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ARTICLE

Antioxidant and dual dose-dependent antigenotoxic and genotoxic properties of an ethanol extract of propolis

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Propolis is a resinous product made by honeybees from plant-derived materials, with high content of polyphenols associated to several beneficial bioactivities with potential of use as natural food additive for preservation and as functional food ingredient. A Portuguese propolis ethanol extract (C.EE) protected *Saccharomyces cerevisiae* cells from loss of viability upon exposure to H_2O_2, both in co- and in pre-incubation experiments. Results obtained with the comet assay suggest that lower concentrations are antigenotoxic while at higher concentrations a genotoxic effect prevails, which correlates with cytotoxicity of high concentrations of C.EE. Flow cytometry analysis with dichlorofluorescein indicates that C.EE induced intracellular antioxidant activity *in vivo*. Overall the results suggest that C.EE is antigenotoxic but is also toxic at higher concentrations. This dual effect could be explained by with the presence of compounds known to interfere with DNA synthesis and/or cell proliferation, such as caffeic acid phenethyl esther (CAPE) and chrysin, together with antioxidants, like kaempferol, pinobanksin and pinocembrin.

Introduction

Propolis is a resinous mixture produced by honeybees from exudates of buds and bark of plants such as poplar (*Populus* sp.), birch (*Betula* sp.), beech (*Fagus* sp.), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus* sp.), Brazilian rosemary (*Baccharis dracunculifolia*), eucalyptus (*Eucalyptus* sp.) and Brazilian pine (*Araucaria angustifolia*). It is generally accepted that propolis is used in the hive mainly as a construction and antiseptic material, repairing mechanical damage and avoiding microbial infections.

Propolis is chemically diverse and its composition varies geographically according to the flora and climate, the season of collection, as well as with the race of the producing bees[1]. Propolis compounds belong to very different chemical groups, such as polyphenols (flavonoids, including flavones, flavonones, flavonols, dihydroflavonols and chalcones), phenolic acids and their esters, terpenoids, steroids and amino acids[2]. Most of these compounds, in particular phenolics such as caffeic acid derivatives and flavonoids, have been associated with propolis biological activities, namely antimicrobial[3], cytotoxic and hepatoprotective[2], radioprotective, antimutagenic[4], antioxidant[5] and as scavenger of free radicals[2].

The widely recognized properties of propolis have been promoting its extensive use in nutraceutics, cosmetics and health care. In addition, the consumers' widely good acceptance of the incorporation of natural products in foods and medicines increases the potential use of propolis as food functional ingredient and as preservative. Hence, propolis has been attracted the attention of researchers to formulate new natural food functional ingredients[6,7] and was also included in several food products with beneficial effects as preservative[8,9]. Still in many countries, honey producers have disregarded propolis due to low yields and lack of knowledge for its economic potential as a valuable co-product. Hence, the demonstration of bioactivities but also cell-protective properties for the development of functional foods and health-care products based on propolis are a major goal.

The antioxidant activity of flavonoids of propolis, in samples from very different provenances, has been reported as a mechanism of protection of genomic DNA against reactive oxygen species[10,11]. Also, genotoxic effects of propolis extracts and of some of its constituents can be found in the literature[12], having been attributed to the pro-oxidant activity of flavonoids[13]. This pro-oxidant activity has been shown to be directly dependent on concentration and is mediated by increased production of superoxide, hydrogen peroxide and hydroxyl radical in human lymphocytes[14]. Therefore, it is conceivable that propolis extracts might have concentration-dependent antagonistic effects, owing to the considerable amount and diversity of flavonoids as constituents. To investigate these effects on genome integrity we studied the antigenotoxic, genotoxic and antioxidant capacity of an ethanol extract of a propolis sample collected in 2010, in the region of Côa (Beira Alta, Portugal), using the eukaryotic experimental model *Saccharomyces cerevisiae*.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

Experimental

Yeast strain, media and growth conditions

In all experiments the haploid *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used. Cultures were grown on liquid YPD medium (1% w/v yeast extract, 1% w/v peptone and 2% w/v glucose), in an orbital shaker at 30 ºC and 200 rpm. Growth was monitored by optical density at 600 nm (OD_{600}).

Propolis ethanol extract

Raw propolis was collected in August 2010 from an apiary set in the region of Côa (Beira Alta, Portugal). A 29 g propolis sample was incubated with 100 mL absolute ethanol in an orbital shaker at 100 rpm, at room temperature, in the dark, for 24 h. The solution was then filtered (Whatman nr. 4) and the residue was re-dissolved in 100 mL absolute ethanol and extracted again. This procedure was repeated three times. The filtrates were pooled and dried in a rotary evaporator (Buchi RE 121), at 40 ºC, under vacuum and gentle rotation (50 rpm), yielding the ethanol extract of Côa propolis (C.EE), which was stored in the dark at 4 ºC until use. Working solutions at the desired concentrations were prepared in ethanol immediately before use.

Viability assays

Yeast cells from exponentially growing cultures were harvested by 2 min centrifugation at 6000 x*g*, 4 ºC, washed twice with the same volume of sterilized deionized H_2O at 4 ºC and suspended in the same volume of S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). The suspension was incubated at 30 ºC, 200 rpm, in the presence of 5 mM H_2O_2, after which aliquots were harvested along time, serially diluted to 10^{-4}, spread on YPD plates and incubated at 30 ºC for 48 h. The percentage of colony-forming units (CFUs) was calculated at each time-point taking as reference the number of colonies obtained before the addition of H_2O_2. In pre-incubation experiments, cells were incubated with C.EE for 20 min, at 30 ºC, 200 rpm and were washed and suspended in S buffer, as described above. In co-incubation experiments C.EE and H_2O_2 were added to the suspension simultaneously. In post-incubation experiments cells were incubated for 20 min with H_2O_2 followed by a washing step, suspension in S buffer and incubation with C.EE for further 20 min. Controls were included by replacing C.EE by the same volume of the solvent (ethanol) and/or H_2O_2 by the same volume of S buffer.

