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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho realizado sob orientação do
Professor Doutor Rui Pedro Soares Oliveira
e da
Professora Doutora Cristina Alexandra de Almeida Aguiar

outubro de 2015

DECLARAÇÃO

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Avaliação de bioatividades numa amostra de própolis (Gerês) de origem Portuguesa

RESUMO

Própolis é uma mistura complexa formada por material resinoso e balsâmico, produzida pelas abelhas a partir de ramos, flores, pólen, brotos e exsudados de árvores, a qual é misturada com secreções salivares das abelhas. As abelhas utilizam o própolis na defesa contra invasores, protegendo a colmeia de infeções resultantes da putrefação. A composição química do própolis pode variar geograficamente, com a flora disponível, o clima, com a altura da colheita e a espécie de abelha. Diferentes grupos de compostos têm sido identificados em própolis, tais como flavonóides, ácidos fenólicos e os seus ésteres. Estes compostos têm sido associados com diversas atividades biológicas, nomeadamente: antimicrobiana; anti tumoral; antioxidante e quelante de radicais livres; anti-genotóxica e genotóxica; e antimutagénica.

O objetivo deste trabalho prende-se com o estudo de amostras de própolis português, particularmente no que diz respeito à sua caracterização química e à avaliação das suas bioatividades, visando a possibilidade da sua utilização/ exploração em aplicações médicas, cosmecêuticas e nutracêuticas. O própolis selecionado para este estudo foi colhido num apiário no Gerês (G), em quatro anos consecutivos, e foi utilizado para preparar extratos etanólicos (EE), que por sua vez foram testados em diferentes ensaios, usando o eucariota simples *Saccharomyces cerevisiae* como modelo biológico.

O ensaio cometa foi realizado para analisar a genotoxicidade/ antigenotoxicidade e os resultados evidenciam que o EE preparado com própolis do Gerês recolhido em 2012 (G12.EE) não apresenta efeito genotóxico significativo. Por outro lado, o própolis do Gerês também não protege as células contra os danos de DNA causados por peróxido de hidrogénio (H_2O_2), um comportamento exibido por qualquer um dos extratos testados (G11.EE, G12.EE, G13.EE e G14.EE. Contudo, células co- ou pré-incubadas com G.EEs e H_2O_2 10 mM exibiram maior viabilidade do que células incubadas apenas com H_2O_2, sugerindo que o propolis protege as células de levedura contra o stresse oxidativo. Esta atividade antioxidante foi também demonstrada por citometria de fluxo - a oxidação do fluorocromo intracelular diacetato de diclorofluoresceina (H_2DCFDA) foi menor em células co- ou pré-incubadas com G.EEs e H_2O_2 do que em células incubadas apenas com H_2O_2 - e corroborada por outros ensaios *in vitro* que demonstraram um efeito quelante de radicais livres por parte dos G.EEs. Foi ainda constatado que os G.EEs, embora revelem baixa citotoxicidade para as células eucariotas testadas, têm atividade antimicrobiana particularmente expressiva contra bactérias Gram-positivas produtoras de esporos, tendo sido igualmente observado um efeito sinérgico com o antibiótico gentamicina. A análise de células tratadas com os vários G.EEs, na presença do fluorocromo rodamina 123, mostrou que o própolis do Gerês exerce influência sobre o potencial da membrana mitocondrial interna.

Todas as amostras de própolis estudadas exibiram um comportamento muito semelhante nas diversas bioatividades avaliadas, o que de um modo geral contraria a variabilidade atribuída a este produto natural quando colhido em diferentes anos, mesmo que proveniente de um só local. Para este perfil de bioatividades mais constante contribui possivelmente o tipo de produção padronizada de própolis usada pelo apicultor responsável, ao contrário do que faz a grande maioria de outros apicultores, particularmente os portugueses. Uma análise química preliminar de G11.EE e G12.EE, releva não haver diferenças significativas em termos do seu perfil em compostos fenólicos, aos quais se atribuem as bioatividades de propolis, justificando assim o comportamento mais constante evidenciado pelos quatro extratos estudados.

PALAVRAS-CHAVE: Própolis - antigenotoxidade - antimicrobiano - sinergismo - antioxidante.

Evaluation of bioactivities of a propolis sample (Gerês) of Portuguese origin

ABSTRACT

Propolis is a complex mixture composed by resinous and balsamic material, produced by bees from branches, flowers, pollen, buds and exudates of trees and mixed with bees' salivary secretions. Bees use propolis in the defense against invaders, protecting the hive from infections resulting from putrefaction. The chemical composition of propolis varies geographically, with the available flora, the climate, the harvesting time and the bee species. Different groups of compounds can be found in propolis extracts, such as flavonoids, phenolic acids and their esters. These compounds have been associated with different biological activities such as antimicrobial; antitumor; antioxidant and free radical scavenger; antigenotoxic and genotoxic; and antimutagenic.

The aim of this work relates to the investigation on Portuguese propolis, particularly with regard to its chemical characterization and the evaluation of biological activities of this product in order to assess the possibility of its use/ exploitation in medical applications, cosmeticeutics and nutraceutics. The propolis samples selected for this study were collected in an apiary from Gerês, over four consecutive years and were used to prepare ethanol extracts (EE) which were tested in different assays, using the simple eukaryote *S. cerevisiae* as biological model.

The comet assay was performed to analyze the genotoxicity/ antigenotoxicity and the results suggest that the EE prepared with propolis from Gerês harvested in 2012 (G12.EE) do not display significant genotoxic effect. On the other hand, propolis from Gerês does not protect cells against DNA damages caused by H_2O_2 either, a behavior displayed by any of the tested extracts (G11.EE, G12.EE, G13.EE e G14.EE). However, cells co- and pre-incubated with G.EE and 10 mM H_2O_2 displayed higher viability than cells incubated only with H_2O_2, suggesting that G.EEs protect yeast cells against oxidative stress. The same antioxidant activity was demonstrated by flow cytometry – a lower fluorescence of the intracellular fluorochrome dichlorofluorescein diacetate (H_2DCFDA) was detected in cells co- and pre-incubated with G.EE and 5 mM H_2O_2 as compared with cells incubated only with H_2O_2 - and corroborated by several other assays *in vitro* that show the free radical scavenging activity of G.EEs. Antimicrobial activity was evaluated by the agar dilution method and the results suggest that G.EEs have antimicrobial activity, especially against Gram-positive spore forming bacteria. A synergistic effect of G.EEs when mixed with gentamicin was also demonstrated in the present work. The analysis of cells treated with G.EEs in the presence of the fluorochrome rhodamine 123 showed that propolis from Gerês has an influence on the inner mitochondrial membrane potential, decreasing the emitted fluorescence.

All the studied propolis samples exhibited a very similar behavior in the different evaluated bioactivities, which in generally is contrary to the variability attributed to this natural product when harvested in different years, even from a single location. For this more constant bioactivities profile possibly contributes the type of standardized production of propolis used by the beekeeper in charge, unlike what makes the great majority of other beekeepers, particularly the Portuguese. A preliminary chemical analysis of G11.EE and G12.EE reveals no significant differences in terms of phenolics profiles, compounds to which the bioactivities of propolis are attributed, thus justifying the more constant behavior evidenced by the four studied extracts.

KEYWORDS: Propolis - antigenotoxicity - antimicrobial - synergism - antioxidant

INDEX

ABBREVIATION LIST

BHA – butylated hydroxyl anisole

BHT – butylated hydroxyl toluene

CAPE – caffeic acid phenyl ester

CAT – catalase

CPE – cytopathogenic effect

DNA – deoxyribonucleic acid

DPPH – 2,2-diphenyl-1-picryl-hydrazyl

EDTA – ethylenediamine tetraacetic acid

EE – ethanol extract

G – Gerês

G11.EE – ethanol extract of propolis from Gerês harvested in 2011

G12.EE – ethanol extract of propolis from Gerês harvested in 2012

G13.EE – ethanol extract of propolis from Gerês harvested in 2013

G14.EE – ethanol extract of propolis from Gerês harvested in 2014

GA – gallic acid

GSH – glutathione

GSH-Px – glutathione peroxidase

GSH-R – glutathione reductase

H_2DCF – dichlorofluorescein

H_2DCFDA – dichlorofluorescein diacetate

HCL – human lung carcinoma

HIV – human immunodeficiency virus

HSV – human simplex virus

LC-MS – liquid chromatography-mass spectrometry

LMA – low melting agarose

MIC – minimum inhibitory concentration

NADH – nicotinamide adenine dinucleotide, reduced form

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

NBT – nitroblue tetrazolium

NMA – normal melting agarose

OD – optical density

P.EE – ethanol extract of propolis

PBS – phosphate buffered saline

PMS – phenazine methosufate

RNA – ribonucleic acid

ROS – reactive oxygen species

RPM – revolutions per minute

SOD – superoxide dismutase

UV – ultra-violet

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1. INTRODUCTION

1.1. Propolis

Throughout history, man has learned to use natural products in medicine. Propolis has been used in traditional medicine since the primordial times of humanity, having acquired popularity among Egyptians, Arabs, Greeks, and many other civilizations (Moreira *et al.*, 2008). For Egyptians, propolis was well known due to its anti-putrefactive properties and its capability to embalm cadavers. Incas used propolis as an anti-pyretic agent and Greek and Roman physicians used it as an oral disinfectant, as antiseptic and to heal wounds, being prescribed for topical treatment of skin and mucosa (Burdock, 1998). Balkans used propolis to treat wounds and burns, sore throat and stomach ulcers. In the Second World War, the Soviets used propolis to treat tuberculosis due to the observed decrease of lung problems (Wollenweber *et al.*, 1990). Registered as an official drug in the pharmacopoeia of London in the 17th century, propolis has become very popular in Europe during the following years in particular due to its antibacterial activity (Fokt *et al.*, 2010).

Propolis, or bee glue as it is also called, is a complex mixture composed by resinous and balsamic material, produced by honeybees (mainly *Apis mellifera* L.) from branches, flowers, pollen, buds and exudates of trees and mixed with salivary enzymes, waxes and other compounds resulting from the metabolism of bees (Fokt *et al.*, 2010). Etymologically the word *propolis* is derived from the Greek *pro* (for 'in front of') and *polis* (for 'community'), meaning that this natural product contributes to the defence of the hive (Sforcin, 2007). In the hive, propolis is used against invaders to immobilize their carcasses, protecting the hive from pests resulting from putrefaction. Another function of propolis is the mechanical and thermal insulation of the hive, being used to fill cracks or openings (Moreira *et al.*, 2008).

The chemical composition and biological activities of raw propolis vary with the source plant species that exist around the hive where propolis is collected (Katalinic *et al.*, 2004; Falcão *et al.*, 2010), the climate characteristics (Falcão *et al.*, 2010), the time of harvest, the technique used to harvest, the species of bee (Pereira *et al.*, 2002) as well as the extraction method (Sheng *et al.*, 2006). Even though, and in general, this complex mixture is composed of around 50 % resins and vegetable balsams, 30 % wax, 10 % essential oils, 5 % pollen and 5 % of other substances, including organic compounds (Fokt *et al.*, 2010).

Propolis has a characteristic and pleasant aromatic odour and its colour can vary from yellow-green, red and dark brown, depending on their origin and age (Bankova *et al.*, 2000). It is hard and brittle when cold, but becomes soft and very sticky when warm (Loutfy, 2006). It is difficult to remove from human skin since it appears to interact strongly with the proteins and oils of the skin (Burdock, 1998).

1.2. Chemical composition and biological activities

There are many compounds that have been identified in different samples of propolis (Marcucci *et al.*, 1995; Bankova *et al.*, 2000; de Castro *et al.*, 2001), and new compounds are yet to be acknowledged during the chemical characterization of new samples. From all the identified compounds, phenolics are the most important. The most common phenolic compounds in propolis from temperate zones are flavonoids like pinocembrin, galangin and chrysin and phenolic acids such as caffeic acid, ferulic acid and cinnamic acid (Marcucci *et al*, 1995; Bankova *et al.*, 2000; Huang *et al.*, 2014). Propolis from tropical areas, especially in South-eastern Brazil, proved to be rich in prenylated phenylpropanoids, and some non-typical compounds such as kaempferide and isosakuranetin have also been found (Bankova *et al.*, 2000).

In European propolis, the main bioactive compounds are flavonoids (flavones, flavonols and flavonones), phenolic acids and their esters (Huang *et al.*, 2014). In a study concerning the phenolic compounds of an ethanol extract of propolis from Northeast Portugal, made by Falcão *et al.* (2010), it was shown that chemical compounds such as flavonoids were also found in the Portuguese sample, side by side with rare pinocembrin or pinobanksin derivatives that contain basic structures of phenolic acids, as well as *p*-coumaric ester derivative dimer (Fokt *et al.*, 2010). Upon analysis by liquid chromatography-mass spectrometry (LC-MS) of the ethanol extract (EE) of propolis from Gerês collected in 2012 (G12.EE), the main compounds found were *p*-coumaric acid, pinocembrin, caffeic acid, quercetin, pinobanksin, chrysin and ferulic acid, among many others and similarly to other chemical profiles of European propolis described (Freitas, 2013).

Certain biological activities are always present in propolis and they can be associated with completely different chemical profiles in samples from diverse geographic and climate areas. Different chemical compositions of propolis from different origins led to the explanation that their

biological properties would be dissimilar, but this is amazingly untrue as samples of different origins can display identical biological activities. The main compounds responsible for propolis biological activities are the flavonoids, aromatic acids, diterpenic acids, phenolic compounds and cinnamic acid derivatives including caffeic acid esters but, very often, different propolis types have distinct main bioactive compounds (Borreli *et al.*, 2002).

Propolis is commercialized in different parts of the world and it is recognized as an important source of compounds with properties for several applications (Moreira *et al.*, 2008). There is a long history of propolis use, that continues today in home remedies and personal products, and that happens because propolis has an endless list of preparations and uses. The demand for this substance is becoming larger due to the growing consumers´ preference for natural products. Propolis can be found in pharmaceutical and cosmetic products such as face creams (vanishing creams and beauty creams), ointments, lotions and solutions. It is also found in dermatological items, useful in wound healing, tissue regeneration, treatment of burns, neurodermatitis, leg ulcers, psoriasis, morphoea, herpes simplex and genitalis and active against dermatophytes (Burdock, 1998). Propolis is commercially available and can be found in the form of capsules (pure or combined with aloe gel, *Rosa canina* or pollen), extracts (hydroalcoholic or glycolic), mouth wash solutions, creams, and many others (Fokt *et al.*, 2010).

It is well known that ethanol and non-ethanol extracts of propolis have different chemical compositions and display diverse biological activities (Majiene *et al.*, 2010; Ramanauskiené and Inkénienė, 2011; Mavri *et al.*, 2012; Kubiliene *et al.*, 2015). In recent years, this product has been the subject of intensive studies, highlighting their biological and pharmacological properties (Falcão *et al.*, 2010; Piccinelli *et al.*, 2013; Kurek-Górecka *et al.*, 2014; Silva-Carvalho *et al.*, 2014, 2015; Boisard *et al.*, 2015; Szweda *et al.*, 2015). Regardless of the plant source (species or geographical origin) and the composition, the biological activity of propolis, particularly the antimicrobial activity, has always been reported. Due to the plant diversity, there are different types of propolis, which contain numerous chemical constituents responsible not only for antimicrobial activity, but also for other valuable bioactivities (Bankova, 2005).

Propolis biological properties include antibacterial activity against various pathogenic bacteria (Burdock, 1998; Kujumgiev *et al.*, 1999; Koo *et al.*, 2000; Borreli *et al.*, 2002; Uzel *et* al., 2005 Falcão *et* al., 2010; Castro *et al.*, 2011, 2012), antifungal (Burdock, 1998; Koo *et al.*,

2000; Borreli *et al.*, 2002; Moreira *et al.*, 2008; Falcão *et* al., 2010; Castro *et al.*, 2011, 2012), anti-protozoan (Castro *et al.*, 2011, 2012), anti-viral (Borreli *et al.*, 2002; Sheng *et al.*, 2006; Moreira *et* al., 2008) as anti-HIV (Falcão *et* al., 2010; Castro *et al.*, 2011, 2012), antioxidant (Banskota *et al.*, 2001b; Borreli *et al.*, 2002; Sheng *et al.*, 2006; Falcão *et* al., 2010), anti-inflammatory (Borreli *et al.*, 2002; Sheng *et al.*, 2006; Lofty *et* al., 2006; Sforcin, 2007, Moreira *et al.*, 2008; Falcão *et* al., 2010), anti-tumor (Grunberger *et al.*, 1998; Sforcin, 2007; Moreira *et al.*, 2008; Valença *et al.*, 2013; Silva-Carvalho *et* al., 2014), hepato-protective (Sheng *et al.*, 2006), anti-neurodegenerative (Chen *et al.*, 2008; Falcão *et* al., 2010), local-anaesthetic (Moreira *et al.*, 2008), anti-tuberculosis (Falcão *et al.*, 2010), free-radical-scavenging (Sheng *et al.*, 2006; Castro *et al.*, 2012), immunostimulating (Borreli *et al.*, 2002; Lofty *et* al., 2006) and cytotoxic (Matsuno *et al.*, 1997). Propolis extracts were also tested as a food preservative due to its bacteriostatic and bactericidal properties (Tosi *et al.*, 2007). Furthermore, Gregoris *et al.* (2011) showed that propolis protects against UV radiation and could be used in the formulation of sunscreens. Propolis is also capable of inhibiting the action of the enzyme hyaluronidase, allowing to retard cell aging (Kim *et al.*, 2005) and, more recently, experimental data showed that propolis can be used to treat fungal infections of *Candida* (Castro *et al.*, 2012).

For all these reasons, this natural product has sparked interest in the pharmaceutical and food industries, being introduced in different products for human consumption as drinks, food and cosmetics, though mostly because of its antioxidant and antimicrobial properties.

1.2.1. Antibacterial activity

Many researchers have studied the antibacterial activity of propolis to evaluate this property against a large panel of Gram-positive and Gram-negative bacteria, normally using one or two of the most popular methods used to evaluate this activity - the disc diffusion method and the broth or agar dilution method (Fokt *et al.*, 2010). Several studies demonstrated that propolis has activity against a wide range of Gram-positive bacteria but had a limited or even no activity against Gram-negative ones (Bankova *et al.*, 2000; Uzel *et al.*, 2005; Lofty, 2006; Jorge *et al.*, 2008; Ramanauskiené and Inkénienė, 2013).

