Stimulation of DNA repair in *Saccharomyces cerevisiae* by *Ginkgo biloba* leaf extract

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Abbreviations[1]

[1] BER, base excision repair; GBE, *G. biloba* leaf extract; H_2DCF, dichlorofluorescein; H_2DCFDA, dichlorofluorescein diacetate; LMA, low-melting agarose; NER, nucleotide excision repair; OD_{600}, optical density at 600nm; ROS, reactive oxygen species; rpm, revolutions per minute; SD, standard deviation.

Abstract

Many extracts prepared from plants traditionally used for medicinal applications contain a variety of phytochemicals with antioxidant and antigenotoxic activity. In this work we measured the DNA protective effect of extracts of *Ginkgo biloba* leaves from oxidative stress using *Saccharomyces cerevisiae* as experimental model. The extract improved viability of yeast cells under oxidative stress imposed by hydrogen peroxide. In accordance with previous reports on antioxidant properties of *G. biloba* extracts, pre-incubation of yeast cells promoted a decrease in intracellular oxidation. We assessed DNA damage by our recently developed yeast comet assay protocol. Upon oxidative shock, DNA damage decreased in a dose-dependent manner in experiments of pre-incubation and simultaneous incubation with the extract, indicating a direct protective effect. In addition, the extract improved DNA repair rate following oxidative shock as measured by faster disappearance of comet tails. This suggests that the extract stimulates the DNA repair machinery in its DNA protective action in addition to directly protect DNA from oxidation. The observed DNA repair depends on the DNA repair machinery since no DNA repair was observed under restrictive conditions in a conditional mutant of the *CDC9* gene (Accession No. Z74212), encoding the DNA ligase involved in the final step of both nucleotide and base excision repair.

Keywords: Oxidative stress, antigenotoxic, antioxidant, yeast comet assay, *Ginkgo biloba, Saccharomyces cerevisiae*

1. Introduction

Extracts prepared from plants traditionally used for medicinal applications have received considerable attention owing to their potential health benefits as therapeutic agents, especially for aging and age-related diseases (Zheng and Wang, 2001; Silva et al., 2005; Manach et al., 2009; Tosetti et al., 2009). Phytochemicals found in these extracts are often difficult to purify and synthesize and biological activity often depends on synergistic effects between compounds.

Ginkgo biloba leaf extracts, some of the best-selling antioxidant medicinal products worldwide, contain ginkgo flavone glycosides (quercetin, kaempferol, isorhamnetin) and ginkgo terpene lactones (ginkgolides, bilobalide), which are believed to be responsible for most of the biological properties (Wei et al., 2000; Tendi et al., 2002; Smith and Luo, 2004; van Beek, 2005; Altiok et al., 2006). Extracts from *G. biloba* leaves have been suggested by several studies to possess numerous beneficial properties, including antioxidant or free radical scavenging (Silva et al., 2005; Wei et al., 2000; Tendi et al., 2002; Altiok et al., 2006; Thiagarajan et al., 2002; Stromgaard and Nakanishi, 2004; Liu et al., 2006; Yeh et al., 2009), antiapoptotic (Wei et al., 2000; Altiok et al., 2006; Thiagarajan et al., 2002; Yeh et al., 2009; Schindowski et al., 2001; Wu et al., 2008), antiaging (Schindowski et al., 2001) and antigenotoxic (Vilar et al., 2009). They have also been described to regulate gene expression (Tendi et al., 2002; Stromgaard and Nakanishi, 2004; Gohil, 2002; Augustin et al., 2009; Bidon et al., 2009).

Reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and H_2O_2, pose a significant threat to cellular integrity. In the presence of redox-active metal ions, such as Fe^{2+}, $O_2^{\bullet}$ and H_2O_2 can undergo Fenton chemistry, generating the

extremely reactive $^{\bullet}$OH, which attacks almost all cell components, including DNA (Henle et al., 1996). High levels of ROS, formed through both endogenous and exogenous routes, and the DNA damage it produces contribute to genetic instability. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain that diffuse out freely through membranes and attack other cellular components, while exogenous ROS occurs through exposure to numerous exogenous agents including ionizing radiation, ultraviolet radiation, chemotherapeutic drugs, environmental toxins and hyperthermia (Salmon et al., 2004). Cells are exposed permanently to oxidative challenge imposed by the environment and by the oxidative metabolism in mitochondria. Incapacity of the cellular defences to cope with the oxidative challenge generates oxidative stress, which can cause oxidative damage in macromolecules, including genomic DNA (Boiteux and Guillet, 2004). DNA lesions such as DNA base modifications, single- and double-strand breaks, and the formation of apurinic/apyrimidinic sites may be formed (Salmon et al., 2004). Once DNA damage is sensed, cell cycle is arrested so that repair mechanisms can operate, or induction of programmed cell death may take place if damage cannot be repaired. The major DNA repair pathways are base excision repair (BER) in the removal of damage of single bases caused by oxidation and nucleotide excision repair (NER), which is involved in repairing bulky DNA lesions caused by ultra-violet light (Friedberg, 2003). A DNA ligase encoded by the mammalian *LIG1* (Accession No. NG_007395.1), orthologous of the budding yeast *CDC9*, completes the repair process for both BER and NER (Wu et al., 1999).

The antigenotoxic activity of many phytochemicals and plant extracts can be attributed to their antioxidant properties, which allows protection of DNA from oxidative damage. Despite the fact that a large number of studies concern the antioxidant properties of *G. biloba* extracts, only few have associated these extracts with antigenotoxicity (Vilar et al., 2009). We investigated viability, intracellular oxidation as well as DNA damage and repair by our

recently developed protocol of comet assay applied to yeast (Azevedo et al., 2010), in cells treated with *G. biloba* extract and exposed to oxidative stress imposed by H_2O_2. Our results show that *G. biloba* leaf extract protects genomic DNA against oxidative stress and suggest that the two modes of action are present, direct protection from oxidation and stimulation of DNA repair.

2. Materials and Methods

2.1. Plant material and extract preparation

Ginkgo biloba leaves were collected in autumn (October) from a specimen located in an urban area of Braga, Portugal (University of Minho in Braga, Campus map coordinates 41.559223,-8.397503). Preparation of the water extract (GBE) was performed as described elsewhere (Ding et al., 2004). Leaves were washed with deionized H_2O, cut to exclude petioles and air-dried at room temperature in the dark for one week. Dried leaves were pulverized with a pestle into a fine powder and stored in nontransparent glass bottles until used for extraction. Five grams of powder were transferred into 200 mL polypropylene centrifuge tubes with 30 mL of boiling deionized H_2O, heated in a water bath at 100ºC for 5 min and centrifuged at 2000 *g* for 15 min. The extraction process was repeated once with the pellet, the supernatants were pooled, cleaned by filtration with 0.5 µm filters, adjusted to pH 6.5 with NaOH and stored in aliquots at -20ºC. The standardized *G. biloba* extract EGb 761 (kindly provided by Schwabe Pharmaceuticals, Germany) was used as benchmark in the comet assay experiments.

2.2. Yeast strains, culture media, and growth conditions

The haploid *Saccharomyces cerevisiae* strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al., 1998), *S. cerevisiae* NK427 (*MATa ura3-52 trp1-289 leu2-3,112 bar1::LEU2 cdc9-1*) carrying a temperature-sensitive mutation in the *CDC9* gene and its reference strain, NK1 (*MATa ura3-52 trp1-289 leu2-3,112 bar1::LEU2*) (Makovets et al., 2004) were used in this work. Stocks of these strains were maintained on standard solid

YPD medium (1% w/v BD Bacto™ yeast extract, 2% w/v BD Bacto™ peptone, 2% w/v glucose) with 2% (w/v) agar at room temperature. Yeast cells were grown on 50 mL liquid YPD medium in an Erlenmeyer flask with air-liquid ratio of 5/1 at 30ºC (strain BY4741) or 23ºC (for strains NK1 and NK427) and 200 revolutions per minute (rpm). Growth was monitored by optical density at 600 nm (OD_{600}).