Comet assay

The yeast comet assay was performed as described before[15]. Briefly, cells from exponentially growing cultures were harvested by centrifugation at 18,000 x*g*, for 2 min at 4 ºC, washed twice with ice-cold deionized H_2O, suspended in S buffer containing 2 mg/mL zymolyase (20,000 U/g; ImmunO™–20T) and 50 mM β-mercaptoethanol and incubated at 30 ºC, 200 rpm for 40 min, in order to obtain spheroplasts. After washing with S buffer, spheroplasts were suspended in S buffer containing 10 mM H_2O_2, incubated for 20 min at 4 ºC and subsequently washed with S buffer. Spheroplasts were embedded in 1.5% low melting agarose (w/v in S buffer) at 35 ºC, spread onto glass slides previously layered with 0.5% (w/v) normal melting agarose, covered with a cover slip and incubated on ice in order to solidify the agarose. The cover slips were then removed and the unwinding of DNA and protein denaturation were made with ice-cold lysing buffer (30 mM NaOH, 1 M NaCl, 50 mM ethylenediamine tetraacetic acid (EDTA), 10 mM tris-HCl, 0.05% (w/v) lauroylsarcosine, pH 10) for 20 min. Samples were subsequently stabilized in ice-cold electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM tris-HCl, pH 10) for 20 min. Glass slides were placed in an electrophoresis chamber and samples were exposed to 0.7 V/cm for 10 min, at 4 ºC, to allow unwound DNA to move out of the nucleoids. The gels were neutralized with 10 mM tris-HCl buffer, pH 7.4, for 10 min at 4 ºC, samples were fixed, firstly in 76 % (v/v) ethanol and then in 96 % (v/v) ethanol, both for 10 min, and the slides were dried at room temperature. After staining with 10 µL of GelRed™ (diluted 10,000 fold from the stock solution; Biotium) comets were visualized by fluorescence microscopy (Leica DMB 5000 with a P&B, Leica, DFC 350FX digital camera) and tail length was measured from at least 20 comets per treatment with the CometScore software. Pre-, co- or post-incubation with C.EE were performed in the spheroplasts suspension as described for viability assays, before embedding in low melting agarose. Controls were also prepared as described in the same experimental procedure.

Flow cytometry

Cells from exponentially growing cultures were harvested as above, washed twice with the same volume of ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), diluted to an OD_{600} of 0.02 and 500 µL were collected for measurement of autofluorescence. Cells were loaded with 50 µM dichlorofluorescein diacetate (H_2DCFDA) by incubation at 30 °C, 200 rpm, during 1 h in the dark, after which they were washed twice with the same volume of ice-cold PBS. Aliquots of 1 mL were mixed with C.EE and 10 mM H_2O_2 in co-incubation experiments, for 20 min, at 30 °C, 200 rpm, in the dark. In pre- and post-incubation experiments cells were incubated, respectively, with the extract and subsequently with H_2O_2 or with H_2O_2 and subsequently with C.EE. Washing steps were included between each incubation step, as described above. Approximately twenty thousand cells of each sample were analysed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green 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 made with the Flowing Software. Controls were included by replacing C.EE by the same volume of ethanol and H_2O_2 by PBS.

Chemical analysis of C.EE

Quantification of total polyphenols. The content in total polyphenols of the extract was determined by the Folin-Ciocalteu colorimetric method[16], with some modifications. Three hundred milligrams of C.EE solution (0.5 mg/g final concentration) were mixed with 2.0 g deionized H_2O, 200 mg Folin-Ciocalteu reagent

(Panreac, Barcelona, Spain), 2.0 g of 10% (w/v) Na_2CO_3, and deionized H_2O to complete 10.0 g final mass of the mixture. The reducing power of the polyphenols in the mixture was evaluated using the OD_{760} measured after 1 h incubation at room temperature. C.EE polyphenol content was calculated using gallic acid as standard and results were expressed as milligrams of gallic acid equivalents per gram of C.EE (mg GAE/g).

Quantification of flavonoids. Total flavonoid content in the extract was determined using a method described by Woisky and Salatino[17]. Five hundred milligrams of 2% (w/v) $AlCl_3 \cdot 6H_2O$ were added to 300 mg of C.EE and ethanol was used to complete 10.0 g of mixture final mass. After 30 min incubation at room temperature, the OD_{420} of the mixture was measured and flavonoids content was calculated by comparison with the standard quercetin (1.2 mg/g) and expressed as milligrams of quercetin equivalents per gram of C.EE (mg QE/g).

LC-MS analysis. One hundred milligrams of C.EE were dissolved with 1 mL of 80% ethanol at 70 °C and filtered through a 0.22 µm nylon filter prior to injection. Standards for gallic acid, protocatechuic acid, chlorogenic acid, vanillic acid, cafeic acid, syringic acid, ferulic acid, *o*-coumaric acid, apigenin, and kaempeferol were acquired from Sigma-Aldrich Co. LLC. Luteolin and gentisic acid standards were acquired from Extrasynthese, France. The chromatographic system consisted of an Agilent 1200 series equipped with 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 at a flow rate of 0.7 mL/min, at 30 °C. Elution was performed by means of a gradient of 0.1% formic acid (eluent A) and acetonitrile (eluent B). The gradient was as follows: started at 10% B, 20% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, at 81 min return to initial conditions and stabilization for 9 min. Electrospray ionization (ESI) was performed with a nitrogen flow of 10 L/min at 300 °C and the MS detector was 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 100 °C, fragmentor energy was 145 V, and cell accelerator voltage was 7 V. Data were acquired and analysed using Masshunter Workstation Software (version B.04.00) Agilent Technologies.

For MS/MS confirmation the same equipment and chromatographic conditions were used. The MS detector was operated in Product Ion scan type, selecting the precursor ions and performing a scan of the fragments in the range 80–500 Da and negative mode was selected. The capillary voltage set to 4.0 kV, the quadrupole temperatures were 100 °C, fragmentor energy was 135 V, cell accelerator voltage was 7 V and collision energy was 15 V. Compounds were identified based on standards retention times and by comparison of the ESI–MS/MS data with the MS/MS data published in the literature.

Statistical analysis

All experiments were done in triplicate and the results are presented as mean ± standard deviation (SD). For the comet assay, the mean was obtained from the mean of three independent experiments. One-way analysis of variance (1-way ANOVA) was used to evaluate treatment effects and Tukey's test was used to perform comparisons between each treatment with the respective control. Asterisks (or §§) indicate statistically significant differences: * means $0.01 < p \leq 0.05$, ** means $0.001 < p \leq 0.01$, and *** means $p \leq 0.001$.

Results & discussion

C.EE polyphenolic content and chemical profile

Polyphenols constitute an important group of biologically active compounds abundant in propolis samples. Particularly, the flavonoids have been associated with the antioxidant properties exhibited by plant and plant-based products, such as propolis[5], but also some phenolic acids and their esters possess antioxidant activity[18]. Hence, to assess C.EE bioactivity potential, an initial quantitative characterization involved the determination of total polyphenols and total flavonoids contents (Table 1).

Table 1. Chemical characterization of Côa propolis ethanol extract (C.EE). The content of total polyphenols and total flavonoids was expressed, respectively, in equivalents of gallic acid (mg GAE) and in equivalents of quercetin (mg QE) per gram of propolis extract.

Sample	Total polyphenols (mg GAE/g)	Total flavonoids (mg QE/g)
C.EE	160.40±16.56	30.21±0.52

Total phenols of C.EE were in the range reported in the literature (120-443 mg GAE/g extract)[19], but in the lower third of the rank. The values are similar to those found for Portuguese samples from the centre of Portugal[20] but are also comparable to those of very distant places, such as India[5]; Anhui, China[21]; and to the red propolis from Brazil[22]. Although still in the range for total flavonoids (25-140 mg QE/g extract) also low levels were obtained for C.EE (Table 1). Again, this content is similar to that of some Portuguese samples from Alentejo[20], but also to red propolis from Cuba[19] and to some samples collected in Anhui, China[21].