Although the propolis mechanism of action for its antibacterial activity is not yet clearly understood, some studies suggest that propolis and some of its cinnamic and flavonoid components were able to uncouple the energy-transducing cytoplasmatic membrane, to inhibit

bacterial motility (Mirzoeva *et al.*, 1997). It was also suggested that propolis inhibits bacterial growth by preventing cell division, resulting in the formation of pseudo-multicellular forms. In addition, propolis also disorganized the cytoplasm, the cytoplasmic membrane and the cell wall, which led to a partial bacteriolysis, and inhibited protein synthesis (Takasi *et al.*, 1994). Other study (Uzel *et al.*, 2005) suggests that the mechanism of action may be related to the inhibition of RNA-polymerase from bacteria.

The antimicrobial activity of propolis may be linked with its complex composition involving a complex mechanism putatively attributed to the synergistic effect of phenolic compounds such as cinnamic acid and ester derivatives including caffeic acid and acid phenyl ester (CAPE). Other compounds such as flavonoids - including quercetin, naringenin (Santos *et al.*, 2002; Boisard *et al.*, 2015), galangin, pinostrobin, and pinocembrin, ferulic acid, hydroquinones - and terpenic acids such as isopimaric, abietic and dehydroabetic acid (Patel *et al.*, 2014) are also suspected to be responsible for this biological activity.

Park *et al.* (1998) reported that an ethanol extract of propolis (P.EE) from various regions of Brazil inhibited the growth of *Streptococcus*, an oral pathogen. Other studies made with periodontitis-causing bacteria, such as *Peptostreptococcus anaerobius* (Santos *et al.*, 2002) *Porphyromonas gingivalis*, *Prevotella intermedia* (Santos *et al.*, 2002; Gebara *et al.*, 2002), *Prevotella melaninogenica*, *Actinobacillus actnomycetemcomitans*, *Fusobacterium nuclatum* and *Capnocytophaga gingivalis* (Gebara *et al.*, 2002) showed the susceptibility of these strains to the EE. Several studies showed the antimicrobial action of P.EE against *Staphylococcus aureus*, a pathogen reported to produce food poisoning (Hegazi *et al.*, 2000; Lu *et al.*, 2005; Ramanauskiené *et al.*, 2009; Ramanauskiené and Inkénienė 2013). Ramanauskiené *et al.* (2009) and Ramanauskiené and Inkénienė (2013) not only showed the antibacterial activity of Lithuanian P.EE against *Staphylococcus aureus*, but also against *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Bacillus subtilis* and *Bacillus cereus.* Wojtyczka *et al.* (2013a) demonstrated the antibacterial activity of Polish P.EE against the methicillin-sensitive (MSSA) and the methicillin-resistant (MRSA) *Staphylococcus aureus,* both clinical isolates. Other study showed the antibacterial activity of a propolis samples from Lebanon against *Staphylococcus aureus* (MRSA) (Chamandi *et al.*, 2015). Antimicrobial activity of Korean propolis was showed by Kim and Chung (2011) against various foodborne pathogens such as *Bacillus cereus*, *Staphylococcus aureus* and *Listeria onocytogenes.*

Polish P.EE showed antibacterial activity against *Staphylococcus epidermidis* reducing the biofilm formation and bacterial growth (Wojtyczka *et al.*, 2013b).

Bianchini and Benedo, (1998) demonstrated the inhibitory effect of aqueous extracts of propolis against some phytophatogenic bacteria such *Agrobacterium tumefaciens*, *Clavibacter michiganensis* subsp. *michiganensis* and *Xanthomonas axonopodis*. Piermann *et al*. (2007) tested an extract of propolis from a commercial product 10% concentrated and showed its antimicrobial activity against eight phytopathogenic bacteria (*Pseudomonas syringae* pv. *tomato*, *Pseudomonas corrugata*, *Clavibacter michiganensis* subsp. *michiganensis*, *Erwinia carotovora* subsp. *Carotovora* and several species of the genus *Xanthomonas*. Other study with phytopathogenic bacteria showed the susceptibility of *Pseudocercospora vitis*, *Elsinoe ampelina* and *Phakopsora euvitis* to alcoholic extracts of propolis (Marini *et al.*, 2012).

A synergistic effect of P.EE with bactericidal anti-tuberculosis drugs, including streptomycin, rifamycin and isoniazide was reported. In the same study, two of the tuberculosis bacilli strains tested, found to be resistant to some of the drugs, lost part of their resistance when treated with P.EE in combination with the drug (Scheller *et al.*, 1999). The synergistic effect between propolis and the antimicrobial drugs ampicillin, ceftriaxone and doxycycline against *Staphylococcus aureus* and with nystatin against *Candida albicans* was noticed by Stepanović *et al*. (2003), authors that also proved that bacterial resistance to antibiotics had no influence on the susceptibility to propolis extracts. Fernandes *et al*. (2005) found a synergistic effect between propolis and antimicrobial drugs against *Staphylococcus aureus*, especially for those agents that interfere on bacterial protein synthesis.

1.2.2. Antifungal activity

The antifungal activity is normally evaluated using the disc diffusion method and/ or the dilution method, as for the estimation of antibacterial activity. For antifungal activity, as well as for antibacterial activity, the effect is associated with the presence of flavonoids and other phenolic components (Farnesi *et al.*, 2009). The propolis mechanism against fungal strains may be related to genes involved in the mitochondrial electron transport chain, vacuole acidification, negative regulation of transcription from RNA polymerase II promoter, regulation of macroautophagy associated with protein targeting to vacuoles, and cellular response to starvation (Castro *et al.*, 2011).

Samples from European propolis demonstrated a fungicidal effect against species belonging to the genera *Candida*, *Microsporum*, *Mycobacteria*, *Trichophyton*, *Fusarium* and other dermatophytes (de Castro *et al.*, 2001). Several other studies demonstrated also the susceptibility of clinical yeasts belonging to *Candida* genus such as *Candida albicans*, (Hegazi *et al.*, 2000; Trusheva *et al.*, 2006; Ramanauskiené *et al.*, 2009; Noori *et al.*, 2012; Ramanauskiené and Inkéniené 2013; Chamandi *et al.*, 2015) as well as of some filamentous fungi, mainly dermatophytes. *S. cerevisiae* and *Trichosporon* sp. showed to be susceptible to propolis as well (Oliveira *et al.*, 2006). The susceptibility of *S. cerevisiae* was also found in a study with Spanish P.EE made by Banvehí and Gutiérrez (2012). Al-Daamy *et al.* (2015) assessed the antifungal activity of propolis from Iraq against the two dermatophytes: *Trischophyton mentagrophytes* and *Trichophyton tonsurans* and on five clinical isolates of *Candida albicans* isolated from oral cavities of different patients, and showed the susceptibility of all the strains. Recently, Szweda *et al.* (2015) studied a sample of P.EE from Poland and showed its fungicidal activity against *Candida albicans*, *Candida glabrata* and *Candida krusei*. D'auria *et al.* (2003) not only showed the antifungal activity of propolis against *Candida albicans* strains but described additionally its inhibitory effect on yeast-mycelial conversion and a reduction on hyphal length. Other study, made by de Castro *et al.* (2013) demonstrated that propolis inhibited the transition from yeast-like to hyphal growth on *Candida albicans* mutants' strains.

1.2.3. Antiviral activity

The methodology normally used to evaluate the antiviral activity is the cytopathogenic effect (CPE) reduction assay (de Castro *et al*, 2001). The data about the antiviral effect of propolis are very few but the studies performed have shown that propolis displays significant antiviral activity at different levels, interfering with the replication of some viruses (de Castro *et al.*, 2001) like herpes simplex types 1 and 2, adenovirus type 2, influenza virus, or human immunodeficiency virus (HIV), among others (Schinitzler *et al.*, 2010; Sartori *et al.*, 2012). Indeed, it was also found that propolis suppressed HIV-1 replication (Hadi and Hedazi, 2002) and inhibited its variants expression (Gekker *et al.*, 2005) too. According to Tait *et al.* (2006), natural and synthetic flavonoids may interfere with picornavirus replication by preventing the decapsidation of viral particles and RNA release within cells or blocking viral RNA synthesis. In fact, apigenin, luteolin, naringenin and quercetin showed to be active aginst enterovirus 71

infections (Ji *et al.*, 2015). Schinitzler *et al.* (2010) analysed the antiviral effect of P.EE and some of the constituents against herpes simplex virus type 1 (HSV-1) and proved that P.EE exhibited high anti-HSV-1 activity and that galangin and chrysin were the main bioactive compounds. Other study made by Shvarzbeyn and Huleihel (2011), who tried to determine which step of Tax oncoprotein-induced NF-kB activation is blocked by propolis and CAPE, showed that both inhibited substantially the activation of NF-kB-dependent promoter by Tax and also that both prevented Tax binding to IkBα and its degradation.

1.2.4. Antioxidant activity

Reactive oxygen species (ROS) such as hydroxyl (HO·), superoxide anion (O_2·), nitric oxide (NO) and hydrogen peroxide (H_2O_2) are continuously generated in the cell due to aerobic metabolism. A free radical can be defined as any molecular species that contains an unpaired electron in an atomic orbital (Lobo *et al.*, 2010). O_2·, normally considered a primary ROS, can be formed by the addiction of an electron to the molecular oxygen (Cadenas and Sies, 1998). Despite not being a very active radical, it is able to interact with other molecules to form other radicals, usually called secondary ROS, such as H_2O_2 and HO· (Ferreira *et al.*, 2007). H_2O_2 can yield HO· when with metal ions, being the ROS that causes more cellular damage due to its strong reactivity (Ferreira *et al*, 2007). In Fenton reaction (Figure 1), iron reacts with H_2O_2, leading to the formation of HO· radicals which have a high redox potential, attacking all the species present in the reaction medium. The high reactivity of HO· results in rapid and non-specific reactions with different substrates, implying that the reaction rate can be limited by the diffusion rate. When Fe^{3+} is used instead of Fe^{2+}, in combination with excess of H_2O_2, other radicals of lower oxidation potential such as hydroperoxyl (HO_2·) and O_2·, are also formed. The proportion in which these radicals are produced is determined by the pH, due to the protonation of the O_2· that occurs in acid medium. The HO· radical can act like an electrophile or like a common nucleophile, attacking organic molecules by the rejection of hydrogen ions or engaging in double bonds and aromatic rings (hydroxylation). The decomposition of H_2O_2 by Fe^{3+} can generate the reduced species Fe^{2+} which also reacts with H_2O_2 and HO· (Aguiar *et al.*, 2007).

ROS can also be generated due to exogenous agents such as heat shock, dehydration, toxic chemicals, ultraviolet and ionizing radiation (Nakajima *et al.*, 2009; Sá *et al.*, 2013; Mitra and Uddin, 2014). At low or moderate concentrations, ROS can be beneficial to the cell, being

involved in several physiological processes such as signalling and redox regulation and defence against infections (Fridovich, 1999). Once produced, most free radicals are removed by the cell antioxidant defences including enzymes and non-enzymatic molecules. Maintaining the balance between free radical production and antioxidant defences is a prerequisite for the normal functioning of the body. However, this balance can be destroyed when generation of ROS overwhelms the cellular antioxidant components or because there is a deficiency in the antioxidant defences of the cell, causing a drastic oxidative stress (Ferreira *et al.*, 2007; Sá *et al.*, 2013). When ROS production exceeds cellular antioxidant capacity, the consequences are oxidative damage of membrane lipids, proteins and nucleic acids, which can lead to cell death or to acceleration in aging and to a number of diseases such as cancer (prostate and colon) (Karamian and Ghasemlou, 2013), Alzheimer, Parkinson or multiple sclerosis (Wilms *et al.*, 2007; Weiner, 2009; Politis *et al.*, 2011).

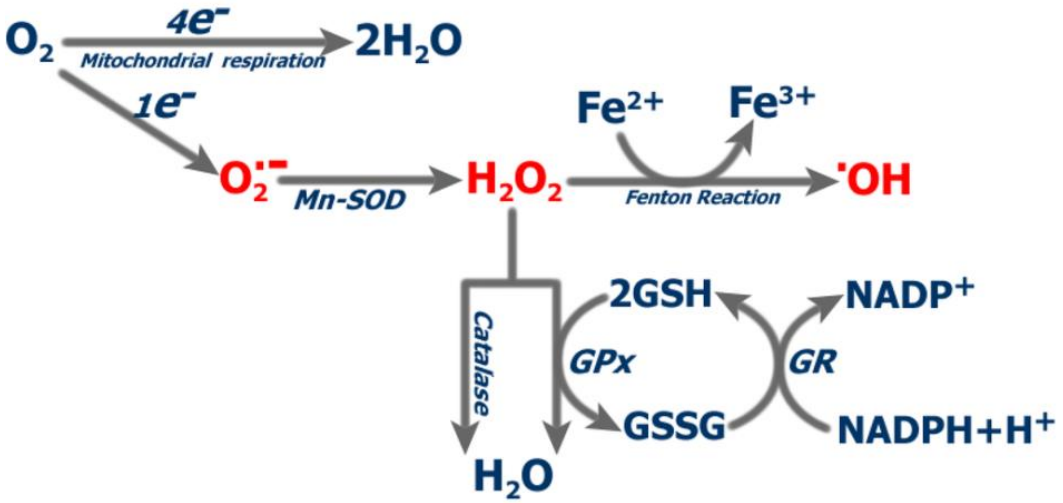

Figure 1 - Cellular reactions which result in the production of HO· - the Fenton reaction. Adapted from Valle *et al.*, 2010.

The exposure of organisms to free radicals led to the selection of those who have developed a number of defence mechanisms. Examples of these defences are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GSH-R). Between the non-enzymatic antioxidant defences are compounds such as glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C), flavonoids and carotenoids (Ferreira *et al*, 2007) (Figure 2) that are mainly related to the elimination and detoxification of the components that can be damaged by ROS (Sá *et al.*, 2013).

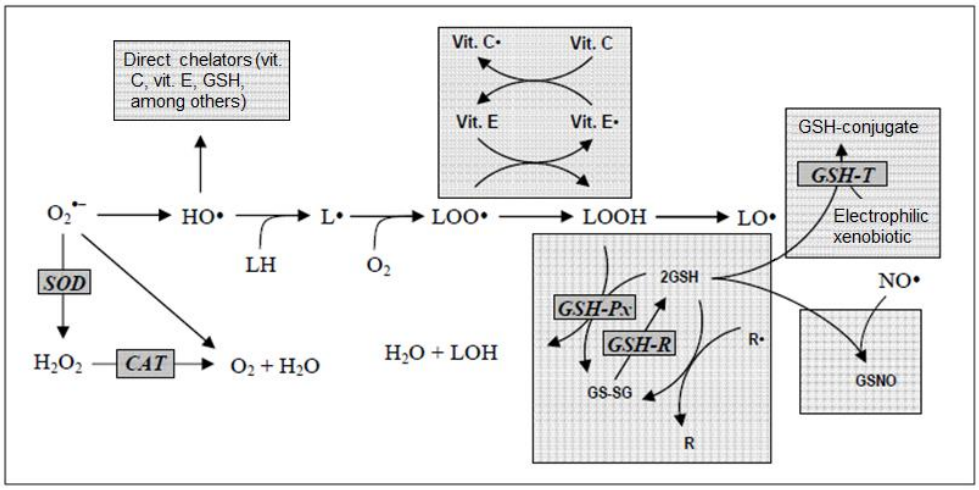

Figure 2 - Main endogenous antioxidant defences of the cell. SOD: superoxide dismutase; CAT: catalysis; GSH: glutathione; GSH-Px: glutathione peroxidase; GSH-R: glutathione reductase; Vit. C: ascorbic acid; Vit. E: α-tocopherol. Adapted from Ferreira *et al.*, 2007.

There are also synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters (Talla *et al.*, 2014), used by food industries to prevent lipid peroxidation and oxidation of food constituents. However, these compounds are suspected to have some negative health effects as liver damage and carcinogenesis. These problems increased the research and the demand for safer natural antioxidants in many applications, renewing the interest on natural products that have been used for centuries for a variety of reasons (Geckil *et al.*, 2005).

Propolis is recognized for being a natural antioxidant agent. The antioxidant potential of propolis is important for immunomodulatory properties because it increases the cellular immune response through the increase of mRNA for interferon-γ and activates the production of cytokines (Fischer *et al.*, 2007). The propolis main mechanisms of action for its antioxidant activity involve the inhibition of the activity of some enzymes which are involved in ROS generation, inhibiting that way the production of ROS; the scavenging activity, interrupting the reactions that result in lipid peroxidation; by chelating metal ions, generally iron and cooper; or potentiating the action of other antioxidants (Kurek-Górecka *et al.*, 2013; Silva-Carvalho *et al.*, 2015).

Polyphenols and flavonoid compounds were the main bioactives reported to be responsible for antioxidant potential in different propolis samples (Kumazawa *et al.*, 2004). According to Orsolić *et al.* (2006), ferulic acid, quercetin, prenylated compounds, apigenin, galangin and *p*-coumaric acid are involved with the antioxidant potential of propolis samples. A study with samples of P.EE from Transylvania showed a positive correlation between high polyphenolic

composition and high antioxidant activity (Mihai *et al.*, 2011). Chen *et al.* (2009) found also CAPE as a component which plays an important role in the antioxidant activity. Other study made with a ethyl acetate fraction of propolis collected in Anhui, China, showed strong scavenging activity and ferric reducing activity and related these activities with the presence of caffeic acid, phenethyl caffeate, cinnamyl caffeate and benzyl caffeate (Yang *et al.*, 2011).

Propolis extracts have been reported to possess a potent antioxidant activity (Viuda-Martos *et al.*, 2008). This activity can be evaluated by various methodologies. By flow cytometry it was possible to observe a decrease in the fluorescence intensity of dichlorofluorescein (H_2DCF) in *S. cerevisiae* propolis-treated cells, which correlated to propolis ability to decrease intracellular oxidation (Cigut *et al.*, 2011). This antioxidant ability of a propolis sample from China was also demonstrated for RAW264.7 cells (Zhang *et al.*, 2015). Sá *et al.* (2013) reported also a potent antioxidant activity of propolis in yeast cells: a reduction of the level of ROS produced when cells were treated with propolis after treatment with H_2O_2.