2.3. Viability measurement

A liquid pre-culture of 5-10 mL was prepared with a single yeast colony and grown overnight. The culture was diluted with fresh medium to a density of $1.2x10^7$ cells/mL and harvested by centrifugation (2 min at 5000 rpm, 4ºC) after two generations. Cells were subsequently washed twice, each time with one culture volume of ice-cold deionized H_2O, and diluted back to $1.2x10^7$ cells/mL in ice-cold S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Pre-treatment with GBE was made by addition of one fifth of a volume GBE in S buffer to the cells suspended in S buffer. Cells and GBE were incubated at 30ºC, 200 rpm for 20 min, washed with one volume of deionized H_2O at 4ºC and resuspended in an equal volume S buffer. One hundred microliters of this suspension was harvested, serially diluted to 10^{-4} in deionized sterilized H_2O and 100 μL were spread on solid YPD medium. Hydrogen peroxide (Merck, Germany) was immediately added to the undiluted suspension (5 mM final concentration) and incubated at 30ºC, 200 rpm. The same procedure for harvesting and plating cells was followed at different time points, all plates were incubated at 30ºC for 48 h and the colonies counted. Survival rates were calculated as percentage of colony forming units at each time point in relation to the beginning of the experiment (0 min).

2.4. Analysis of antioxidant activity

Cells were prepared in the same way as for viability measurement, except that they were diluted to a density of $1.0x10^6$ cells/mL and suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), instead of S buffer. Five hundred microlitres of untreated cells were removed for auto fluorescence measurement. Dichlorofluorescein diacetate (H_2DCFDA; Sigma-Aldrich) (50 µM final concentration) was added to the reminder of the cells and cell suspension was further incubated at 30ºC, 200 rpm for 1 h in the dark, washed twice with the same volume of PBS and distributed in aliquots for the different assay conditions. Cells were subsequently sediment by centrifugation at 5000 rpm, 4ºC for 2 min and suspended in GBE diluted in PBS. Samples were incubated at 30ºC, 200 rpm for 20 min, washed twice with the same volume of PBS and treated with 10 mM H_2O_2 and incubated at 30ºC, 200 rpm for 20 min. Twenty thousand cells of each sample were analyzed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with a 15 mW argon-ion laser emitting at 488 nm. 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 analyzed and histograms were made with the WinMDI 2.8 software.

2.5. Analysis of DNA damage

Analysis of DNA damage was performed with the comet assay as described before (Azevedo et al., 2010). Briefly, cell walls were digested with 2 mg/mL zymolyase (20,000 U/g; ImmunO™ - 20T), spheroplasts were suspended in GBE diluted in S buffer, so that osmotic protection by 1 M sorbitol is maintained, incubated at 30ºC for 20 min and collected by centrifugation at 15300 rpm, 4ºC for 2 min. Treated spheroplasts were then washed, embedded in 1.5% (w/v) low melting agarose (LMA) at 35ºC and distributed by glass slides.

Spheroplasts were then exposed to the oxidant solution (10 mM H_2O_2) for 20 min at 4ºC, washed with S buffer for 5 min and submerged in the lysing buffer (30 mM NaOH, 1 M NaCl, 0.05 % w/v laurylsarcosine, 50 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min in order to lyse spheroplasts. Samples were washed with electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min and samples were then submitted to electrophoresis in the same buffer for 10 min at 0.7 V/cm. After electrophoresis, the slides were incubated in neutralization buffer (10 mM Tris-HCl, pH 7.4) for 10 min, followed by consecutive 10 min incubations in 76 and 96% (v/v) ethanol. Then, the slides were dried at room temperature and were visualized immediately or stored at 4ºC until visualization. For visualization in a fluorescence microscope (Leica Microsystems DM fluorescence) slides were stained with GelRed (10 μg/mL; Biotium) and representative images were acquired at magnification of 400× in order to obtain at least 20 random comets per sample that were analyzed with the CometScore software for the tail length. Error bars represent variability between the mean of at least three different slides obtained from biologically independent experiments.