The sample was further characterized qualitatively by LC-MS analysis (Table 2 and supplementary figure 1). Compounds were identified using standards, or by comparison of MS/MS fragmentation and relative retention times with those described in the literature[23,24,25]. Similarly to what has been described for other propolis samples, C.EE is mostly constituted by flavonoids, phenolic acids and their esters, being flavones, flavonols and flavanones the main flavonoid groups as previously reported[26]. In this sample 43 phenolic compounds were identified: benzoic and hydroxybenzoic acids, caffeic acid esters (caffeic acid isoprenyl esters, caffeic acid benzyl ester, caffeic acid cinnamyl ester and caffeic acid phenethyl ester - CAPE) and derivatives, *p*-coumaric acid and esters (*p*-coumaric methyl ester, *p*-coumaric isoprenyl ester and *p*-coumaric acid-4-hydroxyphenylethyl ester dimer), flavonols (quercetin,

kaempferol, galangin), flavones (luteolin, apigenin, chrysin), flavanones (pinocembrin) and dihydroflavonols (pinobanksin) (Table 2). With the exception of acacetin, all most important flavonoids reported for propolis, apigenin, galangin, chrysin, quercetin, CAPE, luteolin, pinocembrin, pinobanksin and kaempferol[26], are present in this sample. It is also noteworthy that this extract is particularly rich in methoxylated flavonoids (quercetin-3-methyl ether, quercetin-dimethyl ether, quercetin-tetramethyl ether, rhamnetin, isorhamnetin, kaempferol-methoxy-methyl ether, galangin-5-methyl ether, pinocembrin-5-*O*-3-hydroxy-4-methoxyphenyl propionate and 3-hydroxy-5-methoxy flavanone) and in pinobanksin derivatives (pinobanksin-3-*O*-acetate, pinobanksin-3-*O*-acetate, pinobanksin-3-*O*-propionate, pinobanksin-3-*O*-pentanoate). Most of these compounds are generally present in the common temperate propolis type[24], which is the most common in Europe, China and Argentina[25], indicating that, as expected, the C.EE sample fits into this propolis type. In particular, high relative amounts of chrysin, pinocembrin and galangin denote that poplar is an important source of raw material[19]. However, the phenolic composition of this sample displays some distinct features like the presence of uncommon phenolic acids, such as 3,4-dihidroxy vinylbenzene, and the rare non-flavonoid phenolic ellagic acid, reported recently for Portuguese propolis[24].

Table 2. Identification of phenolic compounds from Côa propolis extract (C.EE), by LC-MS/MS. [a] + indicates that TIC peak is not pure. [b] standard – for compounds identified with standards, or m/z (abundance percent) – for compounds confirmed by comparison of MS/MS fragmentation with bibliography. See chromatogram in supplementary Fig. 1.

Peak number	t_R (min) [a]		[M-H]$^-$ m/z	Compound name	Confirmation [b]
1	1,1		169	Gallic acid	Standard
2	2,0	+	153	Protocatechuic acid	Standard
3 +4	3,6		353	Chlorogenic acid	Standard
	3,7		153	Gentisic acid	Standard
5	4,2		177	*p*-Coumaric acid methyl ester	177 (100), 133 (91)
6 +7	4,3		135	3,4-Dihydroxy vinylbenzene	135 (100)
	4,5		179	Caffeic acid	Standard
8	5,0	+	121	Benzoic acid	121 (100), 92 (61)
9	7,0		163	*p*-Coumaric acid	Standard
10	8,3		193	Ferulic acid	Standard
11	9,2		301	Ellagic acid	301 (100)
12	19,0		285	Luteolin	Standard
13	19,4		301	Quercetin	301 (83), 273 (14), 179 (47), 151 (100), 121 (15)
14	21,9		315	Quercetin 3-methyl ether	315 (10), 300 (100), 271 (19), 255 (6)
15	24,2		271	Pinobanksin	271 (100), 253 (30), 225 (6), 215 (6), 197 (21), 151 (9), 125 (7)
16	25,9		269	Apigenin	Standard
17	26,8		299	Kaempferol-methyl ether	299 (6), 284 (100), 137 (7)
18	27,6		285	Kaempferol	Standard
19	29,1		315	Isorhamnetin	315 (65), 300 (100), 151 (7)
20	30,55		299	Unidentified A	299 (10), 28 (100), 255(60), 227 (22)
21	33,0		359	Quercetin-tetramethyl ether	359 (34), 344 (90), 329 (100), 314 (11), 286 (7)
22	38,038	+	315	Rhamnetin	315 (87), 300 (30), 287 (6), 193 (15), 165 (100), 149 (5), 121 (14)
23	38,922		313	Unidentified B	313 (96), 298 (100), 297 (53), 283 (49), 269 (20), 255 (24)
24	40,9		329	Kaempferol-methoxy-methyl ether	329 (91), 314 (100), 299 (75), 285 (15), 271 (12)
25	41,7		329	Quercetin-dimethyl ether	329 (8), 314 (100), 299 (50), 271 (10)
26	42,7		247	Caffeic acid isoprenyl ester (isomer)	247 (25), 179 (60), 161 (25), 135 (83), 134 (100)
27	43,7		247	Caffeic acid isoprenyl ester (isomer)	247 (25), 179 (61), 161 (21), 135 (100)
	44,6		253	Chrysin	253 (100), 209 (5)
28 +29	45,0		247	Caffeic acid isoprenyl ester (isomer)	247 (6), 179 (9), 134 (100)
	45,8		269	Caffeic acid benzyl ester	269 (20), 178 (14), 161 (19), 134 (100)
30 +31	46,1		255	Pinocembrin	255 (100), 213 (29), 211 (7), 187 (6), 185 (6), 171 (12), 151 (13), 107 (7)
32 +33	46,9		283	Galangin-5-methyl ether	283 (45), 268 (100)
	47,2		269	Galangin	269 (100)
34 +35	48,3		313	Pinobanksin-3-O -acetate	313 (9), 271 (11), 253 (100)
	48,5		283	Caffeic acid phenylethyl ester	283 (52), 179 (100), 161 (34), 135 (99)
36	49,5		313	Unidentified C	
37	50,5	+	231	*p*-Coumaric acid isoprenyl ester (isomer 1)	231 (21), 163 (46), 145 (51), 119 (100)
38	51,6		433	Pinocembrin-5-O-3-hydroxy-4-methoxyphenylpropionate	433 (100), 401 (6), 323 (6), 309 (22), 269 (15), 123 (13)
39	51,8		295	Caffeic acid cinnamyl ester	295 (14), 251 (9), 178 (16), 134 (100)
40	52,9		327	Pinobanksin-3-O -propionate	327 (12), 271 (8), 253 (100)
41	53,9		269	3-Hydroxy-5-methoxy flavanone	269 (100), 254 (29), 236 (15), 226 (28), 225 (21), 177 (10), 171 (17), 165 (44), 122 (22)
42	56,7		399	Caffeic acid derivative	399 (100), 178 (19), 134 (9)
43	57,1		399	Caffeic acid derivative (isomer)	399 (100), 179 (33), 134 (32)
44	58,9		565	*p*-Coumaric acid-4-hydroxyphenylethyl ester dimer	565 (25), 417 (6), 283 (100), 269 (7)
45	59,3		355	Pinobanksin-3-O -pentanoate or 2-methylbutyrate	355 (31), 253 (100)
46	60,0		315	Caffeic acid derivative	315 (65), 179 (34), 178 (36), 134 (100)
47	60,9		647	Unidentified D	