Propolis also showed strong reducing power and the ability to chelate metal ions (Miguel *et al.*, 2010; Mavri *et al.*, 2012; Talla *et al.*, 2014) and scavenges free radicals (Moreira *et al.*, 2008; Nakajima *et al.*, 2009; Miguel *et al.*, 2010; Mavri *et al.*, 2012; Campos *et al.*, 2014; Zhang *et al.*, 2015). Geckil *et al.* (2005) reported high metal chelating capacity of propolis and comparable antioxidant activity to the two most widely used synthetic antioxidants, BHA and BHT. Sheng *et al.* (2007) and Talla *et al.* (2014) indicated propolis as a potential natural antioxidant by DPPH free-radical-scavenging activity. Other antioxidant activities of different extracts of propolis were found such as ferric reducing activity (Yang *et al.*, 2011; Piccinelli *et al.*, 2013) and reduction of lipid peroxidation (Valente *et al.*, 2011; Silva *et al.*, 2011; Campos *et al.*, 2014).

A study with the same samples used in this work (G11.EE, G12.EE, G13.EE and G14.EE) using cyclic and differential pulse voltammetry methods, showed significant antioxidant capacity for all the G.EEs in a concentration dependent-manner. The highest antioxidant capacity, in cyclic voltammetry, was found in G12.EE, while G11.EE possesses the lowest antioxidant capacity values, at both concentrations tested. The highest antioxidant capacity, in differential pulse voltammetry, was found in G14.EE, while G11.EE possesses the lowest antioxidant capacity values, at both concentrations tested. The sequence orders of antioxidant capacity on cyclic voltammetry and on differential pulse voltammetry were respectively G12.EE>G14.EE>G13.EE>G11.EE and G12.EE>G13.EE>G14.EE>G11.EE (Sousa, 2015).

1.2.5. Other biological activities

Propolis showed antitumor activity, including cytotoxicity (Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Silva-Carvalho *et al.*, 2015) against several cancer cell lines. Its main mechanism of action involves apoptosis, cell cycle arrest and interference on metabolic pathways (Watanabe *et al.*, 2011; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014). The cytotoxic effect of different flavonoids such as quercetin, chrysin and caffeic acid was shown to be strong against five leukaemia cell lines (MOLT, JURKAT, HL-60, RAJI and U937) (Watanabe *et al.*, 2011). Some diterpenic acids, isolated from propolis, exhibited cytotoxicity towards human lung carcinoma HLC-2 and human carcinoma HeLa cells (Banskota *et al.*, 2001a). Other component of propolis, CAPE, was investigated for its effect on the angiogenesis, tumor invasion, and metastasis and the results showed inhibition of the angiogenesis and of cell proliferation concluding that CAPE has potential as an anti-metastatic agent (Liao *et al.*, 2003). It was also reported that CAPE showed a dose-dependent decrease in cell viability of CT26 colon adenocarcinoma cells (Lofty, 2006). Also, Hernandez *et al.* (2007) showed that CAPE, galangin, xanthomicrol and chrysin had a significant antiproliferative activity on several cancer cells. A study made with a fractionated Portuguese propolis sample from Angra do Heroísmo, Azores Archipelago, on HCT-15 colon cancer cell line showed the ability of propolis to decrease cell viability of colon tumor cells and also a disturbance of cancer cell glycolytic metabolism (Valença *et al.*, 2013). Other study, made by Silva-Carvalho, (2014), with a Portuguese propolis sample, from Pereiro, showed a decrease on cell viability of different tumour cells. The same study showed a decrease on MDA-MB-231 and DU145 cells proliferation and migration, with cell cycle changes.

There are several studies describing the anti-inflamatory activity of propolis (Naito *et al.*, 2007; Funakoshi-Tago *et al.*, 2015; Teles *et al.*, 2015; Valenzuela-Barra *et al.*, 2015; Silva-Carvalho *et al*, 2015). A study showed that P.EE from Croatia suppressed functional activity of macrophages improving psoriatic-like skin lesions on male albino mice (Oršolić *et al.*, 2014). Another studies made on male mice showed that P.EE, from Chile and from Brazil, inhibited the NO release by the macrophages (Valenzuela-Barra *et al.*, 2015) and decreased renal macrophages infiltration (Teles *et al.*, 2015), respectively.

The immunomodulatory effect of propolis was evaluated by Da Silva *et al.* (2013) in *Leishmania* (*Viannia*) *braziliensis* infection, using a propolis sample from Brazil. Propolis was able to increase the interiorization of macrophages and further killing the parasites. The same study

also demonstrated an increase of TNF-α production while IL-12 was downregulated during the infection.

1.3. Biological problem and aim of this work

Over the years, the interest in natural products has been surprisingly increasing because of its potential for the development of new drugs. Also, the industries have revealed an increased interest in these natural products because of its diversified bioactive profiles that can be used in food, in order to replace some usual synthetic compounds that can be toxic, as well as in cosmetic and pharmaceutical industries (Silva-Carvalho *et al.*, 2015). Propolis biological and pharmacological actions have been reported (Silva-Carvalho *et al.*, 2015) and its biological properties have been used by several industries (Marcucci, 1995; Pereira *et al.*, 2002). On the other hand, and unlike the products derived from medicinal plants, propolis has great variability in terms of chemical composition, which is a major problem to its standardization and consequently to its use and acceptance by the medical community (Bankova *et al.*, 2000; Falcão *et al.*, 2010). Propolis purity, regarding the percentage of beeswax or insoluble residues, among others, must be considered as well (Bankova *et al.*, 2000). Propolis composition depends on a variety of factors like the source of plant species, environmental factors (Valença *et al.*, 2013) such climate characteristics (Marcucci, 1995), the time and the technique of harvest, the species of bee (Pereira *et al.*, 2002), as well as the extraction method (Sheng *et al.*, 2006). These factors account for the wide range of compounds found in propolis samples from different regions (Mărghitaş *et al.*, 2013), which in turn constitute a problem for the medical use of propolis and its quality control (Bankova *et al.*, 2000). There is considerable information about the chemical composition and the biological activities of propolis but, in order to be accepted into the health care system, propolis needs chemical analysis performed by some standardized methods (Mărghitaş *et al.*, 2013) and requires systematic investigations of the chemical composition and the biological action, particularly the antimicrobial action (Bankova *et al.*, 2000).

The aim of this work relates to the investigation on Portuguese propolis, particularly with regard to its chemical characterization and the evaluation of biological activities in order to assess the possibility of their use/ exploitation in medical, cosmeticeutics and nutraceutics applications. The propolis samples selected for this study were collected in an apiary from Gerês (G), harvested over four consecutive years (2011, 2012, 2013 and 2014), and used to prepare EE

which were tested in different assays, using the simple eukaryote *S. cerevisiae* as biological model. There are several characteristics that contribute to the choice of the yeast *S. cerevisiae* as the experimental biological model, including the easy handling, non-pathogenicity, and the well-known biology, genetics and metabolism (Guthrie and Fink, 2004). When in rich medium, cultures of *S. cerevisiae* display several distinct stages of growth. After a short period of adjustment, the lag phase, the culture grows exponentially using the energy derived mainly from the fermentation of hexoses such as glucose, with simultaneous repression of genes required for the respiratory metabolism. *S. cerevisiae* is a facultative anaerobe and in the presence of glucose does not perform oxidative phosphorylation. When glucose is exhausted, the repressed genes cease to be, and the cells adjust to the respiratory metabolism. After this phase, cell division is resumed at a reduced rate, via the respiratory metabolism using the final fermentation products like ethanol, acetate, glycerol, and other sources of carbon and energy, thereby performing the aerobic energy metabolism. When these carbon sources are exhausted, the cells stop dividing, thus entering the stationary phase (Winde *et al.*, 1997).

Considering the relative few studies concerning the effects of propolis on yeast DNA, it seemed relevant to evaluate the genotoxic/ antigenotoxic effect of G.EEs in the present work. DNA damages caused by G.EEs alone or in co-incubation conditions with 5 mM H_2O_2 were investigated in *S. cerevisiae* cells by the comet assay. The antimicrobial activity is one of the most important biological activities found on propolis samples and to evaluate the antimicrobial potential of propolis from Gerês, the antimicrobial activity of G.EEs was determined against a panel of bacteria and yeast strains, being expressed by the minimum inhibitory concentration (MIC) of propolis for which no microbial growth was detected. With microbial antibiotic resistance becoming a significant world health problem in recent years, strategies to overcome microbial resistance as well as new antimicrobial drugs are urgently needed. Being the synergistic effect of propolis with several antibiotics already reported, a possible synergistic effect of G.EEs and the antibiotic gentamicin was also studied in this work, being evaluated against the bacteria *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA).

The antioxidant activity is another important activity found in propolis, and naturally it was important to evaluate if propolis samples from Gerês also have potential to be used as an antioxidants. I*n vitro* antioxidant assays were carried out to measure the G.EEs ability to: scavenge DPPH free radical (by the DPPH assay); to quench the superoxide anions ($O_2^{\cdot}$) (by the

superoxide anion scavenging activity) and chelate Iron (Fe^{2+}) (by iron chelation activity). The intracellular oxidation and the antioxidant activity were also analysed *in vivo* by flow cytometry with the intracellular redox-sensitive fluorochrome dichlorofluorescein diacetate in yeast cells incubated with G.EE alone and in co- and pre-incubation conditions with 5 mM H_2O_2.

Propolis influence on the inner mitochondrial membrane potential was also investigated as such effect was described for other propolis samples. The influence of G.EE on the inner mitochondrial membrane potential was evaluated by flow cytometry and using rhodamine 123 as probe, which stains the mitochondria directly and distributes electrophoretically into the mitochondrial matrix in response to the mitochondrial electrical potential, making possible to detect alterations in mitochondrial distribution (Ludovico *et al.*, 2001). In parallel, in order to overcome the problem of propolis standardization and given the need to chemically characterize and compare propolis samples from Gerês, G1.EE was analyzed by LC-MS to characterize its profile in phenolic compounds.

2. MATERIALS AND METHODS

2.1. Propolis samples and preparation of propolis extracts

The propolis samples used in this work are from Gerês (G) and were kindly provided by the beekeeper Amadeu Fortunas whose apiary is located near the Cávado River, between the villages of Paradela and Sirvozelo, in Montalegre, Gerês, Portugal (41^{0}45'41.62'' N; 7^{0}58'03.34'' W). Four samples of propolis were collected in different years - 2011 (G11), 2012 (G12), 2013 (G13) and 2014 (G14) – and were used to prepare ethanol extracts (EE).

For alcoholic extraction, approximately 15 g of propolis were incubated with 80 mL of absolute ethanol in an orbital shaker at 25 ^{0}C, 100 revolutions per minute (rpm), for 24 h in the dark. The resulting solution was filtered with Macherey-Nagel filter papers, using a Buchner funnel and a Kitasato system attached to a vacuum pump. The residues were collected and extracted again, with 50 mL of absolute ethanol. The resulting filtrates were polled and dried in a Büchi Rotavapor RE 121 with a water bath (Büchi 461), at 40 ^{0}C, 40 rpm, yielding the ethanol extracts of propolis – G11.EE (Carvalho, 2012; Pereira, 2013), G12.EE (Pereira, 2013), G13.EE (Araújo, 2014) and G14.EE (this work) - which were stored at 4 ^{0}C, in the dark, until further use. The stock solutions prepared for the following described assays were performed by diluting the propolis extracts in the same solvent used for the extraction.

2.2. Chemical analysis of G.EE

Propolis chemical analysis was performed by Paulo Antunes at Centro de Apoio Tecnológico Agro-Alimentar (CATAA), Castelo Branco. Briefly, samples were homogenized and diluted with 80 % ethanol, at 70 ^{0}C for 1 h. The resulting mixture was filtered directly to a vial, through a nylon 0.22 µm filter. Standards for gallic acid, siriginc acid, ferulic acid, *p*-coumaric acid, apigenin and kaempferol were acquired from Sigma-Aldrich Co. Luteolin and gentisic acid standards were acquired from Extrasynthese, France. The chromatographic system consists of an Agilent 1200 series equipped with a model of a triple quadrupole mass spectrometer Agilent 6400. A Sorbax SB-C18 (50 mm x 4.6 mm i.d. x 1.8 µm particle diameter – Agilent technologies) column was used for the separation of the components of a flow rate of 0.7 mL/ min, at 30 ^{0}C. Elution was performed using a gradient of 0.1 % formic acid (eluent A) and acetonitrile (eluent B). The gradient was as follows: started at 10 % of B, then 20 % of B in 10

min, 40 % of B in 40 min, 60 % of B in 60 min, 90 % of B in 80 min and at 81 min return to the initial conditions, stabilizing for 9 min. ESI operated with a nitrogen flow of 10 L/ min, at 300 ^{0}C. MS detector operated in MS2-Scan, scan type in the range 80-1000 Da, and negative mode was selected. The capillary voltage was set to 4.0 kV, the quadrupole temperatures were 100 ^{0}C, the fragmentation energy was 145 Kjmol^{-1} and the cell accelerator voltage was 7 kV. Data were acquired and analysed using Masshunter Workstation Software (version B.04.00) from Agilent technologies.

For MS/ MS confirmation, the same equipment and chromatographic conditions were used. MS detector operated in Product Ion scan type, selecting the precursor ions and performing a scan of the fragments in the range 80-500 Da, and the negative mode was selected. The capillary voltage was set to 4.0 kV, the quadrupole temperatures were 100 ^{0}C, the fragmentation energy was 135 Kjmol^{-1}, cell accelerator voltage was 7 kV and the collision energy was 15 eV. Compounds were identified (Antunes, P., personal communication) based in standards retention times and by comparison of the ESI-MS/MS with the data from MS/MS published in the literature, such as in Falcão *et al*. (2010).

2.3. Yeast strains, media and growth conditions

In all experiments the haploid yeast strain *S. cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used. Yeast cell cultures were prepared on liquid YPD medium (1 % (w/ v) yeast extract DB Bacto™, 2 % (w/ v) peptone DB Bacto™, 2 % (w/ v) glucose) or YPE (the same as liquid YPD with 2 % (v/ v) of ethanol instead of 2 % glucose) depending on the experiments, while bacterial cultures were grown on liquid LB medium (Luria-Bertani – 0.5 % (w/v) yeast extract DB Bacto™, 1 % (w/ v) tryptone Bacto™, 1 % (w/ v) NaCl). For solid media (YPDA and LBA) 2 % agar (w/ v) was added to the same recipes. For liquid cultures a ratio flask/medium volume of 5/1 was used and incubation was performed at 30 ^{0}C, for yeasts, or at 37 ^{0}C, for bacteria, both at 200 rpm. The cultures growth was monitored by optical density at 600 nm (OD_{600}).

To prepare the cells to the experiments an overnight microbial culture was diluted in fresh medium to an OD_{600} = 0.1 (10 mL final volume) and incubated at 30 ^{0}C for 4 h, for yeasts, or at 37 ^{0}C for 2 h, for bacteria, and 200 rpm until OD_{600} reached 0.4-0.8 (at least two generation times, exponential phase).

2.4. Genotoxic and antigenotoxic properties of propolis

A volume of 1 mL of a yeast culture obtained as described in the above section was harvested by centrifugation at 14500 rpm, 4 ^{0}C for 2 min and washed twice with the same volume of deionized water at 4 ^{0}C. The resulting pellet was suspended in S buffer (sorbitol 1 M, KH_2PO_4 25 mM, pH 6.5) with 6600 U lyticase (66 $U\mu l^{-1}$) and 50 mM β-mercaptoethanol, being incubated at 30 ^{0}C, 200 rpm for 30 min in order to obtain spheroplasts. The spheroplasts were collected by centrifugation at 14500 rpm, 4 ^{0}C for 2 min, washed twice with the same volume of S buffer and resuspended in 1 ml S buffer. The suspension was divided by 100 µL aliquots and centrifuged under the same conditions. Supernatants were discarded and spheroplasts were treated with 5, 10, 25, 50 or 100 μgmL^{-1} of G12.EE to evaluate the genotoxic effects of propolis. For negative and positive controls similar cell suspensions were treated with ethanol or ethanol and H_2O_2 (10 mM), respectively. All aliquots were incubated at 30 ^{0}C, 200 rpm, during 20 min. After incubation, spheroplasts were collected from each sample by centrifugation, at 14500 rpm for 2 min, washed with S buffer and each pellet was resuspended in 40 µL of low melting agarose (LMA) 1.5 % (w/ v in S buffer) at 35 ^{0}C. The mixture was spread onto glass slides pre-coated with normal melting agarose (NMA) 0.5% (w/ v) and covered with cover slides. Glass slides were placed on ice for 5 min to solidify the agarose. The cover slips were removed and the glass slides were submerged in ice-cold lysing buffer (30 mM NaOH, 1 M NaCl, 50 mM EDTA, 10 mM Tris-HCl, 0.05 % (w/ v) laurylsarcosine, pH 10) for 20 min, followed by 20 min immersion in ice-cold electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10). The slides were then placed in the electrophoresis tank filled with electrophoresis buffer and an electric field of 0.7 V/ cm was applied for 10 min. Samples were fixed with 76 % (v/ v) ice-cold ethanol and subsequently with 96 % (v/ v) ice-cold ethanol, both for 10 min. The gels were dried at room temperature and stored at 4 ^{0}C until observation. Slides were analysed by fluorescence microscopy (Leica DM5000B+CTR5000+ebq100) after staining with 10 µL of GelRed™ (3.300x diluted; Biotium), with 400x magnification. Comets´ tail length was measured by informatic analysis of the images.

To assess the antigenotoxicity of propolis, the procedure was the described above with the exception of the incubation step, being the cells incubated with each of the four G.EEs simultaneously with H_2O_2 (10 mM).

2.5. Assessment of propolis cytotoxicity

To assess propolis cytotoxicity a yeast culture (see 2.3.) was divided into 1 mL aliquots and treated with 25, 50, 100, 200, 500 or 750 μgmL^{-1} of each of the four G.EEs. The same volume of ethanol was added to a similar cell suspension for the negative control. Immediately after G.EE or ethanol addition, 100 µL of each suspension were removed and serially diluted to 10^{-4}. This procedure was repeated after 30, 60 and 90 min incubation at 30 ^{0}C, 200 rpm. After dilutions, drops of 5 µL of each sample were transferred to YPDA plates and then incubated at 30 ^{0}C for 48 hours.

