroxidation and DNA damage induced by morin and narigenin in isolated rat liver nuclei". *Food Chem. Toxicol*. **35**: 443-447 (1997)

Schijlen, E.G.W.M.; De Vos, C.H.R.; Van Tunen, A.J.; Bovy, A.G. "Modification of flavonoid biosynthesis in crop plants". Phytochemistry. 65: 2631-2648 (2004)

Sforcin, J.M.; Orsi, R.O.; Bankova, V., "Effects of propolis, some isolated compounds and its source plant on antibody production". *Journal of Ethnopharmacology*. **98**: 301-305 (2005)

Sforcin, J.M., "Propolis and the immune system: a review". *Journal of Ethnopharmacology*. **113**: 1-14 (2007)

Sforcin, J.M.; Bankova, V., "Propolis: Is there a potential for the development of new drugs?". *Journal of Ethnopharmacology*. **133**: 253-260 (2011)

Sicili, S.; Kutluca, S., "Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region". *Journal of Ethnopharmacology*. **99**: 69-73 (2005)

Simões, L.M.C.; Gregório, L.E.; Da Silva Filho, A.A.; de Souza, M.L.; Azzolini, A.E.C.S.; Bastos, J.K.; Lucisano-Valim, Y.M., "Effect of brazilian green propolis on the production of reactive oxygen species by stimulated neutrophils". *Journal of Ethnopharmacology*. **94**: 59-65 (2004)

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., "A simple technique for quantization of low levels of DNA damage in individual cells". *Exp. Cell. Res*. **175**:184-191 (1988)

Slupphaug, G.; Kavli, B.; Krokan, H.E. "The interacting pathways for prevention and repair of oxidative DNA damage". *Mutat. Res*. **531**: 231-251 (2003)

Stone, W.L.; LeClair, I.; Ponder, T.; Baggs, G.; Reis, B.B. "Infants discriminate between natural and synthetic vitamin E". *The American Journal of Clinical Nutrition*. **77**: 899-906 (2003)

Tran, L.-T.; Miki, T.; Kamakura, M.; Izawa, S.; Tsujimoto, Y.; Miyabe, S.; Inoue, Y.; Kimura, A., "Oxidative stress response in Yeast. Induction of glucose-6-phosphate dehydrogenase by lipid hydroperoxide in *Hansenula mrakii*". *Journal of Fermentation and Bioengeneering*. **80**: 606-609 (1995)

Tavares, D.C.; Barcelos, G.R.M.; Silva, L.F.; Tonin, C.C.C.; Bastos, J.K., "Propolis-induced genotoxicity and antigenotoxicity in Chinese hamster ovary cells". *Toxicology in Vitro*. **20**: 1154-1158 (2006)

Usia, T.; Banskota, A.H.; Tezuka, Ya; Midorikawa, K.; Matsushige, K., Kadota, Sh. "Constituents of Chinese propolis and their antiproliferative activity". *J Nat Prod.* **65**: 673–76 (2002)

Valente, M.J., Baltazar, A.F., Henrique, R., Estevinho, L., Carvalho, M., "Biological activities of Portuguese propolis: Protection against free radical-induced erythrocyte damage and inhibition of human renal cancer cell growth *in vitro*". *Food and Chemical Toxicology*. **49**: 86-96 (2011)

Varanda, E.A., Monti, R., Tavares, D.C., "Inhibitory effect of propolis and bee venom on mutagenicity of some direct- and indirect-action mutagens". *Teratogenesis, Carcinogenesis, and Mutagenesis*. **19**: 403-413 (1999)

von Borstel, R.C.; Higgins, J.A. "Janus carcinogens and mutagens". *Mutation Research*. **402**: 321-329 (1998)

Wang, D.; Kreutzer, D.A.; Essigmann, J.M. "Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions". *Mutation Research*. **400**: 99-115 (1998)

Zheng, W.; Wang, S.Y. "Antioxidant activity and phenolic compounds in selected herbs". J*. Agri. Food Chem.* **49** (11): 5165-5170 (2001)