Despite the low total flavonoid content (Table 1), that may partially be explained by an underestimation of flavanones and dihydroflavonols by the use of the aluminium chloride-ethanol method, the results obtained from chemical characterization,

revealing flavonoid diversity and in high relative abundance, suggest that C.EE may exhibit some of the widely reported propolis biological activities, namely the antioxidant. Indeed, flavonoids (such as pinobanksin, kaempferol, pinocembrin and galangin) and phenolic acids (caffeic acid and CAPE) have been the classes of phenolic substances most extensively reported to have antioxidant activity. On the other hand, a close correlation between antioxidant potential and the total content in flavonoids is not always observed (see the reviews[19,21]), the presence of specific compounds in propolis composition being more indicative of its bioactivity profile.

Relevant bioactivities have been described for most of these compounds, such as anti-proliferative, anti-tumor, antimicrobial and antioxidant, but also pro-oxidant effects were reported for some flavonoids, depending on the redox state of the environment[27]. For this a set of assays were performed to study the antioxidant properties of C.EE at the cell and DNA levels using the yeast model and different technical approaches.

Effects of C.EE on cell viability under oxidative stress conditions

To test the hypothesis that C.EE is capable of protecting cells against oxidative stress, yeast cells were pre-incubated with C.EE and their viability was assessed, as CFUs, in the presence of H_2O_2 for 90 min. Also, co-incubation and post-incubation experiments were performed to investigate the antioxidant activity of the extract in the presence of the stressor agent and its participation in cell recovery from oxidative damage, respectively.

When cells were pre-incubated with C.EE at 100 µg/mL (Fig. 1A) and 300 µg/mL (Fig. 1B), viability loss was reduced when compared to control cells (pre-treated with ethanol) (p = 0.0018 and p = 0.0011 after 60 min incubation, respectively), during the 90 min of exposure to 5 mM H_2O_2. Cells treated only with S buffer or pre-treated with ethanol or C.EE, and afterwards with water, displayed a nearly constant viability throughout the experiment (Fig. 1A and 1B). These results suggest that C.EE triggers a protection mechanism in yeast cells that allows increased resistance against oxidative stress.

To investigate a direct effect on the oxidant agent (H_2O_2) and/or early antioxidant effects of C.EE we have determined the viability in co-incubation experiments. As depicted in Fig. 1D, 100 µg/mL C.EE significantly decreased the rate of viability loss (p = 0.0097 after 60 min incubation) of cells exposed to 5 mM H_2O_2, while 25 µg/mL and 300 µg/mL C.EE had no significant effect (Fig. 1C and 1E respectively). In addition, unlike cells treated with 2% ethanol or with S buffer, cells incubated with 100 µg/mL C.EE (p = 0.0029) or 300 µg/mL C.EE (p = 0.005) displayed increased loss of viability (Fig. 1D and 1E). The differences regarding pre-incubation experiments with the same concentrations of C.EE are consistent with the fact that while cells were in contact with C.EE for 20 min in pre-incubation experiments, in co-incubation experiments cells contacted with C.EE for 90 min. Together these observations suggest that there is a range of concentrations where C.EE protects cells against oxidative stress and a concentration threshold above which C.EE has a toxic effect on yeast cells.

The hypothesis that propolis could also improve recovery of cells after oxidative shock was tested by performing post-incubation experiments, which are based on a previous treatment with H_2O_2 for 20 min, wash of the cells to remove the toxicant and a subsequent 20 min treatment with C.EE. As expected, considering the short term incubation, cells without any treatment or treated only with C.EE or 2% ethanol (solvent control) showed a nearly constant survival rate throughout the experiment (Fig. 1F, 1G and 1H). After treatment with 5 mM H_2O_2 for 20 min, when yeast cells were incubated with 2% ethanol or C.EE (see Fig. 1F, 1G and 1H after minute 20), the viability loss rate did not change significantly (Fig. 1F and 1G), except in the experiment with 300 µg/mL C.EE, which promoted a reproducible although not statistically significant faster loss of viability when compared with the respective control (Fig. 1H). These results proved that C.EE could not improve recovery from oxidative stress but also that at the highest concentration tested (300 µg/mL; Fig. 1H) C.EE increased the loss of viability. In fact, when comparing viability loss after 20 min in the presence of 300 µg/mL C.EE in all experiments (time-point 40 min in Fig. 1H), in the post-incubation experiment the C.EE effect was higher (Fig. 1B, 1E and 1H). These results may be explained by an exacerbated pro-oxidant effect of C.EE in cells that were exposed previously to oxidative challenge by H_2O_2 (Fig. 1H). It should not be excluded however non-oxidative mechanisms of C.EE triggering general stress response in yeast cells.

Tsai et al.[13] proposed that flavonoids from propolis could be pro-oxidant and genotoxic by reaction with metal ions. Besides C.EE flavonoid total content being relatively low, it is also known that some flavonoids, like quercetin, are more prone to oxidation than others, especially at physiological pH[28]. Therefore the composition must also be taken into account when analysing these properties and considering that quercetin is one of C.EE components (Table 2) it is tempting to explain the decrease of cell viability by a significant pro-oxidant activity of C.EE. This is also supported by post-incubation experiments with the highest concentration tested (300 µg/mL; Fig. 1H), where the increased rate in viability loss with C.EE after a previous incubation period with H_2O_2 could be explained by further accumulation of oxidative damage in the cells. In studies also with propolis of Portuguese origin, cytotoxicity was observed in human tumour cell lines. Our results could help to explain such activity. In all cases however, a link between cytotxicity and pro-oxidant activity was not reported, being the mechanism either not studied[29,30] or associated with a disturbance of glycolytic metabolism[31].

The pro-oxidant activity of propolis extracts could be associated to the cytotoxicity in human tumour cell lines however, in studies where propolis of Portuguese origin were used the mechanism was not uncovered[29,30], except for the disturbance of glycolytic metabolism detected in one sample from Azores[31].

Globally, the results suggest that C.EE can protect yeast cells against an induced oxidative stress, possibly by direct scavenging and reduction of free radicals and by improving adaptation of cells to stress. In addition C.EE can have cytotoxic effects at higher concentrations, especially in already compromised yeast cells by a previous exposure to oxidative stress.