2.5.1. Evaluation of propolis protective effects against oxidative stress

A possible protective effect of propolis against oxidative stress was evaluated in similar viability assays but in the presence of hydrogen peroxide. In co-incubation conditions, the procedure was the described above with the exception of a simultaneous incubation with 5 mM H_2O_2 and a particular concentration of each G.EE. In pre-incubation conditions, the procedure was also basically the same but a previous incubation with G.EE for 20 min was followed by incubation with 5 mM H_2O_2, both at 30 ^{0}C and 200 rpm. A positive control for both co- and pre-incubation experiments was prepared adding 5 mM H_2O_2 to a similar cell suspension, which followed the experimental procedure herein described.

2.6. Evaluation of the antimicrobial properties of propolis from Gerês

To evaluate the antimicrobial properties of propolis from Gerês, the MIC (minimum inhibitory concentration) values of all the G.EEs studied in the present work were determined against two yeast strains and six bacterial strains, one gram-negative and five gram-positive (Table 1) using an adaptation of the agar dilution method (Sforcin *et al.*, 2000). The cultures of yeasts and bacteria were grown on YPD and LB medium, respectively (see 2.3.). Overnight cultures were diluted with fresh medium to OD_{600} = 0.1 and incubated until OD_{600} = 0.4-0.8, to reach the exponential phase. Thereafter, 100 µL of each suspension were removed and serially

diluted to 10^{-4}. After dilutions, drops of 5 µL of each sample were transferred to YPDA or LBA plates containing different concentrations of G.EE varying from 10 to 2000 µgmL^{-1}, or only absolute ethanol (same volume as the extract), used as a control. Plates were incubated at 30 ^{0}C for 48 h, for yeasts, or at 37 ^{0}C for 24 h, for bacteria. The MIC values against each microorganism were obtained by observation of the presence/absence of growth.

Table 1 - Strains used in this work as indicator strains in the antimicrobial assay

Strains	Strain reference
Yeasts	
Saccharomyces cerevisiae	BY4741/Y00000
Candida albicans	53B
Gram-negative bacteria	
Escherichia coli	CECT 423
Gram-positive bacteria	
Bacillus subtilis	48886
Bacillus cereus	ATCC 7064
Bacillus megaterium	932
Staphylococcus aureus	ATCC 6538
Staphylococcus aureus	M746665/Methicillin Resistant-MRSA

2.6.1. Evaluation of the synergistic effect between G.EE and an antimicrobial drug

A possible synergistic effect between propolis and the antibiotic gentamicin was evaluated using the bacteria for which MIC values were calculated (Table 3), and taking in consideration the MIC values of gentamicin also obtained in this work against the same strains, and determined by a procedure similar to the one described in 2.6.. Briefly, a bacterial overnight culture was refreshed in new medium, incubated until OD_{600} = 0.4-0.8, serially diluted to 10^{-4} and used to inoculate LBA plates containing different concentrations of gentamicin (varying from 0.01 to 1.5 µgmL^{-1}). After incubation at 37 ^{0}C for 24 h, the MIC values against each microorganism were determined.

To evaluate a possible synergistic effect, microbial cultures were grown on LB medium (see 2.3.), and 100 µL of each suspension were removed and serially diluted to 10^{-4}. Drops of 5

µL of each sample were transferred to LB plates containing mixtures of different sub-MIC concentrations of all of the four G.EEs and gentamicin, or only supplemented with absolute ethanol (same volume as the extract; control). After incubation at 37 ^{0}C for 24 h, MIC values against each tested microorganism were determined upon observation of the presence/absence of growth.

2.7. *In vitro* evaluation of the antioxidant activity of G.EEs

2.7.1. DPPH scavenging activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge giving it a deep violet colour. This compound shows strong absorption at 517 nm which disappears due to its radical scavenging capability that can be followed spectrophotometrically by an absorbance loss when the yellow non radical form (DPPH-H) is produced during the reaction of a hydrogen-donating antioxidant (Mitra and Uddin, 2014).

G.EEs were dissolved in absolute ethanol to obtain concentrations ranging from 1 to 50 µgmL^{-1}. Then, 1 mL of dissolved extract was mixed with 2 mL of DPPH (0.04 %) and incubated at room temperature in the dark for 20 min. The absorbance of the reaction was measured at 517 nm and ethanol was used as a blank. The control was prepared with DPPH and ethanol in place of sample extract. The scavenging activity of G.EEs was calculated and compared to the antioxidant standard gallic acid (0.2-1.5 µgmL^{-1}). Results were expressed as a percentage decrease with respect to control values according to the following equation:

$$(\%)Reduction = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

A_{sample} is the absorbance of the sample extract and $A_{control}$ is the absorbance of control with DPPH and ethanol, after 20 min of reaction. IC50 (µgmL^{-1}) was defined as the concentration of an antioxidant extract, in this case, which was required to scavenge 50 % of the initial DPPH· under the given experimental conditions, and it was obtained by interpolation from linear regression analysis. The total antioxidant capacity of the G.EEs was also calculated and expressed as gallic acid equivalents.

2.7.2. Superoxide anion scavenging activity

To assess the G.EEs scavenging capacity of the radical $O_2^{\bullet-}$, a non-enzymatic system was used through the reaction of phenazine methosulfate (PMS) with NADH (nicotinamide adenine dinucleotide, reduced form) and molecular oxygen, resulting in the production of $O_2^{\bullet-}$. This radical is able to reduce the nitroblue tetrazolium (NBT) to formazan, a blue compound that shows strong absorption at 562 nm but disappears due to the radical scavenging capability, which can be followed spectrophotometrically by an absorbance loss during the reaction with an antioxidant, such as propolis, capable of quenching the superoxide anion, inhibiting the production of formazan and consequently decreasing colour intensity (Zhang *et al.*, 2014).

Propolis extracts were dissolved in phosphate buffer (19 mM KH_2PO_4, pH 7.4) to obtain concentrations ranging from 50 to 1000 μgmL^{-1}. Then, 50 µL of dissolved propolis was mixed with 50 µL of NADH (166 µM), 150 µL of NBT (43 µM) and 50 µL of PMS (2.7 µM) and incubated, at room temperature, for 5 min. The absorbance of the reaction was measured at the wavelength of 562 nm, with SpectraMaxPlus micro plate reader. The control was prepared with a similar mixture where phosphate buffer replaced propolis extract. The blank was prepared as described, but with phosphate buffer instead of PMS. The scavenging activity was calculated and results were expressed as a percentage decrease with respect to control values according to the following equation:

$$(\%)Reduction = \frac{[A_{control} - (A_{sample} - A_{blank})]}{A_{control}} \times 100$$

A_{sample} is the absorbance of the sample extract, $A_{control}$ the absorbance of control and A_{blank} the absorbance of the blank, after 5 min of reaction. IC50 (μgmL^{-1}) values, corresponding to the concentration of each G.EE required to quench 50 % of the initial $O_2^{\bullet-}$ under the experimental conditions, were obtained by interpolation from linear regression analysis.

2.7.3. Iron chelating activity

The iron chelating activity assesses the ability of antioxidants to chelate Fe^{2+} (iron (II) sulphate). In this assay, ferrozine (3-(2-pyridyl)-5,6.diphenyl-1,2,4-triazine-4',4''-disulphonic acid sodium salt) was used to produce a red chromophore complex, with a strong absorption at 515 nm which disappears due to the chelating capability that can be followed spectrophotometrically

by an absorbance loss during the reaction with a chelating agent, such as propolis, with the ability to chelate Fe^{2+}, leading to a decrease of colour intensity (Geckil *et al.*, 2005; Oktyabrsky *et al.*, 2009).

Propolis extracts, from an ethanolic stock solution, were dissolved in phosphate buffer (19 mM KH_2PO_4, pH 7.4) to obtain concentrations ranging from 50 to 1000 μgmL^{-1}. Then, 50 µL of dissolved propolis was mixed with 50 µL of $FeSO_4$ (0.06 mM in ultrapure water) and 50 µL of ferrozine (0.3 mM in ultrapure water) and incubated for 10 min, in the dark, at room temperature. The absorbance of the reaction was measured at 562 nm, with SpectraMaxPlus micro plate reader. Control was prepared with ultrapure water instead of propolis extract. The blank was also prepared with ultrapure water instead of ferrozine. The scavenging activity was calculated and results were expressed as a percentage decrease with respect to control values according to the following equation:

$$(\%)Reduction = \frac{[A_{control} - (A_{sample} - A_{blank})]}{A_{control}} \times 100$$

A_{sample} is the absorbance of the sample extract, $A_{control}$ the absorbance of control and A_{blank} the absorbance of the blank, after 20 min of reaction. IC50 (μgmL^{-1}), here defined as the concentration of extract required to chelate 50 % of the initial Fe^{2+} under the experimental conditions, was obtained by interpolation from linear regression analysis.

2.8. Evaluation of the antioxidant activity of G.EE by flow cytometry

Flow cytometry experiments were carried out using H_2DCFDA as redox-sensitive fluorescent probe. This substance is able to diffuse freely through the plasma membrane into the cells where esterases promote its deacetylation to H_2DCF, which is impermeable to membranes, staying inside the cells. In the presence of oxidants, H_2DCF oxidizes and forms DCF, which is fluorescent and detectable by flow cytometry (Sá *et al.*, 2013).

A yeast cell culture (see 2.3.) was distributed in 500 µL aliquots, centrifuged at 14.500 rpm, 4 ^{0}C, for 2 min and washed with the same volume of PBS (137 mM NaCl, 27 mM KCl, 4.3 mM Na_2HPO_4, 1.47 Mm KH_2PO_4, pH 7.4). Three aliquots were treated with 50, 100 or 200 μgmL^{-1} of each G.EE and further incubated at 30 ^{0}C, 200 rpm for 1 h in the dark. Ethanol (same volume as the extract) was added to a similar cell suspension for a negative control. An aliquot

was removed for auto fluorescence measurement and kept on ice until cytometry analysis. Dichlorofluorescein diacetate (50 µM final concentration) was added to each cell suspension for 1 h further incubation at 30 ^{0}C, 200 rpm in the dark. Around 20000 cells were analysed by flow cytometry in an Epics XL™ cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. Data were analysed and histograms were built with the Flowing Software.

This experimental protocol was performed in the presence of H_2O_2, both in co-and pre-incubation conditions. In co-incubation conditions an incubation of each G.EE at particular concentration was performed simultaneous with 5 mM H_2O_2. In pre-incubation conditions, the exception to the procedure above described was a previous incubation with each G.EE, followed by incubation with 5 mM H_2O_2. A similar cell suspension was only treated with hydrogen peroxide to be used as positive control.

2.9. Evaluation of the G.EEs influence on inner mitochondrial membrane potential by flow cytometry

Flow cytometry assays were performed to investigate possible G.EE effects on yeast cells inner mitochondrial membrane potential. The protocol followed for this assay was adapted from Ludovico *et al.* (2001) and the concentration of rhodamine 123 used (50 nM) was much lower than that described in other studies (Johnson *et al.*, 1980; Juan *et al.*, 1994). This fluorochrome stains mitochondria directly and distributes into the mitochondrial matrix in response to the membrane potential, which allows detection of changes therein (Ludovico *et al.*, 2001).

A *S. cerevisiae* cell suspension prepared from a culture in YPE (see 2.3.) was diluted to OD_{600}= 0.02 in sterilized deionized water and distributed into 500 µL aliquots, one of them corresponding to the autofluorescence control, that was kept on ice after addition of fluorochrome and until cytometry analysis. Cell suspensions were washed twice with the same volume of sterilized deionized water and then treated with different G.EEs for final concentrations of 50, 100 or 200 µgmL^{-1} and further incubated at 30 ^{0}C, 200 rpm for 1 h. Ethanol was also added to a similar cell suspension to be used as negative control. Rhodamine 123 (50 nM final concentration) was added to each aliquot followed by an incubation at 30 ^{0}C, 200 rpm for 20

min, in the dark. A volume of 20 µL of each aliquot was removed for observation by fluorescence microscopy (Leica DMB 5000). Around 20000 cells were analysed by flow cytometry in an Epics XL™ cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. Data were analysed and histograms were built with the Flowing Software.

2.10.Statistical analysis

Unless otherwise stated, the experiments were done at least in triplicate and results are presented either as one representative experiment or as a mean of a certain parameter from 3 experiments±standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison of more than two means and Turkey's test to multiple comparisons. Asterisks indicate differences considered statistically significant: * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$, when compared to the respective controls.

3. RESULTS AND DISCUSSION

3.1. Effects of propolis on *S. cerevisiae* DNA

To evaluate possible propolis genotoxic effects in *S. cerevisiae* different concentrations (2.5, 10, 25, 50, 100 or 200 μgmL^{-1}) of an ethanol extract of propolis from Gerês - G12.EE – were used to treat yeast cells and DNA damage was assessed by the comet assay (Oliveira and Johansson, 2012). Two controls - one with ethanol (C-), representative of the extract solvent and the cells used in the experiment, and another with 10 mM H_2O_2 (C+) - were also included to assess the damage caused by H_2O_2 alone. In Figure 3 the difference between the DNA damage found in the two typical controls can be observed in the tail length of the comets without DNA damage (C-, Figure 3A) and of the comets resulting from DNA damage caused by 10 mM H_2O_2 (C+, Figure 3B).

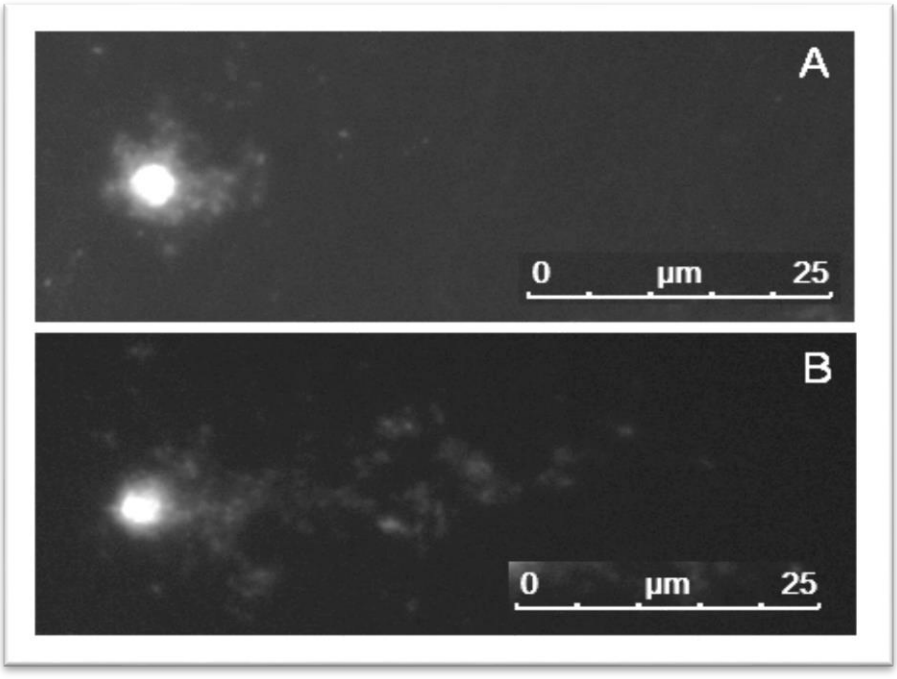

Figure 3 - Examples of comets observed with a fluorescence microscope after DNA labeling with GelRed. (A) *Saccharomyces cerevisiae* cells untreated (C-) or (B) treated with 10 mM H_2O_2 (C+).

As can be seen in Figure 4, cells treated only with ethanol (C-) have a low level of DNA damage, while cells treated with 10 mM H_2O_2 show a significant increase in the tail length, resulting in the highest value of DNA damage. It can also be seen that increasing concentrations of G12.EE caused an increase in the tail length; however this increase was not statistically significant, suggesting that G12.EE has no genotoxic effects.

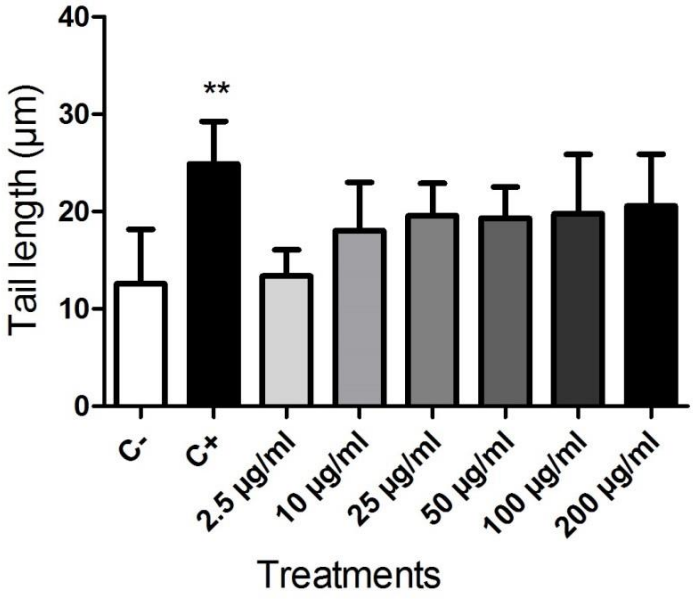

Figure 4 - Assessment of propolis genotoxic effects by the comet assay. Incubation of *S. cerevisiae* with 5, 10, 25, 50, 100 or 200 µgmL^{-1} G12.EE. (C-) cells treated with ethanol in the same volume as the extracts. (C+) cells treated with 5 mM H_2O_2. Mean±SD values of comet tail length are from three independent experiments (* means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$).

3.1.1. G.EEs do not protect yeast cells from DNA damage caused by H_2O_2

Given the previous results for G12.EE (Figure 4) and the evidence found by Russo *et al.* (2006) that reported a propolis ability to decrease DNA damages induced by H_2O_2, it seemed relevant to test if propolis from Gerês protects yeast cells against similar DNA damage caused by oxidative stress. For that cells were treated, in co-incubation conditions, with different concentrations of G.EEs (2.5, 10, 25, 50, 100 or 200 µgmL^{-1}) and 10 mM of H_2O_2, simultaneously. As propolis diversity is well known, both in chemical profiles and bioactivities spectra, which depend on the source plant species, the climate and the time of harvest among other factors, all the four G.EEs were tested for this property. Data were converted in percentage of DNA damage, considering 100 % the damage caused by H_2O_2 (C+). All the other treatments were compared to this positive control. Besides a statistically significant difference between the positive and negative control, a significant difference was also found for G14.EE, at the lowest concentration tested, suggesting some protective effect (Figure 5). However this effect is lost with increasing propolis concentrations. For all the other remaining G.EEs, no statistically significant

differences were found for propolis-treated cells when compared with cells treated only with H_2O_2 suggesting that this propolis from Gerês has no significant antigenotoxic effects.