C.EE protects yeast cells from DNA damage induced by H_2O_2

It is conceivable that at least some of the antioxidant effects reported for propolis[5,32] have an impact in genomic DNA integrity under oxidative challenges by avoiding DNA damage. As the propolis sample herein studied also displayed antioxidant activity, it was decided to investigate its antigenotoxicity.

Yeast spheroplasts were pre-treated for 20 min with 25 µg/mL, 100 µg/mL or 300 µg/mL C.EE in S buffer, to maintain osmotic protection, and then exposed to 10 mM H_2O_2. Exposure to H_2O_2, after pre-incubation with 2% ethanol (extract solvent), increased dramatically comet tail length (Fig. 2A) confirming that the H_2O_2 concentration used in the experiments was genotoxic. However, when yeast spheroplasts were pre-treated with C.EE before exposure to H_2O_2, a statistically significant decrease in comet tail length was observed even for the lowest concentration tested (Fig. 2A). This result indicates that C.EE has a potent antigenotoxic activity by protecting cells against oxidative-induced DNA damage and is consistent with the increase in yeast viability previously observed in the pre-incubation experiment (Fig. 1 A and B).

Acknowledging the cytotoxic properties of C.EE, a possible explanation for its antigenotoxic effect is that a mild genotoxic effect of C.EE could trigger adaptation of cells against oxidative stress by H_2O_2. To investigate this hypothesis, spheroplasts were incubated with different C.EE concentrations and then with S buffer instead of H_2O_2. A statistically significant increase in comet tail length was observed only with 300 µg/mL C.EE when compared with cells treated with 2% ethanol (Fig. 2B), indicating that C.EE has also genotoxic activity. DNA damage induced by 300 µg/mL C.EE alone (Fig. 2B) is similar to that found in C.EE pre-treated cells when subjected to H_2O_2 (Fig. 2A), suggesting that the potential for genoprotection is constrained by its genotoxicity. These results seem to support the hypothesis that C.EE may exert its antioxidant/antigenotoxic activity through a mild geno-insult enabling cells to adapt to subsequent genotoxic oxidative stresses like that induced by H_2O_2.

Similarly to what was done in the cell viability assays, co-incubation experiments using yeast spheroplasts were performed to investigate if C.EE can protect DNA from damage under oxidative stress conditions, presumably by direct ROS detoxification. In this experiment spheroplasts were incubated with C.EE and 10 mM H_2O_2 for 20 min, after which DNA damage was analysed as before. As observed in pre-incubation experiments, H_2O_2 increased dramatically comet tail length (Fig. 2C) and when yeast spheroplasts were treated with H_2O_2 and C.EE simultaneously, a significant decrease of DNA damage was observed (Fig. 2C). Hence these results suggest that reducing/scavenging activities by some C.EE compounds may be protecting cells against H_2O_2-induced oxidative DNA damage. Genotoxicity of C.EE was also investigated after incubation of spheroplasts with propolis extract without H_2O_2 and omitting the subsequent step of incubation of 20 min that allows DNA damage repair. A significant increase in tail length was observed for all concentrations tested (with S buffer) when compared with the ethanol control (Fig. 2D), indicating that C.EE acted as a genotoxic agent to *S. cerevisiae* cells. These results together with the decrease of viability observed for 100 µg/mL and 300 µg/mL C.EE (Fig. 1D and 1E) indicate that DNA damage is possibly involved in C.EE-induced loss of cell viability. In fact, propolis flavonoids, as effective scavengers of free radicals and other reactive species *in vitro*, may explain a decrease in oxidative DNA damage *in vivo*[33]. So, antigenotoxicity and genotoxicity were observed in both pre-incubation and co-incubation experiments, which strongly supports a dual activity of C.EE propolis regarding DNA integrity. A similar behaviour regarding genotoxic and antigenotoxic dual role of propolis has been previously reported by Tavares and co-workers[34] for a Brazilian propolis sample. However, in that study, the antigenotoxic activity was observed against the DNA intercalating chemotherapeutic drug doxorubicin.

It is interesting to note that lower concentrations of C.EE (25 µg/mL and 100 µg/mL) displayed genotoxicity only in co-incubation experiments where damage was analysed immediately after incubation unlike the pre-incubation experiments, which included an additional incubation with S buffer (Figs. 2B and 2D). Repair of DNA damage during this incubation may be the reason explaining the difference observed in both experiments. In fact, DNA repair activity during incubation of yeast cells with S buffer, as has been reported before[15], could eliminate DNA injuries caused by C.EE in pre-incubation up to a maximum C.EE concentration, after which the amount of damage caused being presumably in excess to the repairing capacity of cells.

Genotoxicity detected in the alkaline comet assay (Figs. 2B and 2D) argues also in favour of a pro-oxidant activity. Longer comet tails are caused by single strand and double strand breaks, the former being one of the DNA lesions typically detected in the alkaline version of the comet assay as a result of oxidative DNA damage[35]. Nevertheless, the possibility of a different non-oxidative stressing activity by propolis cannot be disregarded since these complex mixtures might contain genotoxic compounds that could lead to similar results as those presented in this work. The finding that propolis extract can enhance the antitumor activity of irinotecan, which induces DNA double-strand breaks by a non-oxidative mechanism[36] provides support to this idea. Also, many propolis compounds, namely most of those found in C.EE, have been reported to have anti-proliferative, anti-tumour and anticancer effects. For instance, caffeic acid, CAPE, quercetin, apigenin, kaempferol, chrysin and galangin all exhibit antitumor activity (for a thorough review see[33]). Quercetin 3-methyl ether, a methoxylated flavonoid, has potent anticancer-promoting activity by inducing cell cycle G2–M phase accumulation[37]. Chrysin inhibits DNA synthesis by G1 cell cycle arrest in C6 glioma cells[38]. Even at low concentrations ellagic acid interacts synergistically with quercetin enhancing the anticarcinogenic activity of the individual counterparts[39]. Pro-oxidant activity of C.EE may be responsible for its toxicity, but could also have an indirect role in protection due to induction of antioxidant defences and xenobiotic metabolizing enzymes by the imposition of a mild oxidative stress, which may contribute to a more effective cytoprotection[40].

C.EE decreases intracellular oxidation in pre-incubation and co-incubation experiments

To investigate if the antioxidant effect of C.EE in the presence of H_2O_2 is mediated by a decrease in intracellular oxidation level, cells under co-incubation conditions were analysed using flow cytometry and H_2DCFDA as the fluorescent redox-sensitive probe. This lipophilic compound permeates the cells and is deacetylated to dichlorofluorescein by intracellular esterases. The deacetylated

form is hydrophilic and becomes trapped inside the cells. In the presence of oxidants, it oxidizes and fluoresces with a maximum of excitation at 485 nm and of emission at 530 nm. As depicted in Fig. 3 the presence of H_2O_2 induced a significant peak displacement towards higher levels of fluorescence (Figs. 3A and 3D), revealing an increase in intracellular oxidation. Treatment with C.EE decreased this intracellular fluorescence in a dose-dependent manner (Figs. 3G, 3J and 3M). Inspection of cells by bright-field (Figs. 3B, 3E, 3H, 3K and 3N) and fluorescence (Figs. 3C, 3F, 3I, 3L and 3O) microscopy confirmed both, the intracellular origin of fluorescence and the dose-dependent effect on fluorescence decrease by the extract.

The same approach was used to investigate the antioxidant activity of C.EE in pre-incubation and post-incubation conditions, similarly to experiments of viability and the comet assay (Fig. 4). The antioxidant activity of the extract was still present in pre-incubation experiments in all concentrations tested (Figs. 4C, 4D and 4E). Interestingly, prior incubation of cells with the extract completely abolished the oxidative effect of H_2O_2 as the intracellular fluorescence of dichlorofluorescein was similar (25 µg/ml C.EE; Fig. 4C) or lower (100 and 300 µg/ml C.EE; Figs. 4D and 4E, respectively) when compared to the negative control. These results correlate with the viability assays with pre-incubation of C.EE, where a decrease in the rate of loss of viability was observed (Figs. 1A and 1B) when compared with cells pre-incubated with the solvent (2% ethanol). As suggested by the loss of viability in co-incubation experiments in cells incubated only with C.EE (Figs. 1D and 1E), a pro-oxidant activity of C.EE is compatible with a mechanism of induction of the cellular response against oxidative stress. In addition, pre-incubation with 300 µg/ml C.EE did not decrease further the intracellular oxidation as it should be anticipated in a dose-dependent activity (Fig. 4E), suggesting that at this concentration the pro-oxidant activity might be too strong to yield an increase in the protective effect, which correlates with the absence of increase in viability protection when the concentrations of 100 and 300 µg/ml are compared (Figs. 1A and 1B). In post-incubation experiments an increase in intracellular oxidation was observed for all concentrations tested (Figs. 4H, 4I and 4J), which is in line with a pro-oxidant activity of C.EE.

The dose-dependent intracellular antioxidant activity in co-incubation experiments is in accordance with the observed protective effect in cell viability (Fig. 1) and the antigenotoxic activity upon oxidative shock (Fig. 2) and with the view of some compounds acting directly in quenching oxidative species[2]. The evaluation of propolis antioxidant potential is generally performed by *in vitro* assays (eg. DPPH, ABTS)[41,42,43]. Here, the antioxidant effects of C.EE were evaluated *in vivo* using an eukaryotic cell model, as others did before[44,45], which is of higher significance when considering applications in human cells and tissues or in a whole-body physiological context. However, in our study the antioxidant activity was investigated in cells under highly challenging conditions by exogenously added H_2O_2, which highlights the antioxidant potency of this propolis extract.

Conclusions

Globally, these results fit in the so-called "Janus" effect, which is used to classify compounds or mixtures that have a dual effect, one positive and one negative[46]. These dual and opposite effects of propolis have been also reported before by Tavares et al.[34] for Brazilian green propolis, which also acts as antigenotoxic against the DNA intercalating chemotherapeutic drug doxorubicin at low concentration and as genotoxic at high concentration. Here we provide evidence that the Janus effect of propolis is also present when cells are challenged with an oxidation-mediated DNA damaging agent such as H_2O_2 and that detection can be made with the alkaline version of the comet assay. In conclusion, depending of the dose, the studied propolis extract exhibits both genotoxic and antigenotoxic activities and acts as an intracellular antioxidant in cells challenged with H_2O_2. These conclusions highlight the need for careful formulation of propolis-based food and medical products and for biological monitoring of these products to avoid undesirable harming effects.

Acknowledgements

The authors are thankful to Cristina Ribeiro (Department of Biology, University of Minho) for her collaboration in flow cytometry experiments and to Pedro Fernandes, from Mel do Abel, who kindly collected and provided the propolis sample for the study. This work is supported by national funds by FCT - Portuguese Foundation for Science and Technology, under the project PEst-OE/AGR/UI4033/2014.

Notes and references

‡ The authors declare that there are no conflicts of interest.

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Journal Name

ARTICLE

Legends to Figures

Fig. 1. Influence of pre-incubation (A and B), co-incubation (C, D and E) and post-incubation (F, G and H) with C.EE on the kinetics of loss of viability of *S. cerevisiae* cells exposed to oxidative stress. Yeast cells were incubated with C.EE (25 µg/mL, C and F; 100 µg/mL, A, D and G; or 300 µg/mL, B, E and H) and with 5 mM H_2O_2 (see Experimental for details). At each time-point an aliquot was collected, diluted and spread on YPD plates. Colonies were counted after 48 h of incubation at 30 ^oC and viability was calculated as percentage, taking time 0 min as reference (100% viability). Data are the mean±SD of three independent experiments. ** means $0.001 < p \leq 0.01$ between samples treated with H_2O_2 and samples treated with H_2O_2 and C.EE (pre-incubation and co-incubation). §§ means $0.001 < p \leq 0.01$ between C.EE-treated samples and those treated with the solvent of C.EE (pre-incubation).

Fig. 2. Antigenotoxicity of C.EE in *S. cerevisiae* cells exposed to oxidative stress (A and C) and genotoxicity of C.EE in *S. cerevisiae* cells (B and D). Yeast spheroplasts were pre-treated with C.EE (25 µg/mL, 100 µg/mL and 300 µg/mL) for 20 min, washed with S buffer and incubated with 10 mM H_2O_2 (A) or S buffer (B) for further 20 min. In co-incubation experiments, spheroplasts were incubated with C.EE (25 µg/mL, 100 µg/mL or 300 µg/mL) and 10 mM H_2O_2 for 20 min (C) or with C.EE and S buffer for 20 min, which was used instead of H_2O_2 (D). DNA damage was analysed with the yeast comet assay (see Experimental section). Controls included untreated cells as well as cells treated with the solvent of C.EE (ethanol; 2% final concentration as in the assays with C.EE). Mean±SD values are from three independent experiments (* represents $0.01 < p \leq 0.05$, ** represents $0.001 < p \leq 0.01$ and *** $p \leq 0.001$).

Fig. 3. Intracellular oxidation of *S. cerevisiae* cells exposed to H_2O_2 is decreased in the presence of C.EE. Cells were loaded with H_2DCFDA and treated simultaneously with 2% ethanol (A, B and C), 10 mM H_2O_2 (D, E and F) or with 10 mM H_2O_2 and C.EE (G, H and I: 25 µg/mL; J, K and L: 100 µg/mL; M, N and O: 300 µg/mL) simultaneously for 20 min and analysed for fluorescence by flow cytometry (A, D, G, J and M), bright-field microscopy (B, E, H, K and N) and fluorescence microscopy (C, F, I, L and O). Data are from a representative experiment from three independent experiments. Bar = 10 µm.