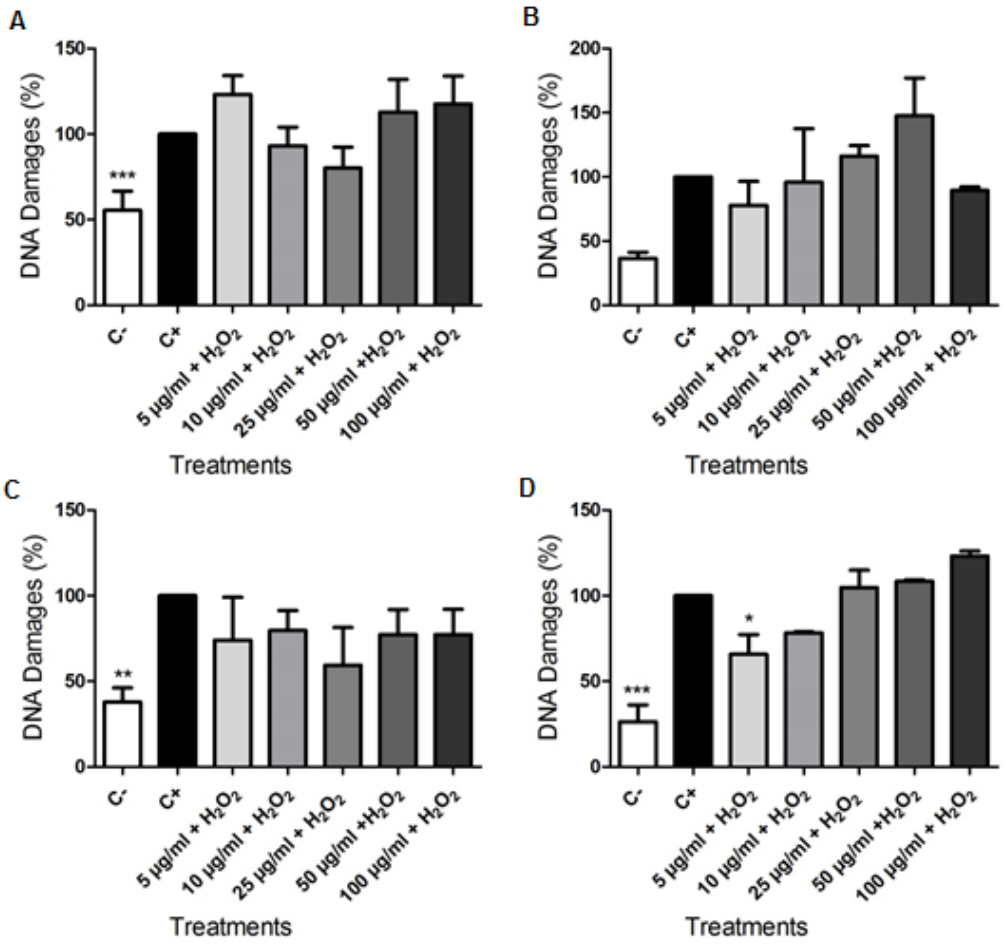

Figure 5 - Comet assay to evaluate propolis protective effects on DNA damage induced by oxidative stress. Co-incubation of *S. cerevisiae* with 5, 10, 25, 50 or 100 µgmL^{-1} of G11.EE (A), G12.EE (B), G13.EE (C) and G14.EE (D). Propolis from Gerês does not seem to protect cells from DNA damage under oxidative stress caused by H_2O_2 (10 mM). (C-) cells treated with ethanol, the solvent used for extraction. (C+) cells treated with H_2O_2. Mean±SD values are from three independent experiments (* means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$).

These results are not in agreement with those found by Cruz (2011) for a propolis sample of Portuguese origin (Côa; C.EE) which displayed a genotoxic effect on yeast cells at concentrations of 25, 100 and 300 µgmL^{-1}. In parallel, a protective effect was also evidenced by a decrease of the comet tail length observed in yeast spheroplasts co-treated with C.EE and H_2O_2. This dual pattern of activity was previously described by Tavares *et al.* (2006) who showed a genotoxic activity of propolis at higher concentrations whereas the same extract displayed a chemopreventive effect at lower concentrations.

3.2. Effects of G.EE on cell viability

Propolis is a recognized complex mixture of pharmacological interest (Silva-Carvalho *et al.*, 2015) but it is important to investigate its toxicity before proposing any application for this natural product. Loss of viability was studied in *S. cerevisiae* and detected by reduction of the number of colonies on YPDA plates after propolis treatment during 0, 30, 60 and 90 minutes. A control tube, containing cells and ethanol was included to determine the viability of untreated cells. As can be seen in Figure 6, a nearly constant viability was observed during the 90 min of incubation, either if the cells were treated with G.EE (and with any G.EE) in concentrations ranging from 100 to 750 μgmL^{-1} (Figure 6.I, B-E, 6.II, B-E, 6.III, B-E and 6.IV, B-E) or if they were in the presence of ethanol (Figure 6.I, A, 6.II, A, 6.III, A and 6.IV, A). From the above results, it can be concluded that propolis from Gerês does not display cytotoxic effects against the eukaryotic unicellular microbe *S. cerevisiae*.

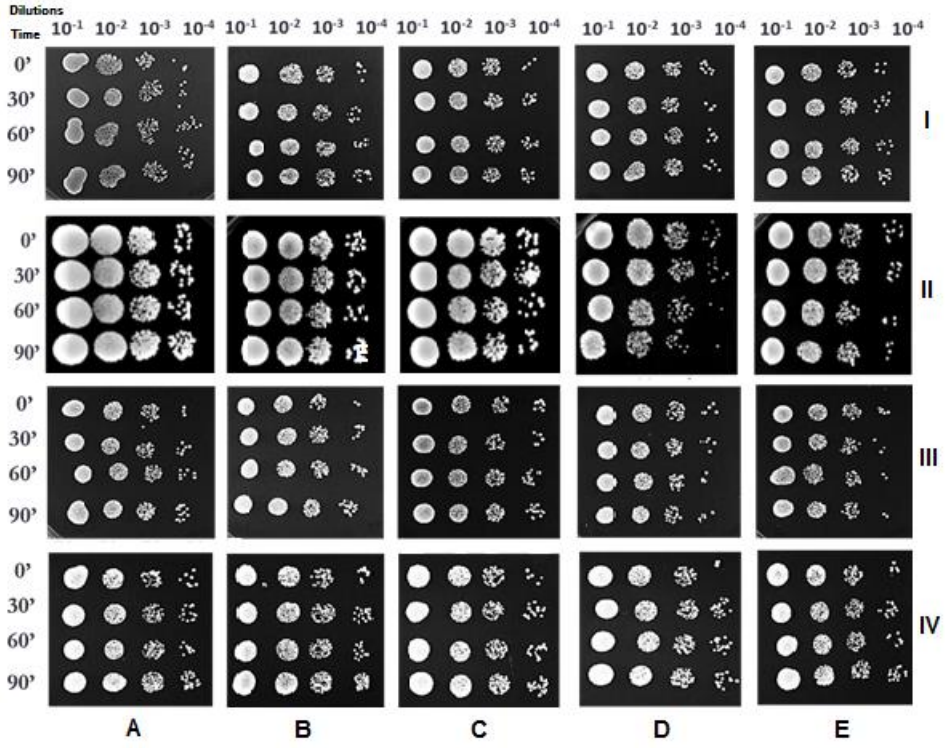

Figure 6 - Viability of *S. cerevisiae* cells after 30, 60 and 90 min of incubation with (A) ethanol alone (control) or 100 μgmL^{-1} (B); 200 μgmL^{-1} (C); 500 μgmL^{-1} (D) and 750 μgmL^{-1} (E) of G11.EE (I), G12.EE (II), G13.EE (III) and G14.EE (IV).. Data are from a representative experiment from three independent experiments.

Viability of the yeast *S. cerevisiae* was also not affected in cells exposed to Brazilian propolis in concentrations ranging from 25 to 100 μgmL^{-1} (Sá *et al.*, 2013). The effect of propolis

in *S. cerevisiae* cells was also assessed by Cigut *et al.* (2011) but no significant differences were found between cell growth of the control and of treated cells. However, experiments made by Bonvehí and Gutiérrez (2012) with P.EE from Spain showed inhibition of *S. cerevisiae* growth and a MIC value between 500 and 1500 μgmL^{-1} was found, contradicting the above results and the ones obtained in this work.

3.2.1. G.EE protects yeast cells under stress conditions caused by H_2O_2

As G.EEs did not display genotoxic effects and did not affect cell viability as well, it was investigated whether propolis extracts could have protective effects against oxidative stress caused by an oxidizing agent such H_2O_2, the most abundant ROS species *in vivo*, being continuously produced as a by-product of aerobic metabolism (Kakinuma *et al.*, 1979). Experiments were performed along time with 5 mM H_2O_2 in co- (Figure 7) and pre-incubation (Figure 8) conditions with all samples of G.EE.

When exposed to 5 mM H_2O_2, yeast cells had a significant reduction in viability after 20 min (Figures 7I.B, 7II.B, 7III.B and 7IV.B). In cells simultaneously incubated with 5 mM H_2O_2 and 25 to 750 μgmL^{-1} of G11.EE (Figure 7I.C-H), G12.EE (Figure 7II.C-H), G13.EE (Figure 7III.C-H) or G14.EE (Figure 7IV.C-H)), it can be seen that viability continued to decline dramatically except for concentrations above 25-50 μgmL^{-1} of G.EE. These results suggest that under conditions of co-incubation, all the G.EEs had some protective effect on yeast cells against oxidative stress caused by H_2O_2. This protective effect is more pronounced for G14.EE (7IV.C-H) while it seems weaker and similar to each other in the remaining three G.EEs.

In pre-incubation experiments, cells were first incubated with G.EE, ranging from 25 to 750 μgmL^{-1}, washed once with sterile deionized water and then exposed to 5 mM H_2O_2, being the viability measured over time. As depicted in Figure 8, there is a high protective effect of G.EE from all samples although this protection is more pronounced for the two highest concentrations, 500 and 750 μgmL^{-1} (Figure 8I to 8IV, panels G and H), where it can be observed a few colonies in more diluted suspensions, even after 60 min of incubation. Regarding the extracts, the protective effect is more pronounced for G14.EE (Figure 8IV.C-H) as it is possible to observe a few colonies even in the more diluted suspensions after 60 min of incubation. G13.EE (Figure 8III.C-H) is the extract displaying the least pronounced protective effect: a few colonies can only

be observed after 60 min of incubation and just in the first two dilutions of the intermediate concentrations tested (Figure 8III.E and F). The protective effect was similar for G11.EE (Figure 8I.C-H) and G12.EE (Figure 8II.C-H).

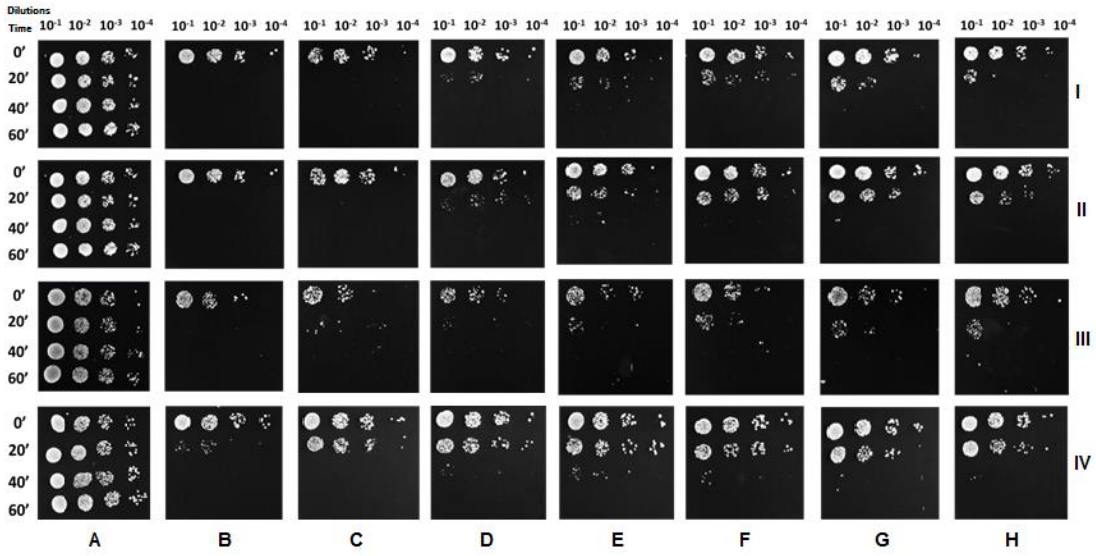

Figure 7 - Viability of *S. cerevisiae* cells after 20, 40 and 60 min of co-incubation with H_2O_2 (5 mM) and (C) 25 μgmL^{-1}; (D) 50 μgmL^{-1}; (E) 100 μgmL^{-1}; (F) 200 μgmL^{-1}; (G) 500 μgmL^{-1} or (H) 750 μgmL^{-1} of G11.EE (I), G12.EE (II), G13.EE (III) and G14.EE (IV). (A) Cells treated with ethanol alone; (B) cell treated with 5 mM H_2O_2 to assess the damage caused by the oxidizing agent alone. Data are from a representative experiment from three independent experiments.

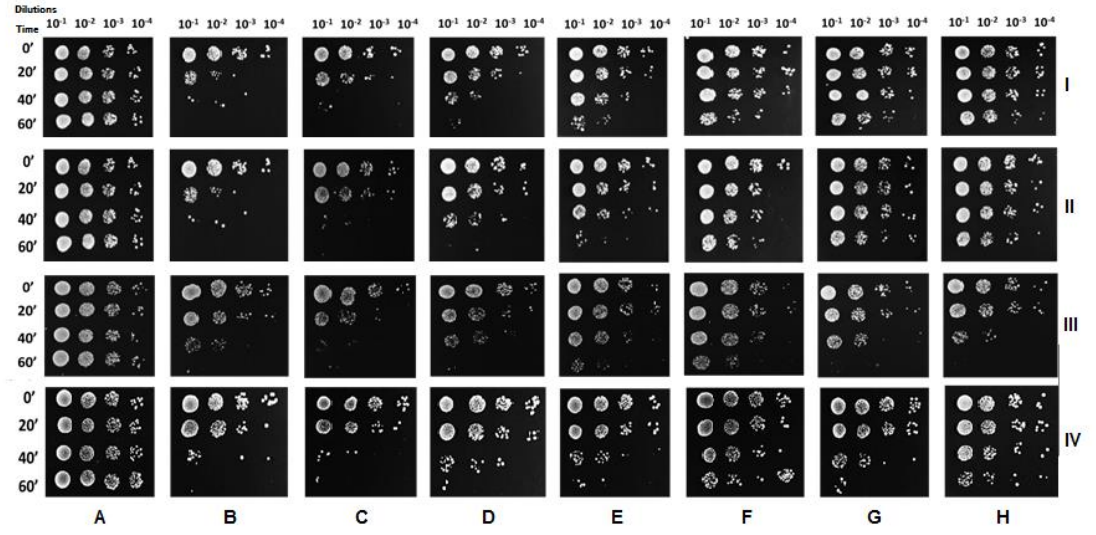

Figure 8 - Viability of *S. cerevisiae* cells previously incubated with (C) 25 μgmL^{-1}; (D) 50 μgmL^{-1}; (E) 100 μgmL^{-1}; (F) 200 μgmL^{-1}; (G) 500 μgmL^{-1} or (H) 750 μgmL^{-1} of G11.EE (I), G12.EE (II), G13.EE (III) or G14.EE (IV) for 20 min, washed with deionized water and suspended in YPD medium, and subsequently, incubated with H_2O_2 (5 mM), for 20, 40 and 60 min. (A) Cells treated with ethanol alone; (B) cell treated with 5 mM H_2O_2 to assess the damage caused by this oxidizing agent alone. Data are from a representative experiment from three independent experiments.

Brazilian propolis also increased the survival of *S. cerevisiae* cells, almost 3 times, after direct exposure to H_2O_2 (Sá *et al.*, 2013). Hydrogen peroxide frequently induces oxidative damages in biomolecules such as lipids, proteins and DNA (Nery *et al.*, 2008) but some propolis can reduce lipid and protein oxidation in cells pre-treated with this natural product and subsequently exposed to H_2O_2 (Sá *et al.*, 2013).

3.3. Antimicrobial activity of G.EE

Antimicrobial activity of the G.EEs was evaluated by the agar dilution method, MIC values being estimated against each of the test organisms, by the observation of the lowest concentration, which resulted in growth abolishment. All the G.EE exhibited the same MIC values for all the tested strains, so the results presented are representative of the four G.EEs studied in this work (Table 2).

Tested bacteria are more susceptible to G.EEs than the yeasts chosen as indicator strains for the assay and, among bacteria the gram-positive seem to be the more susceptible. The higher susceptibility of Gram-positive over Gram-negative bacteria is a common and almost generalized result reported in several works (Bankova *et al.*, 2000; Uzel *et al.*, 2005; Ramanauskiené *et al.*, 2013). The highest activity of propolis from Gerês was displayed against the Gram-positive bacteria of genus *Bacillus* (MIC values of 50 μgmL^{-1}) followed by *Staphylococcus aureus* (200 μgmL^{-1}). Similar MIC values (MIC>2000 μgmL^{-1}) were obtained against MRSA, the dreaded methicillin resistant *Staphylococcus aureus* strain, the Gram-negative *Escherichia coli* and the yeasts *S. cerevisiae* and *Candida albicans*. The higher antibacterial activity of G.EEs against *Bacillus* sp., in accordance with other works (Sorkun *et al.*, 1997; Ramanauskiené *et al.*, 2009) indicating that spore-forming bacteria like *Bacillus cereus* are more susceptible to propolis compared to bacteria that do not produce spores, can suggest a specific activity spectrum of G.EEs against Gram-positive spore-forming bacteria.

Propolis diversity can be easily detected analyzing a few works regarding the evaluation of this bioactivity. In fact, different propolis samples display different MIC values against the same microbial strains and diverge in terms of bioactivity spectra, both in terms of number and type of hit strains. For instances, Ramanauskiené *et al.* (2013) also reported that *Bacillus subtilis* and *Bacillus cereus* were the most susceptible microorganisms to an EE of propolis from Lithuania, although with a MIC value much more lower (0.06 μgmL^{-1}).