Fig. 4. Intracellular oxidation of *S. cerevisiae* cells pre-incubated with C.EE and exposed to H_2O_2 is decreased while post-incubation with C.EE aggravates intracellular oxidation. Cells were loaded with H_2DCFDA, incubated with 3% ethanol (A and B), or C.EE (C: 25 µg/ml; D: 100 µg/ml; E: 300 µg/ml), washed and incubated with H_2O (A) or 10 mM H_2O_2 (B, C, D and E). Alternatively, H2DCFDA-loaded cells were incubated with H_2O (F), or 10 mM H_2O_2 (G, H, I and J), washed and incubated with 3% ethanol (F and G) or C.EE (H: 25 µg/ml; I: 100 µg/ml; J: 300 µg/ml). All cells were analysed for fluorescence by flow cytometry. Data are from a representative experiment from three independent experiments.

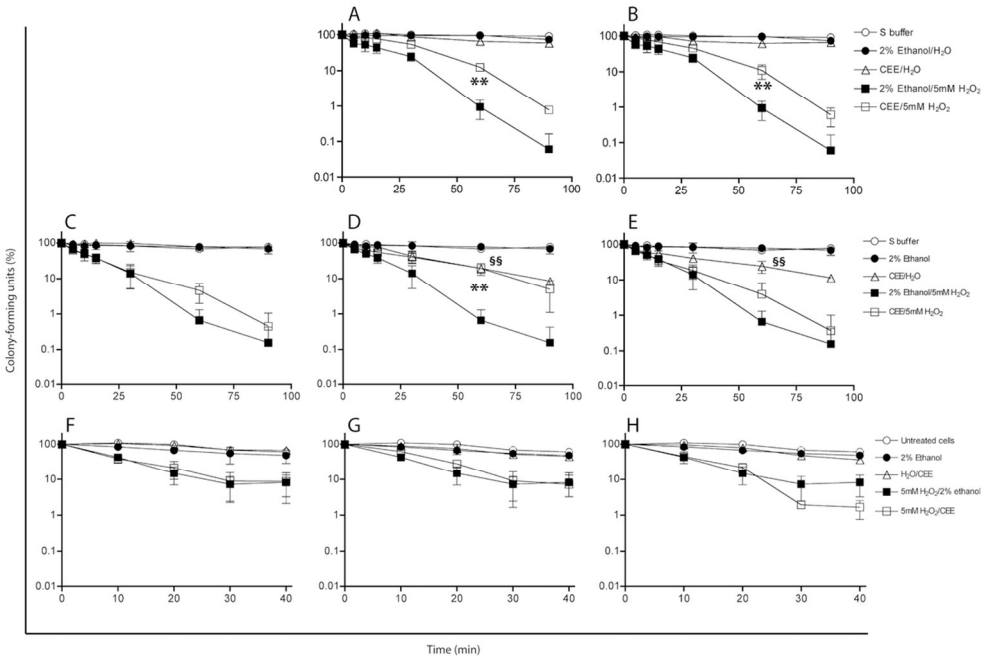

Fig. 1. Influence of pre-incubation (A and B), co-incubation (C, D and E) and post-incubation (F, G and H) with C.EE on the kinetics of loss of viability of S. cerevisiae cells exposed to oxidative stress. Yeast cells were incubated with C.EE (25 µg/mL, C and F; 100 µg/mL, A, D and G; or 300 µg/mL, B, E and H) and with 5 mM H2O2 (see Experimental for details). At each time-point an aliquot was collected, diluted and spread on YPD plates. Colonies were counted after 48 h of incubation at 30 ºC and viability was calculated as percentage, taking time 0 min as reference (100% viability). Data are the mean±SD of three independent experiments. ** means $0.001 < p \leq 0.01$ between samples treated with H2O2 and samples treated with H2O2 and C.EE (pre-incubation and co-incubation). §§ means $0.001 < p \leq 0.01$ between C.EE-treated samples and those treated with the solvent of C.EE (pre-incubation).
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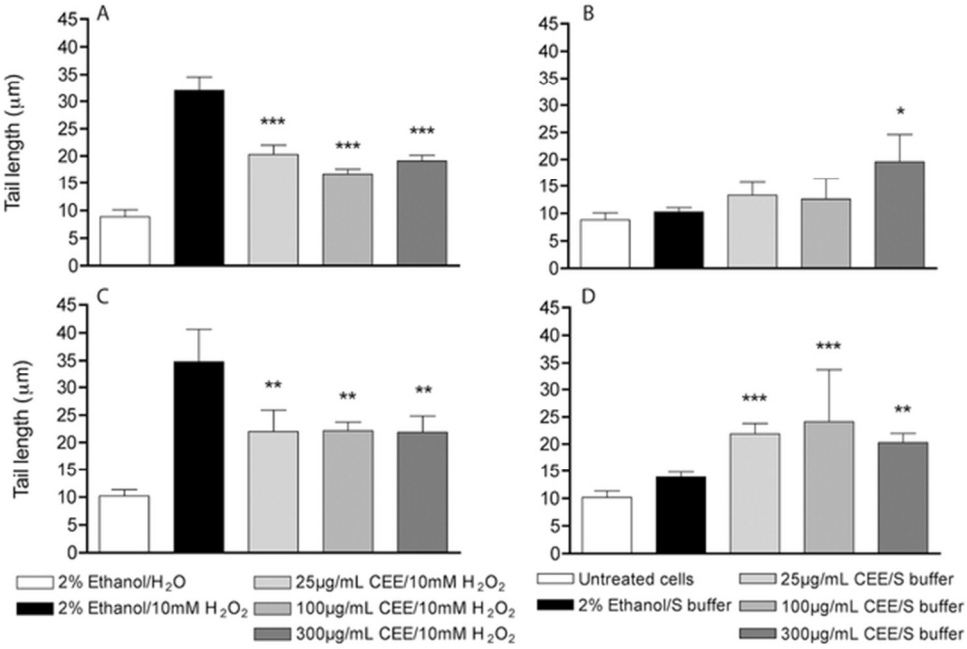