Table 2 - MIC values (μgmL^{-1}) of G.EEs against the panel of susceptibility indicator strains. Results are the same for G11.EE, G12.EE, G13.EE and G14.EE.

Strains	**G.EEs MIC (μgmL^{-1})**
Gram-positive bacteria	
Bacillus megaterium	50
Bacillus subtilis	50
Bacillus cereus	50
Staphylococcus aureus	200
Staphylococcus aureus (MRSA)	>2000
Gram-negative bacteria	
Escherichia coli	>2000
Yeasts	
S. cerevisiae	>2000
Candida albicans	>2000

Also unlike what was found for G.EEs, Ramanauskiené *et al*. (2013) reported that the same EE displayed antifungal activity and an equal MIC values against *Candida albicans*. However, another study made by Ramanauskiené *et al*. (2009) report a closer MIC to the value found in this work for *Bacillus subtilis* (MIC = 34±2.2 μgmL^{-1}). Other authors (Patel *et al*., 2014) studied a P.EE with a higher MIC value (500 μgmL^{-1}) against *Staphylococcus aureus* and a lower MIC value (400 μgmL^{-1}) against *Candida albicans*. Velikova *et al*. (2000) tested different P.EE from Bulgaria, Greece, Turkey and Algeria and showed a good antibacterial activity against *Staphylococcus aureus* but a week or lacking effect against *Escherichia coli*. Rahman *et al*. (2010) showed that a propolis sample inhibited *Staphylococcus aureus* and *Escherichia coli* growth with MIC values of 3500±960 and 5410±960 μgmL^{-1}, respectively. The antimicrobial activity of a Polish P.EE was evaluated against methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus* clinical isolates by Wojtyczka *et al*. (2013a), who showed the efficiency of propolis against several strains, Similar results were obtained by Boisard *et al*. (2015) with a French P.EE. However, none of the G.EEs studied in this work was active against MRSA at the tested concentrations. Also, in contrast with a study made by Bonvehí and Gutiérrez (2012) with a P.EE from Spain which inhibits *S. cerevisiae* growth with MIC values ranging from 500 to 1500 μgmL^{-1}, no G.EE inhibited the *S. cerevisiae* strain tested in the present work. Increasing concentrations of a Brazilian propolis did not affect the survival of *S. cerevisiae* too (Sá *et al*., 2013).

These differences between antibacterial activities may be due to the complex and variable propolis composition and consequently explained by the active compounds present in this mixture that can differ in quantity and quality (Santos *et al.*, 2002). It is also important to highlight that different technics are frequently used to determine MIC values and microbial susceptibility to P.EEs, being also tested different concentration ranges as well as strains, even of the same species, complicating comparison between the results of different works.

3.3.1. Synergistic effect between G.EE and gentamicin

Synergistic effects between propolis and antibiotics, which can allow reduction of the dose of therapeutic drugs, have been described against a large spectrum of both Gram-positive and Gram-negative bacteria (Scheller *et al.*, 1999; Stepanović *et al.*, 2003; Fernandes *et al.*, 2005; Davies and Davies, 2010; Noori *et al.*, 2012; Al-safi, 2014). In this work, the synergistic effect between G.EE and the broad spectrum antibiotic gentamicin, which inhibits bacterial protein synthesis by irreversible binding to the 30S subunit of the ribosome (Morgan, 2014), was evaluated. Susceptibility of microorganisms to G.EEs and to gentamicin was determined upon observation of the lowest concentration for which no growth was detected. Synergism assays were carried out on six bacteria, five of them gram-positive, using sub-MIC concentrations of both gentamicin (Table 3) and G.EE (Table 2). As shown in Table 3, *Bacillus megaterium* was the most susceptible strain to the antibiotic (MIC = 0.25 μgmL^{-1}) followed by *Bacillus subtilis* and *Bacillus cereus*, for which gentamicin has MIC values of 1.25 μgmL^{-1}. The less sensitive strains to the antibiotic were *Escherichia coli and Staphylococcus aureus* (MRSA): MIC value is higher than 1.5 μgmL^{-1}.

Table 3 - MIC values (μgmL^{-1}) of gentamicin against the panel of tested bacteria.

Strains	**Gentamicin MIC (μgmL^{-1})**
Gram-positive bacteria	
Bacillus megaterium	0.25
Bacillus subtilis	1.25
Bacillus cereus	1.25
Staphylococcus aureus	1.5
Staphylococcus aureus (MRSA)	>1.5
Gram-negative bacteria	
Escherichia coli	>1.5

A synergistic effect between all the G.EEs and gentamicin was detected for all the tested strains (Tables 4-7). The synergistic effect of G.EEs and gentamicin against *Bacillus megaterium* can be seen in the decrease of gentamicin MIC to less than half of its initial value (from 0.25 μgmL^{-1} to 0.01 μgmL^{-1} when combined with a sub-MIC concentration of G.EE) (Table 4).

Table 4 - *Bacillus megaterium* growth in the presence of sub-MIC concentrations of G.EEs and gentamicin. Results were the same for all the four studied G.EEs.

Gentamicin	**G.EEs (25 μgmL^{-1})**
0.01 μgmL^{-1}	**-**
0.05 μgmL^{-1}	**-**

(+) growth; (-) without growth

Results obtained for the combined antimicrobial effect of G.EEs and gentamicin against *Bacillus subtilis* and *Bacillus cereus* are described on Table 5. The MIC of gentamicin against both bacteria was 1.25 μgmL^{-1} but none of the bacterial species was viable on the plates when 25 times less antibiotic was used in combination with 25 μgmL^{-1} of G.EEs. Other study made with different propolis samples from various origins mixed with different antibiotics such as ampicillin, tetracycline, chloramphenicol, ciprofloxacin and erythromycin did not demonstrate significant synergistic effect against *Bacillus subtilis* (Oliveira, 2015), but the authors used different antibiotics and propolis samples.

Table 5 - *Bacillus subtilis* and *Bacillus cereus* growth in the presence of sub-MIC concentrations of G.EEs and gentamicin. Results were the same for all the four studied G.EEs.

Gentamicin	**G.EEs (25 μgmL^{-1})**
0.01 μgmL^{-1}	**+**
0.05 μgmL^{-1}	**-**

(+) growth; (-) without growth

In Table 6, it is possible to see the combined effect of G.EE and gentamicin against *Staphylococcus aureus*. The MIC of gentamicin for this strain was 1.5 μgmL^{-1} but when using only a third of this concentration in combination with 25 μgmL^{-1} of G.EE no growth was observable (Figure 9).

Synergistic effect of propolis and antimicrobial drugs against this strain was also found by Fernandes *et al*. (2005) especially when propolis was in combination with agents that interfere on bacterial protein synthesis, which is precisely the case of gentamicin, the antibiotic used in this work. Other study performed by Scazzocchio *et al*. (2006) showed this synergistic effect between a P.EE and gentamicin and the MIC of the gentamicin (MIC = 12.5 μgmL^{-1}) decreased to 0.1 μgmL^{-1}.

Table 6 - *Staphylococcus aureus* growth in the presence of sub-MIC concentrations of G.EEs and gentamicin. Results were the same for all the four studied G.EEs.

Gentamicin	**G.EEs (25 μgmL^{-1})**	**G.EEs (100 μgmL^{-1})**
0.01 μgmL^{-1}	**+**	**+**
0.05 μgmL^{-1}	**+**	n.t.
0.5 μgmL^{-1}	**-**	n.t.
0.75 μgmL^{-1}	**-**	n.t.
1.0 μgmL^{-1}	**-**	**-**

(+) growth; (-) without growth; (n.t.) not tested

The MIC of gentamicin for *Escherichia coli* and *Staphylococcus aureus* (MRSA) was greater than 1.5 μgmL^{-1} but when a third of this concentration was used in combination with 25 μgmL^{-1} of G.EE no bacterial growth was detectable (Table 7). However, in the presence of 1.5 μgmL^{-1} of gentamicin in combination with 2000 μgmL^{-1} of G.EE both bacteria grew. A synergistic effect against *Escherichia coli* was demonstrated when a propolis sample in combination with honey, both collected in Saudi Arabia, was used (AL-Waili *et al*., 2012).

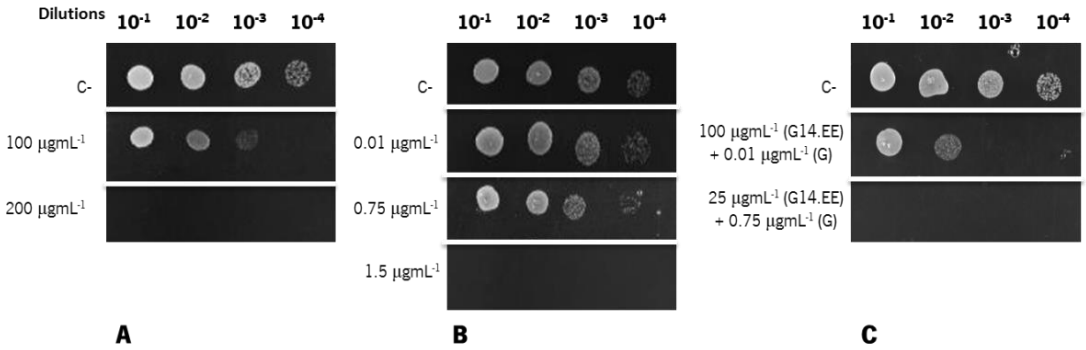

Figure 9 - Detail of the assays to detect synergism between propolis and gentamicin. Drops of a *Staphylococcus aureus* prepared as described in section 2.3 were placed on top of LBA plates supplemented with 100 μgmL^{-1} or 200 μgmL^{-1} G.EE (A), gentamicin (B) or G.EE and gentamicin (C) (100 μgmL^{-1} G.EE and 0.01 μgmL^{-1} gentamicin or 25 μgmL^{-1} G.EE and 0.75 μgmL^{-1}gentamicin). (C-) LBA plate supplemented with ethanol. Data are from a representative experiment from three independent experiments.

Table 7 - *Escherichia coli* and *Staphylococcus aureus* (MRSA) growth in the presence of sub-MIC concentrations of G.EEs and gentamicin.

Gentamicin	**G.EEs (25 µgmL^{-1})**	**G.EEs (100 µgmL^{-1})**	**G.EEs (2000 µgmL^{-1})**
0.01 µgmL^{-1}	**+**	**+**	n.t.
0.05 µgmL^{-1}	**+**	n.t.	n.t.
0.5 µgmL^{-1}	**-**	n.t.	n.t.
0.75 µgmL^{-1}	**-**	n.t.	n.t.
1.0 µgmL^{-1}	**-**	**-**	n.t.
1.5 µgmL^{-1}	n.t.	n.t.	**+**

(+) growth; (-) without growth; (n.t.) not tested

Propolis is a very complex mixture and as such it is expected that its biological compounds could exhibit either synergistic or antagonist effects. Sometimes maybe the additive effects prevail, other times the antagonistic compounds may overcome possible synergistic effects and interfere negatively with the antibiotic itself, depending on the proportions of each component and thus having different effects on cell viability. Even though, all these results (Tables 4-7) suggest that propolis from Gerês has a strong synergistic effect when used in combination with gentamicin, which is very interesting considering the worldwide problem of antibiotic resistance and the need to rethink antibiotic therapy. However it is important to understand not only the complexity of this mechanism but to test other antibiotics too, in order to find other possible synergistic effects.

3.4. G.EE has significant DPPH radical scavenging activity

Given the different types of ROS and the multiple ways ROS can act on living organisms, it is difficult to define a universal method by which the antioxidant activity could be measured accurately and quantitatively (Alves *et al.*, 2010). This question is even more problematic in the case of chemically complex and diverse natural products like propolis, for which no single method is considered the best or the most suitable. Thus, other commonly used methods to evaluate the antioxidant activity *in vitro* - DPPH assay, superoxide anion scavenging activity and iron chelating activity - were also used in the present work to evaluate G.EEs antioxidant potential.

DPPH is a free-radical compound and has been widely used to test free-radical-scavenging ability of antioxidant compounds. The DPPH assay, as the method is named, is based on the

reduction of DPPH in ethanol solution which in the presence of a hydrogen-donating antioxidant leads to the formation of the non-radical form DPPH-H (Mitra and Uddin, 2014).

Sheng *et al*. (2007) claim that P.EEs have potential natural antioxidant once exhibit DPPH free-radical-scavenging activity. G.EEs DPPH free radical scavenging activity was determined using gallic acid (GA) (IC50 = 0.803±0.02 μgmL^{-1} (Annex: Table 13) as standard and the respective IC50 values were calculated (Table 8).

Table 8 - Percentage of reduction of DPPH free radical by the four G.EEs tested.

Propolis ethanol extracts	**Concentration (μg/ml)**					**IC50 (μg/ml)**
	1	**5**	**10**	**25**	**50**	
G11.EE	3.64±2.05	16.70±1.79	32.44±2.90	68.18±2.26	94.23±0.34	17.77±0.78
G12.EE	3.48±1.97	19.17±2.18	40.01±3.12	84.38±2.53	94.43±0.20	14.41±0.56
G13.EE	1.18±0.92	9.24±2.37	20.08±2.89	49.94±3.87	84.79±2.16	25.24±2.45
G14.EE	2.45±2.26	13.59±3.14	26.55±3.65	77.89±1.45	90.49±3.69	16.47±0.75

All the G.EEs showed significant radical scavenging activity, and in a concentration-dependent manner. The highest antioxidant activity was found in G12.EE (IC50 = 14.41±0.56). G11.EE and G14.EE had similar radical scavenging activities whit IC50 values 17.77±0.78 and 16.47±0.75, respectively, while G13.EE possesses the lowest radical scavenging activity, at all concentrations (IC50 value of 25.24±2.45). At the highest concentration (50 μgmL^{-1}), however, this difference was less significant, as the extracts showed relatively similar radical scavenging activity values, with the exception of G13.EE for which a lower value was obtained. With these results, it is possible to establish an ordered sequence according to the G.EE antioxidant capacity evaluated by DPPH assay: G12.EE>G14.EE>G11.EE>G13.EE. The antioxidant capacity of G.EEs was compared with the antioxidant activity of the standard control, GA. For that, the total antioxidant activity of G.EE was also determined using the linear regression equation ($y = -0.3465x + 0.7702$, $R^2 = 0.9842$; where x is GA concentration in μgmL^{-1} and y is absorbance) of the calibration curve (Annex: Figure 16) and results were expressed as GA equivalents (Table 9).

As can be seen in Table 9, G12.EE is the extract with the highest activity in terms of GA equivalents (0.068±0.001 μgmL^{-1}), while G13.EE is the one with the lowest activity (0.035±0.003

μgmL^{-1}). This means that, for example and for G13.EE, 1 μgmL^{-1} has an antioxidant activity equivalent to 3.5 % of the activity observed for 1 μgmL^{-1} of GA.

Table 9 - Total antioxidant activity of G.EEs expressed as GA equivalents, in μgmL^{-1}.

Propolis ethanol extracts	GA equiv μgmL^{-1}	GA equiv %
G11.EE	0.060±0.008	6.0±0.8
G12.EE	0.068±0.001	6.8±0.1
G13.EE	0.035±0.003	3.5±0.03
G14.EE	0.050±0.008	5.0±0.8

Zhang *et al.* (2015) reported that a P.EE from China possesses a strong free radical scavenging activities, obtaining an IC50 value of 32.35±2.84, a propolis sample from Cameroon showed an IC50 value calculated (2800 μgmL^{-1}) (Talla *et al.*, 2014) and Miguel *et al.*, 2010 studied P.EEs samples from Algarve collected in winter and in spring obtaining IC50 values of 27±10 and 31±10 μgmL^{-1}, respectively. Although propolis can be very diverse in this bioactivity too, as the IC50 values for G.EEs were lower, a stronger DPPH free radical scavenging activity is displayed by propolis from Gerês making this propolis very attractive for some applications.

3.5. G.EE has superoxide anion scavenging activity

The superoxide anion ($O_2^{\cdot-}$) radical can be generated by different enzymatic systems: through the mitochondrial respiratory membrane, the xanthine oxidase and NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) oxidase in activated phagocytes. The production of $O_2^{\cdot-}$ is essential to the defense of living organisms and acts in the cell signaling between living cells. On the other hand, its overproduction causes oxidative stress and it is involved in various pathologies (Alves *et al.*, 2010). G.EEs display $O_2^{\cdot-}$ scavenging activity in a concentration dependent manner, and exhibit IC50 values ranging from 161.73±11.13 to 251.83±6.07 μgmL^{-1}. G12.EE displayed the highest antioxidant activity, while G13.EE displayed the lowest values, at all concentrations (Table 10). According to the G.EE antioxidant capacity on $O_2^{\cdot-}$ scavenging assay, the ethanol extracts of propolis from Gerês can be ranked by the following sequence G12.EE>G14.EE>G11.EE>G13.EE, which is exactly the same obtained in the DPPH assay.

Table 10 - Superoxide anion scavenging activity displayed by the four G.EEs studied in the present work.

Propolis ethanol extracts	Concentration(μgmL^{-1})				IC50 (μg/ml)
	100	**150**	**300**	**500**	
G11.EE	22.51±2.76	36.75±3.50	54.61±1.71	89.01±3.42	247.44±18.44
G12.EE	34.28±1.21	51.50±4.64	70.64±0.97	98.41±1.09	161.73±11.13
G13.EE	19.68±0.35	30.64±1.17	46.86±1.35	95.66±1.04	251.83±6.07
G14.EE	32.98±2.40	43.68±1.05	56.54±1.61	85.63±2.38	178.18±9.29

Nakajima *et al.* (2009) also demonstrated that P.EE exhibit strong antioxidant effects against $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$. And a study made by (Zhang *et al.*, 2015) showed the antioxidant effect of China propolis against $O_2^{\cdot-}$ by its radical scavenging activity, corroborating the results obtained in this work. Superoxide anion scavenging activity was evaluated for P.EEs of samples collected in Algarve and the IC50 value of 34±10 μgmL^{-1} was obtained for a sample collected in winter and a sample harvested in spring (Miguel *et al.*, 2010). In general, and taking into consideration the values of IC50 that have been reported for propolis samples, propolis from Gerês has less antioxidant capacity.

3.6. G.EE has iron chelating activity

Iron is an essential ion because it is required for oxygen transport, respiration and activity of many enzymes (Cheng *et al.*, 2013; Karamian and Ghasemlou, 2013; Choonpicharn *et al.*, 2014). However, iron is a highly reactive metal involved in the production of ROS, consequently causing changes in lipids, proteins and other cellular components (Cheng *et al.*, 2013). Chelating agents may have a stabilizing effect on transition metals, leading to the inhibition of generation of radicals and consequently reducing free radical damage. The main strategy to avoid ROS generation associated with redox active metal catalysis involves the chelation of metal ions by a chelate agent, such propolis. Iron chelating activity assays were carried out to study possible G.EEs effects on transition metal ions involved in ROS production. In the iron cheating assay, ferrozine can quantitatively form complexes with Fe^{2+} which can be disrupted in the presence of chelating agents, such as propolis (Geckil *et al.*, 2005). As can be seen in Table 11, G.EEs interfered with the formation of Fe^{2+} and ferrozine complex. G.EE in concentrations ranging from 100 to 500 μgmL^{-1} significantly chelate Fe^{2+}, observed by the decrease of the absorbance when compared to Fe^{2+} alone, suggesting that G.EEs capture Fe^{2+} before ferrozine. Taking into

consideration the IC50 values of the chelating effect of G.EE on the complex formation, it can be concluded that G12.EE displayed the highest iron chelation activity (IC50 = 118.87±6.90 μgmL^{-1}) while G13.EE displayed the lowest (IC50 = 158.14±7.78 μgmL^{-1}). An ordered sequence for G.EE antioxidant capacity on iron chelating assay would be G12.EE>G14.EE>G11.EE>G13.EE.

Table 11 - Iron chelating activity displayed by the four G.EEs studied.

Propolis ethanol extracts	**Concentration (μgmL^{-1})**				**IC50 (μg/ml)**
	100	**150**	**300**	**500**	
G11.EE	45.17±1.6	52.70±2.17	60.65±2.06	95.67±6.14	127.68±6.49
G12.EE	49.26±0.77	53.56±3.25	65.76±3.55	84.20±2.59	118.87±6.90
G13.EE	39.31±1.50	47.19±1.17	66.73±2.45	90.27±4.80	158.14±7.78
G14.EE	47.21±2.36	54.90±4.13	65.87±9.97	99.37±17.39	120.89±7.43

Geckil *et al*. (2005) argue that P.EEs have high metal chelating capacity and comparable antioxidant activity to the two most widely used synthetic antioxidants, BHA and BHT. The iron chelating activity of a P.EE from Cameroon was demonstrated by Talla *et al*. (2014). Metal chelating activity was evaluated for a P.EEs samples from Algarve collected in winter and another in spring and IC50 values were calculated, in percentage, as 39.9±0.8 and 49.9±0.8, respectively (Miguel *et al*., 2010). The quenching of the ferrozine-Fe^{2+} complex by propolis was evaluated by the iron chelating activity on Bornes and Fundão samples and IC50 values were calculated as being 10300 and 17800 μgmL^{-1}, respectively (Moreira *et al*., 2011). Thus, different Portuguese propolis samples display great diversity in what concerns this bioactivity, having propolis from Gerês lower antioxidant capacity than Portuguese samples collected in the south Miguel *et al*. (2010), but higher antioxidant capacity than the samples studied by Moreira *et al*. (2011).

3.7. G.EEs decreases intracellular oxidation

Taking in consideration the results obtained in viability assays, with cells under oxidative stress caused by H_2O_2, the question if and how G.EE influences the intracellular oxidation in the absence/ presence of H_2O_2 emerged. As expected, increasing concentrations of G.EEs decreased

cell fluorescence suggesting that all the G.EEs have antioxidant activity inside the cells (Figure 10).

These results are in line with those obtained by Cigut *et al.* (2011) which reported that Slovenian propolis was able to reduce the intracellular oxidation levels in wild type *S. cerevisiae* by 42 % when cells were exposed to 50 μgmL^{-1} of propolis, for 1 h. Zhang *et al.* (2015) demonstrated also that P.EE reduced the intracellular oxidation levels in RAW264.7 cells.

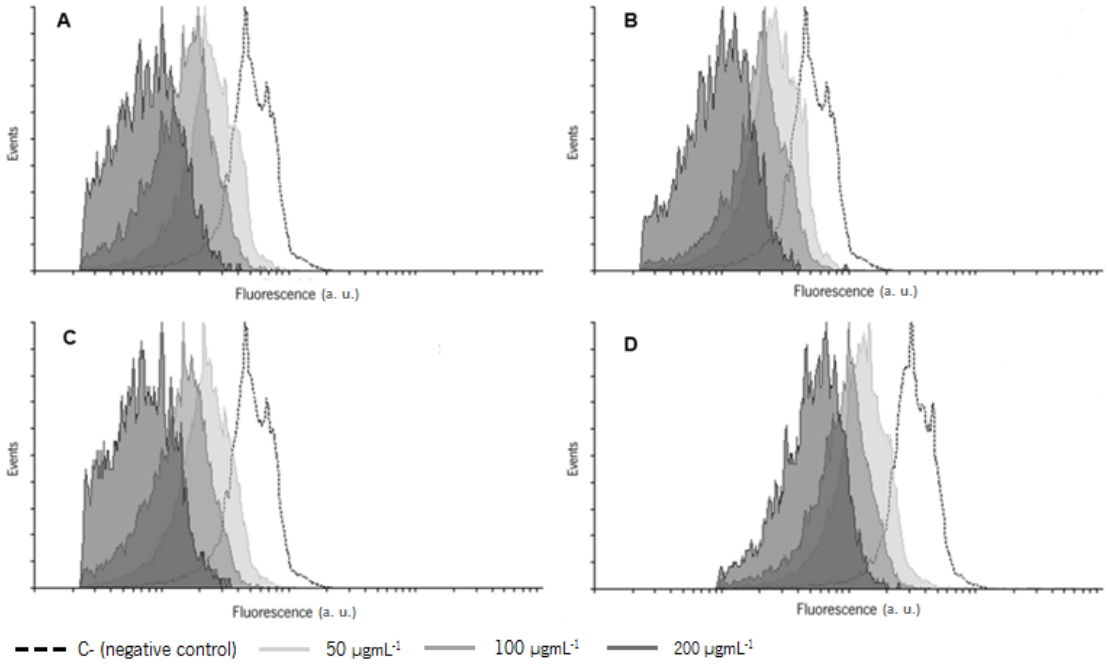

Figure 10 - Intracellular oxidation of *S. cerevisiae* cells loaded with H_2DCFDA, incubated for 1 h with different concentrations (50. 100 or 200 μgmL^{-1}) of G11.EE (A), G12.EE (B), G13.EE (C) or G14.EE (D) and analysed for fluorescence by flow cytometry. The control, (C-), representative of the extract solvent and the cells used in the experiment, only had ethanol. Data are from a representative experiment from three independent experiments.

As all the G.EEs showed potential to reduce the oxidation by endogenous ROS of yeast cells, it was investigated if propolis from Gerês protects from oxidative stress caused by externally added H_2O_2. For experiments in co-incubation conditions, cells were loaded with H_2DCFDA and then simultaneously incubated with each G.EE, ranging from 50 to 200 μgmL^{-1}, and 5 mM H_2O_2, before measurement of the fluorescence in the cytometer. After 20 min of incubation with H_2O_2, yeast cells had a significant increase in fluorescence (Figure 11), due to the increase of intracellular oxidation. On the other hand, cells co-treated with all the G.EEs showed a decrease in fluorescence in a dose-dependent manner, compared with cells treated only with H_2O_2, which shows that under co-incubation conditions propolis can protect cells against oxidative stress

caused by H_2O_2, decreasing the oxidation state of the cells, and corroborating the protective effect seen in the viability assays in the presence of H_2O_2 (Figure 7).

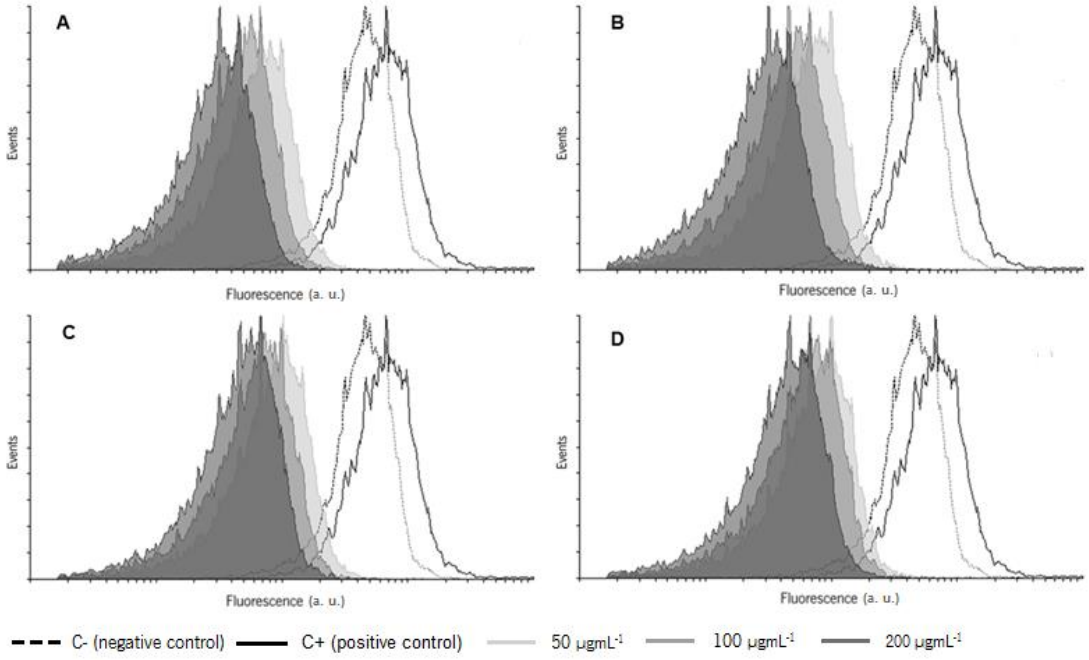

Figure 11- Intracellular oxidation of *S. cerevisiae* cells loaded with H_2DCFDA and analyzed for fluorescence by flow cytometry, after co-incubation with 5 mM H_2O_2 and different concentrations (50. 100 or 200 µgmL^{-1}) of G11.EE (A), G12.EE (B), G13.EE (C) or G14.EE (D) for 20 min. Two controls, one with ethanol (C-), representative of the extract solvent and the cells used in the experiment, and another with H_2O_2 5 mM (C+), to assess the damage caused by H_2O_2 alone, were included. Data are from a representative experiment from three independent experiments.

For pre-incubation, cells loaded with H_2DCFDA were previously incubated with G.EEs, ranging from 50 to 200 µgmL^{-1}, washed once with PBS and then treated with 5 mM H_2O_2. The same tendency observed in co-incubation conditions is observed in pre-incubation conditions (Figure 12), with the exception of G14.EE, for which the concentration of 100 µgmL^{-1} displays less fluorescence than 200 µgmL^{-1}, while for concentrations of 50 and 200 µgmL^{-1} the fluorescence is similar. This ability of G.EEs to decrease intracellular oxidation caused by H_2O_2, both in co- and in pre-incubation conditions, possibly explain the increased viability of yeast cells subjected to oxidative stress but treated (previously or concomitantly) with propolis from Gerês (Figures 7 and 8).

Studies made on RAW264.7 cells demonstrated that a P.EE from China decreased the ROS level, produced by H_2O_2, in a dose-dependent manner as observed by the decrease in fluorescence intensity (Zhang *et al.*, 2015). Sá *et al.* (2013) evaluated the H_2DCF fluorescence

increase when cells were exposed to menadione, a naphtoquinone used as an oxidative stress generator displaying strong ability to produce $O_2^{\cdot-}$ inside the cells. The authors demonstrated a potent antioxidant activity of propolis by the reduction of H_2DCF oxidation in *S. cerevisiae* cells after propolis treatment, suggesting that propolis protect yeast cells by reducing the levels of ROS.

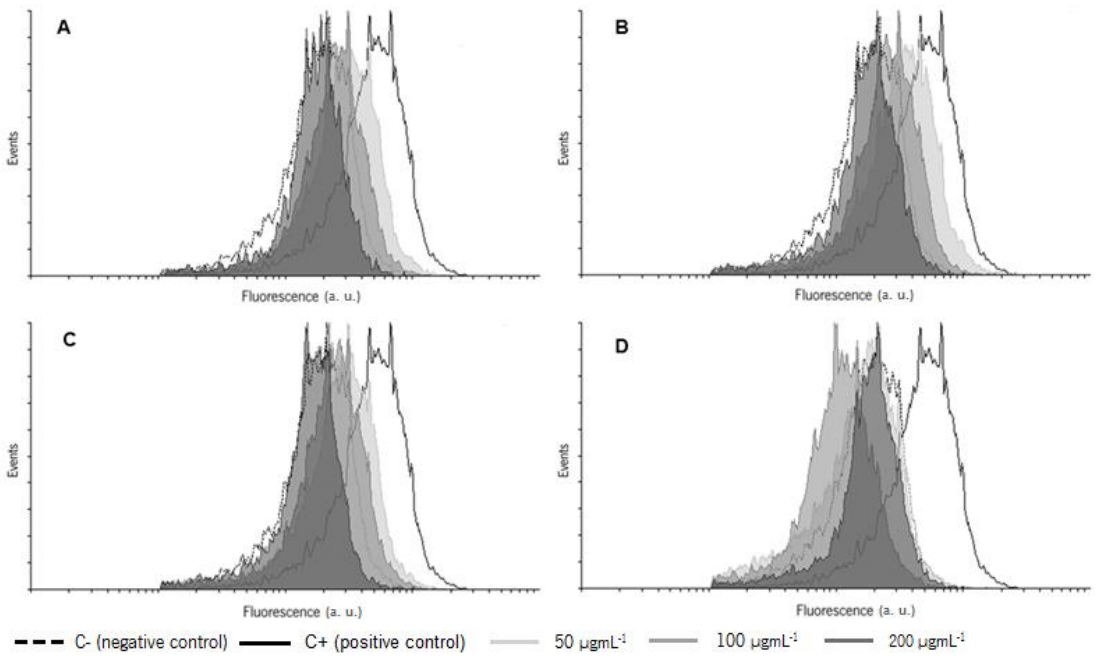

Figure 12 - Intracellular oxidation of *S. cerevisiae* cells loaded with H_2DCFDA, previously incubated with different concentrations (50. 100 or 200 µgmL^{-1}) of G11.EE (A), G12.EE (B), G13.EE (C) or G14.EE (D) for 20 min, washed once with PBS and subsequently, incubated with 5 mM H_2O_2 for 20 min and analyses for fluorescence by flow cytometry. Two controls, one with ethanol (C-), representative of the extract solvent and the cells used in the experiment, and another with H_2O_2 5 mM (C+), to assess the damage caused by H_2O_2 alone, were included. Data are from a representative experiment from three independent experiments.

3.8. Influence of G.EE on inner mitochondrial membrane potential

Considering the observations that G.EEs neither have genotoxic effects nor affect the viability of yeast cells, and taking into consideration the findings of Castro *et al.* (2011) regarding the increase of *S. cerevisiae* susceptibility to propolis when several genes related to energy derivation by oxidation of organic compounds, mitochondrial genome maintenance and the mitochondrial electron transport chain are deleted, it seemed relevant to test the hypothesis of G.EE toxicity on yeast cells performing the respiratory function.

A decrease in fluorescence emission was observed with increasing concentrations (50, 100 or 200 µgmL^{-1}) of any of the G.EEs, compared to the negative control (C-), where cells were treated with ethanol, suggesting that all the G.EEs influence the potential of the inner mitochondrial membrane (Figure 13).

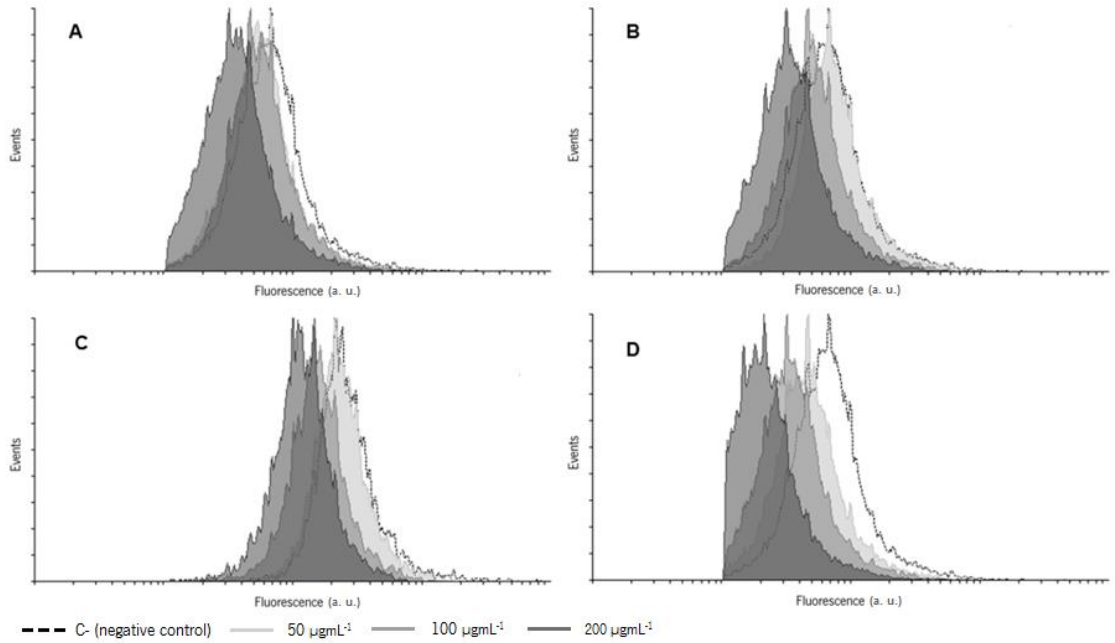

Figure 13 - Inner mitochondrial membrane potential state of *S. cerevisiae* cells loaded with rhodamine 123 after treatment with different concentrations (50, 100 or 200 µgmL^{-1}) of G11.EE (A), G12.EE (B), G13.EE (C) and G14.EE (D) and analyzed for fluorescence by flow cytometry. A negative control, of cells treated with ethanol was included. Data are from a representative experiment from three independent experiments.