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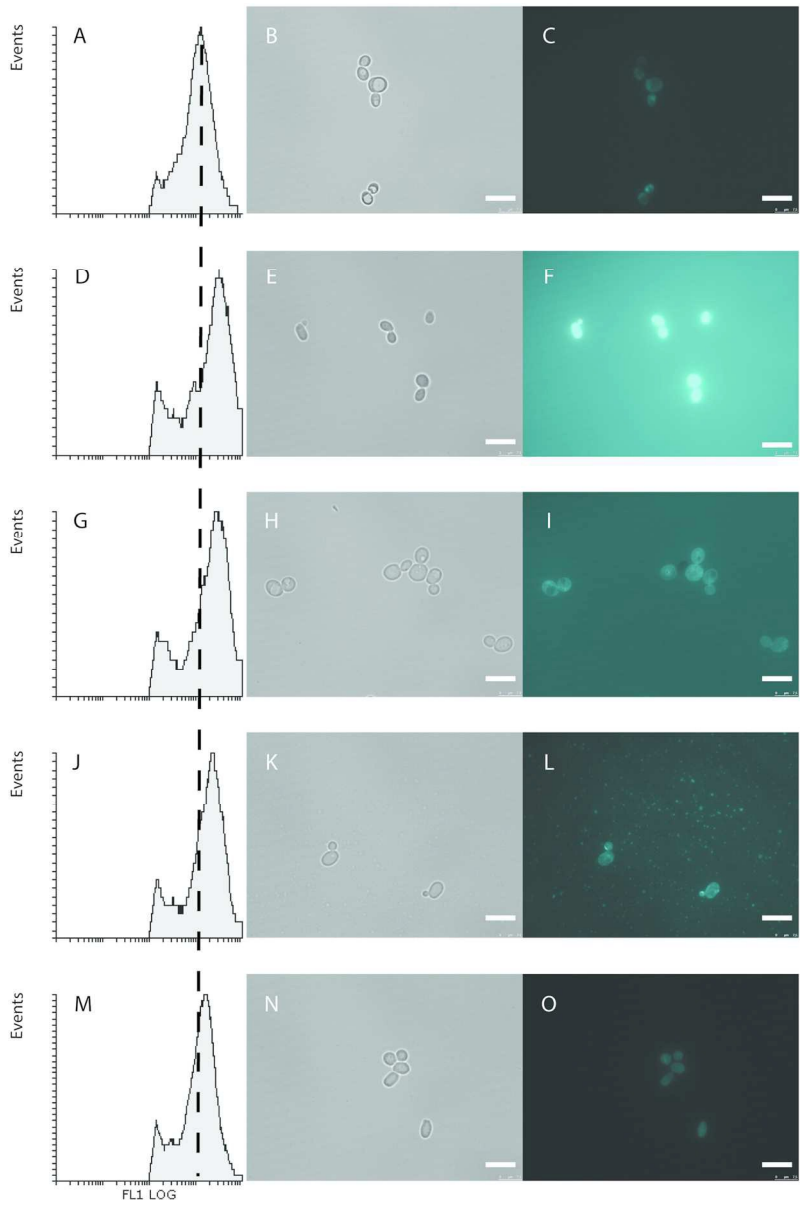

Fig. 3. Intracellular oxidation of S. cerevisiae cells exposed to H2O2 is decreased in the presence of C.EE. Cells were loaded with H2DCFDA and treated simultaneously with 2% ethanol (A, B and C), 10 mM H2O2 (D, E and F) or with 10 mM H2O2 and C.EE (G, H and I: 25 µg/mL; J, K and L: 100 µg/mL; M, N and O: 300 µg/mL) simultaneously for 20 min and analysed for fluorescence by flow cytometry (A, D, G, J and M), bright-field microscopy (B, E, H, K and N) and fluorescence microscopy (C, F, I, L and O). Data are from a representative experiment from three independent experiments. Bar = 10 µm.
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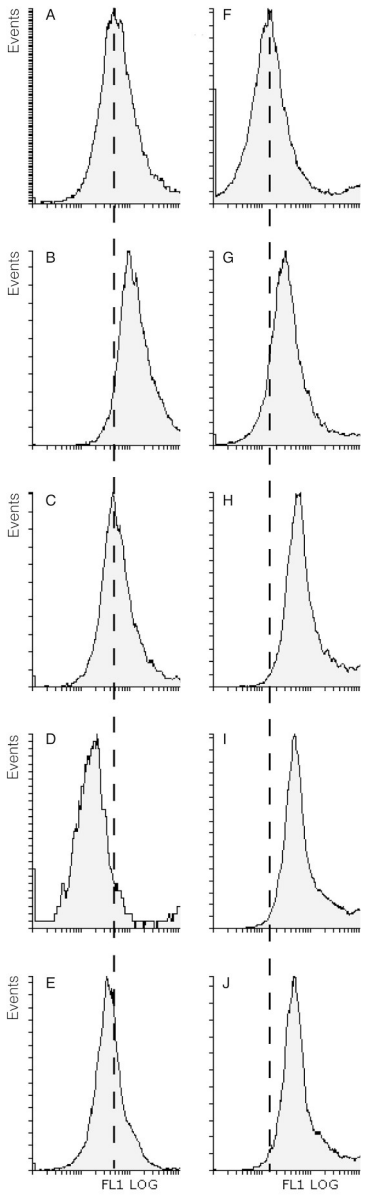

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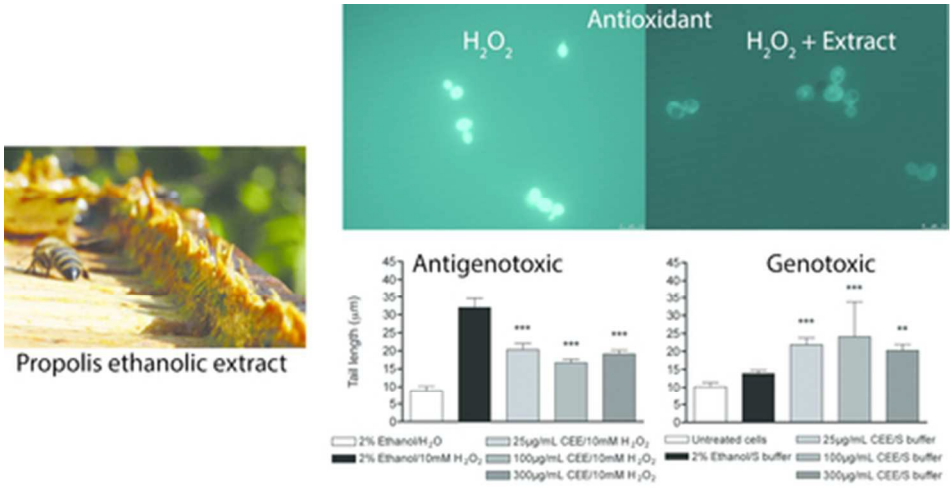
Abstract

Propolis is a resinous product made by honeybees from plant-derived materials, with high content of polyphenols associated to several beneficial bioactivities with potential of use as natural food additive for preservation and as functional food ingredient. A Portuguese propolis ethanol extract (C.EE) protected *Saccharomyces cerevisiae* cells from loss of viability upon exposure to H_2O_2, both in co- and in pre-incubation experiments. Results obtained with the comet assay suggest that lower concentrations are antigenotoxic while at higher concentrations a genotoxic effect prevails, which correlates with cytotoxicity of high concentrations of C.EE. Flow cytometry analysis with dichlorofluorescein indicates that C.EE induced intracellular antioxidant activity *in vivo*. Overall the results suggest that C.EE is antigenotoxic but is also toxic at higher concentrations. This dual effect could be explained by with the presence of compounds known to interfere with DNA synthesis and/or cell proliferation, such as caffeic acid phenethyl esther (CAPE) and chrysin, together with antioxidants, like kaempferol, pinobanksin and pinocembrin.



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