To verify if the fluorescence measured in the cytometer had mitochondrial origin an aliquot of cells from each sample was analyzed in a fluorescence microscope. Rhodamine 123 has been used to evaluate the mitochondrial respiratory function including the perception between respiratory-competent and –deficient cells because of its direct staining into the mitochondria and its electrophoretic distribution into the mitochondrial matrix occurs in response to the potential (Ludovico *et al.*, 2001). As depicted in Figure 14, it is possible to see more fluorescence in cells treated with 100 µgmL^{-1} (A) than with 200 µgmL^{-1} (B) of G12.EE and infer that propolis has influence on the inner mitochondrial membrane potential. In accordance with the results in Figure 14, cell fluorescence corresponds, essentially, to the fluorescence of the mitochondria that in yeast have tubular shape, forming a mitochondrial network. These results are in agreement with those find by Castro *et al.* (2011) suggesting the toxic effect of propolis on respiration by the observation of a 1/10 decrease of the initial NADH oxidase activity.

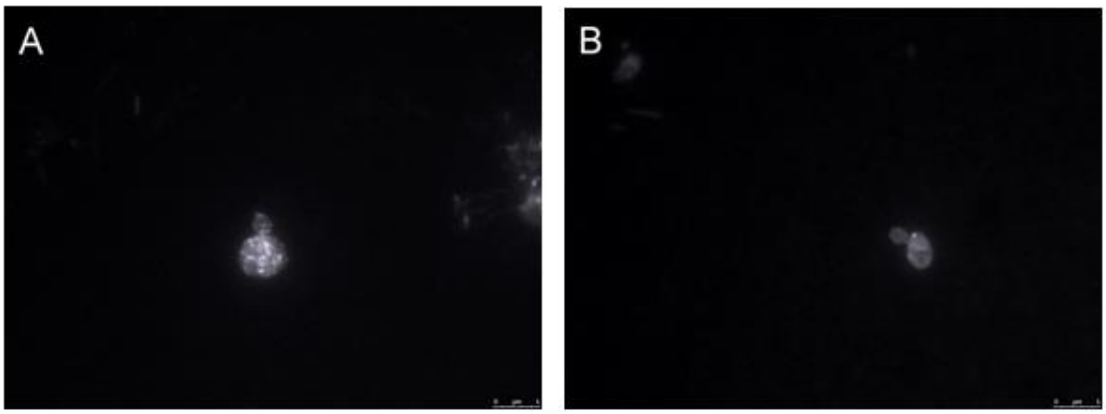

Figure 14 - Microphotographs of fluorescence microscopy of *S. cerevisiae* loaded with rhodamine 123 after incubation with G12.EE 100 μgmL^{-1} (A) and 200 μgmL^{-1} (B).

3.9. Chemical composition of G.EE

Considering the results described in this work for all the ethanol extracts prepared from four propolis samples collected at Gerês in subsequent years, it is possible to conclude that all the G.EEs have similar biological activities, suggesting that they may also have similar chemical profiles. As a chemical analysis of G12.EE by LC-MS was already performed (Freitas, 2013), a second ethanol extract - G11.EE -was selected to be further characterized in terms of chemical composition (Antunes P., personal communication).

Identical compounds to those found in European samples from other countries (Falcão *et al.*, 2010) and very similar, almost equal, to those described for G12.EE were found in G11.EE (Table 12 and Figure 15). This similarity at the chemical profile of phenolic compounds supports the resemblance between all the G.EEs at the level of bioactivities. The slight differences observed between the extracts, eventually more perceptible in terms of the antioxidant activity, could possibly be linked to the different quantities of some compounds found in the two samples of G.EEs. For example, apigenin, kaempferol, galangin and caffeic acid isoprenil ester (isomer) are compounds mostly reported to be responsible for the antioxidant activity (Kumazawa *et al.*, 2004; Orsolić *et al.*, 2006; Chen *et al.*, 2009; Mihai *et al.*, 2011; Yang *et al.*, 2011). These compounds are present in both G11.EE and G12.EE but in marginally larger amounts in G12.EE, which displayed higher antioxidant capacity than the other extracts studied. Sousa (2015) also demonstrated the higher antioxidant capacity of G12.EE by voltammetry. The *p*-coumaric acid derivatives that are also involved in antioxidant activity of propolis (Fokt *et al.*, 2010) are also present in the analyzed propolis samples of Gerês.

Compounds related to propolis antimicrobial activity such as pinocembrin, galangin, ferulic acid, quercetin, CAPE and caffeic acid cinnamyl ester, among others (Santos *et al.*, 2002; Patel *et al.*, 2014; Boisard *et al.*, 2015) as well as compounds that showed to have antiviral activity such apigenin, galanging, quercetin, CAPE, luteolin and chrysin (Schinitzler *et al.*, 2010; Shvarzbeyn and Huleihel, 2011; Ji *et al.*, 2015) were also found in G11.EE and G12.EE.

Table 12 - Chemical composition of G11.EE obtained by LC-MS.

Nº	t_R (min)	$[M-H]^-$ m/z	Composto	Confirmação (NºAmostra 1300389)
1	1,1	169	Gallic acid	Padrão
2	2,0	153	Protocatechuic acid	NA
3	3,6	353	Chlorogenic acid	NA
4	3,7	153	Gentisic acid	NA
5	4,2	177	p-Coumaric acid methyl ester	177 (100), 133 (19), 89 (31)
6	4,3	135	3,4-Dihydroxy vinylbenzene	135 (100), 107 (14), 89 (8)
7	4,5	179	Caffeic acid	Padrão
8	5,0	121	Benzoic acid	121 (100), 92 (39)
9	7,0	163	p-Coumaric acid	Padrão
10	8,3	193	Ferulic acid	NA
11	9,2	301	Ellagic acid	301 (100)
12	19,0	285	Luteolin	Padrão
13	19,4	301	Quercetin	301 (72), 273 (7), 179 (59), 151 (100), 121 (24)
14	21,9	315	Quercetin 3-methyl ether	315 (8), 300 (100), 271 (25), 255 (6)
15	24,2	271	Pinobanksin	271 (100), 253 (30), 225 (8), 197 (25), 161 (12), 151 (13), 125 (6)
16	25,9	269	Apigenin	Padrão
17	26,8	299	Kaempferol-methyl ether	299 (6), 284 (100)
18	27,6	285	Kaempferol	Padrão
19	29,1	315	Isorhamnetin	315 (58), 300 (100)
20	33,0	359	Quercetin-tetramethyl ether	359 (37), 344 (100), 329 (64), 314 (6), 301 (9), 286 (7)
21	38,2	315	Rhamnetin	315 (51), 300 (23), 287 (6), 207 (6), 193 (20), 165 (100), 151 (5), 121 (18)
22	40,9	329	Kaempferol-methoxy-methyl ether	329 (87), 314 (100), 299 (42), 285 (18), 271 (7)
23	41,7	329	Quercetin-dimethyl ether	329 (21), 314 (100), 299 (46), 271 (6)
24	42,7	247	Caffeic acid isoprenyl ester (isomer)	247 (36), 179 (65), 161 (40), 135 (100), 134 (92), 133 (24)
25	43,7	247	Caffeic acid isoprenyl ester (isomer)	247 (19), 179 (57), 161 (30), 135 (100)
26	44,6	253	Chrysin	253 (100), 209 (7)
27	45,0	247	Caffeic acid isoprenyl ester (isomer)	247 (6), 179 (12), 134 (100)
28	45,8	269	Caffeic acid benzyl ester	269 (20), 178 (19), 161 (15), 134 (100)
29	46,1	255	Pinocembrin	255 (100), 213 (25), 211 (14), 187 (8), 171 (13), 151 (24), 107 (8)
30	46,9	283	Galangin-5-methyl ether	283 (34), 268 (100)
31	47,2	269	Galangin	269 (100)
32	48,3	313	Pinobanksin-3-O -acetate	313 (9), 271 (10), 253 (100)
33	48,5	283	Caffeic acid phenylethyl ester	283 (77), 179 (97), 161 (36), 135 (100)
34	50,5	231	p-Coumaric acid isoprenyl ester (isomer 1)	231 (20), 163 (38), 145 (54), 119 (100)
35	51,6	433	Pinocembrin-5-O-3-hydroxy-4-methoxyphenylpropionate	433 (100), 401 (12), 309 (22), 269 (11)
36	51,8	295	Caffeic acid cinnamyl ester	295 (14), 211 (8), 178 (22), 161 (6), 134 (100)
37	52,9	327	Pinobanksin-3-O -propionate	327 (11), 271 (9), 253 (100)
38	53,9	269	3-Hydroxy-5-methoxy flavanone	269 (100), 254 (25), 226 (40), 225 (18), 177 (16), 171 (12), 165 (48), 163 (11), 122 (22)
39	56,7	399	Caffeic acid derivative	399 (100), 355 (6), 179 (20), 134 (22)
40	57,1	399	Caffeic acid derivative (isomer)	399 (74), 355 (21), 178 (55), 134 (100) mto ruído!!
41	58,9	565	p-Coumaric acid- 4-hydroxyphenylethyl ester dimer	565 (16), 417 (7), 344 (11), 283 (100), 269 (11)
42	59,3	355	Pinobanksin-3-O -pentanoate or 2-methylbutyrate	355 (30), 253 (100)
43	60,0	315	Caffeic acid derivative	315 (24), 179 (31), 134 (100)

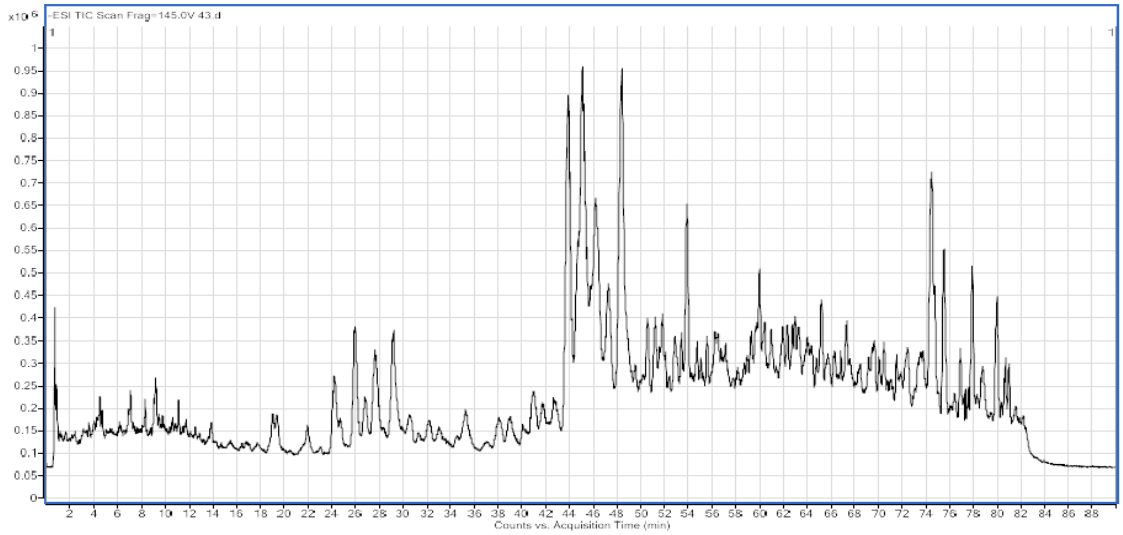

Figure 15 - Chromatographic profile of G11.EE. Each peak in the figure represents a different compound, corresponding to the compounds showed in Table 11.

4. CONCLUSIONS

Through the analysis of the results obtained by the evaluation of the genotoxic/ antigenotoxic activity it is possible to sustain that, at least, one of the four ethanol extracts of propolis from Gerês, namely G12.EE, has no genotoxic effect on yeast DNA. Other studies to assess the possible genotoxic effect of the other G.EEs (G11.EE, G13.EE and G14.EE) are still needed to clarify the absence of genotoxicity in the remaining propolis samples. The hypothesis of a possible antigenotoxic activity for this propolis from Gerês was discarded as no significant decrease of the tail length was observed for cells co-treated with H_2O_2 and any of the four G.EEs.

In what concerns antimicrobial activity all the G.EEs exhibited the same activity spectra and identical MIC values against the panel of tested strains. Propolis from Gerês primarily displays antibacterial activity; the antifungal action was not detected in the range of tested concentrations. Gram-positive spore forming bacteria showed to be the most susceptible species to all of the four G.EEs tested and these results seem to suggest a very particular activity spectrum. It will important to confirm this hypothesis in the near future, testing other bacteria, either Gram-negative or Gram-positive, but particularly other spore-forming strains. Gram-positive bacteria spore forming bacteria includes the genus *Bacillus* as well as *Clostridium* (Saujet *et al.*, 2014) because of their morphological and molecular similarities in sporulation (Paredes *et al.*, 2005; Saujet *et al.*, 2014) and strains of these two genera should be included in a future screening. Moreover, it will be important to test other fungal species too, in order to clarify if propolis from Gerês only displays antibacterial activity or if an antifungal action is only expressed at higher concentrations.

Antimicrobial synergism is a subject of great interest for scientists/researchers and clinicians as antimicrobial combinations can be effective in treating polymicrobial infections, in preventing the selection of resistant microorganisms when a high mutation rate of the causal organism exists to the antibiotic indicated and in reducing a possible dose-related toxicity. A synergistic effect with the antibiotic gentamicin was observed for all of the four G.EEs studied and against all the bacteria. However, for a clinical isolate of *Staphylococcus aureus* methicillin-resistant strain and for *Escherichia coli* this synergistic effect disappears when 2000 μgmL^{-1} of G.EE was used in combination with 1.5 μgmL^{-1} of gentamicin, which was observed for all the four G.EEs. A possible synergism between certain compounds of propolis with gentamicin, only detectable when both parts are present in certain proportions, could possibly explain the absence of the synergistic effect for particular combinations. This hypothesis needs to be tested in future assays, manipulating the proportion of G.EEs and gentamicin in the antimicrobial combination.

As none of the four G.EEs was cytotoxic for *Saccharomyces cerevisiae*, experiments to evaluate the protective effect of propolis on yeast cells co- or pre-treated with an oxidizing agent were performed. All the G.EEs had some protective effect on yeast cells against oxidative stress caused by H_2O_2 in both co- and pre-incubation conditions. Such protective effects could be linked with the antioxidant activity of G.EEs as all showed ability to scavenge the DPPH free radical in a dose-dependent manner, as well as $O_2^{\cdot-}$ and an iron chelating activity. An ordered sequence for G.EE antioxidant capacity was established from each of the methodss and is shared between the three *in vitro* assays: G12.EE>G14.EE>G11.EE>G13.EE. The antioxidant activity of propolis was also evaluated *in vivo* by flow cytometry and all the G.EEs decrease the oxidation state of the cells with increasing concentrations and protect yeast cells against oxidative stress caused by H_2O_2 in both co- and pre-incubation conditions.

Despite being collected in different years, G11.EE and G12.EE chemical profiles showed a huge similarity in terms of the type of phenolic compounds, some of which have been linked to propolis biological properties. This supports in certain degree the results obtained in this work regarding the great resemblance between all the G.EEs at the level of bioactivities. To confirm these inference it will be necessary to analyze the remaining two G.EEs and to perform a more complete analysis of the compounds present in all the G.EEs. This is also an important finding considering the renowned difficulty of propolis standardization of solutions and consequent commercialization and acceptance by the medical community. The similarity between all the four G.EEs in terms of the bioactivity profiles evaluated in this work, provided it is supported by similar chemical profiles as it is suggested by the analysis of two G.EEs, can open several applications' opportunities for propolis do Gerês.

Any application of G.EEs requires that the mechanism of propolis action should be clarified. Given the results found by de Castro *et al.* (2011) suggesting that *S. cerevisiae* susceptibility to propolis depends on the mitochondrial function, a possible role of propolis from Gerês on the potential of the inner mitochondrial membrane was investigated. G.EEs have influence on the inner mitochondrial membrane, in a dose-dependent manner.

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Annexs

Table 13 - Antioxidant activity (%) by the free radical DPPH scavenging by gallic acid concentration (µgmL^{-1}).

GA concentration (µgmL^{-1})	Reduction (%)	IC50 (µgmL^{-1})
0.2	15.48±2.12	0.803±0.02
0.375	22.73±2.55	
0.5	30.35±2.73	
0.75	46.81±3.34	
1.5	63.79±4.60	
3.0	81.36±1.97	
6.0	91.09±0.76	

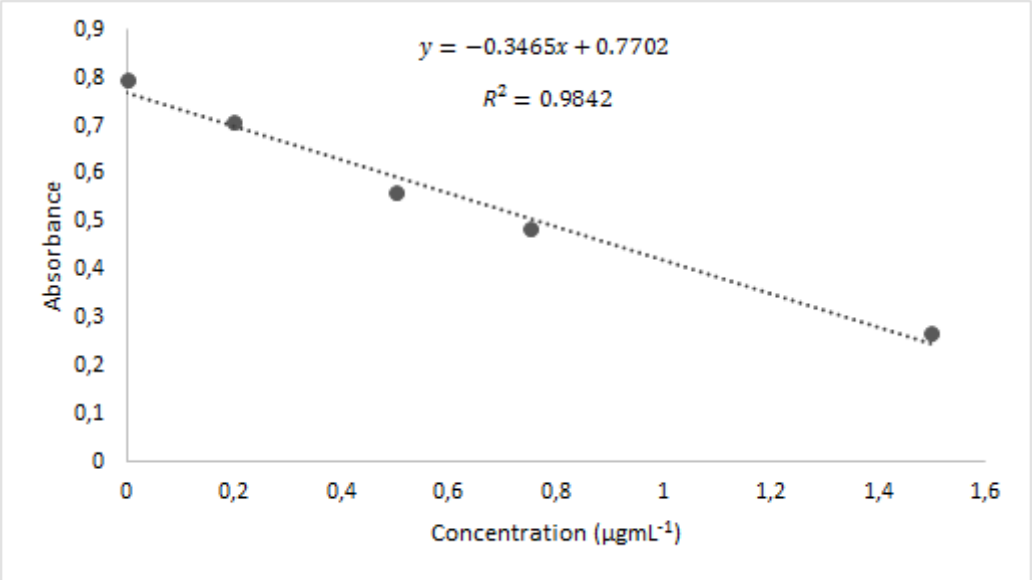

Figure 16 - Percentage of reduction in absorbance of DPPH (517 nm) by adding increasing concentrations of gallic acid.