2.6. Analysis of DNA Repair

Analysis of DNA repair was performed with the comet assay as described before (Azevedo et al., 2010). Here, treatments were all performed with spheroplasts in suspension before embedding in LMA. After an incubation of 20 min at 4ºC with 10 mM H_2O_2 (in S buffer), spheroplasts of each sample were collected by centrifugation at 15300 rpm, 4ºC for 2 min, washed with 80 μL of S buffer and then resuspended in 80 μL of GBE diluted in S buffer (or only S buffer for the control). Samples were incubated at 37ºC for different periods of time until 20 min to allow DNA repair, collected by centrifugation at 15300 rpm, 4ºC for 2

min and incorporated in LMA. The described procedure for the comet assay (Azevedo et al., 2010) was followed afterwards. When using the *cdc9* conditional mutant, incubation at the restrictive temperature of 37ºC (Makovets et al., 2004) for 1h was performed before treatment with H_2O_2 to allow complete inactivation of Cdc9p.

2.7. Statistical analyses

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

3. Results

3.1. GBE increases viability of S. cerevisiae cells under oxidative stress

To investigate the protective effect of GBE against oxidative stress we assessed viability of yeast cells in the presence of H_2O_2. *Saccharomyces cerevisiae* cells were incubated for 20 min with GBE diluted 6 fold in S buffer and washed with S buffer without GBE. Hydrogen peroxide in S buffer was subsequently added to a final concentration of 5 mM before incubation at 30ºC. Aliquots of the suspension were harvested at different time points, diluted and plated on rich medium in order to count colonies after 48 h incubation at 30ºC. Relative cell survival at each time point was calculated as the percentage in relation to

the beginning of the experiment (0 min). Yeast cells exposed to 5 mM H_2O_2 lost viability after little more than 50 min treatment (Fig. 1). Pre-incubation with GBE rescued the cells so that 1% viable cells remained even after 200 min. As expected, cells without any treatment or GBE alone (results not shown) displayed a nearly constant viable count during the 200 min incubation.

3.2. GBE decreases intracellular oxidation

The observed higher rate of survival of GBE-treated yeast cells under oxidative stress could be due to an antioxidant effect as reported in previous studies (Silva et al., 2005; Wei et al., 2000; Tendi et al., 2002; Altiok et al., 2006; Thiagarajan et al., 2002; Stromgaard and Nakanishi, 2004; Liu et al., 2006; Yeh et al., 2009). We investigated the antioxidant activity of GBE by flow cytometry with the fluorochrome H_2DCFDA. The diacetate form of dichlorofluorescein is able to diffuse freely through the plasma membrane into cells where intracellular esterases deacetylate H_2DCFDA to dichlorofluorescein (H_2DCF), which is accumulated since it does not permeate membranes. Oxidation increases fluorescence of H_2DCF so whole cell fluorescence can be used as a marker for intracellular oxidation.
To investigate if GBE protects cells generally from innate oxidative stress, we incubated mid-log growth phase cells with H_2DCFDA, subsequently with GBE diluted two fold in PBS for 20 min and then measured fluorescence in the flow cytometer. As can be seen in Fig. 2 (inset), fluorescence of the population of cells shifted down after incubation with GBE, indicating that the effect of the extract causes a general decrease of the oxidation state of the cells. Oxidative shock by H_2O_2 induces an increase of intracellular oxidation and, hence, higher fluorescence compared to non-treated cells (Figs. 2A and B). When cells were treated with GBE diluted two fold in PBS before the oxidative shock, fluorescence decreased

significantly, suggesting that GBE neutralize or stimulate protection in yeast cells against oxidative shock (Fig. 2C). Antioxidant activity was also present when GBE was further diluted in PBS: 4, 10 and 20 fold (not shown).

3.3. GBE protects yeast cells against DNA damage by H_2O_2

We used our recently developed protocol of comet assay applied to yeast cells (Azevedo et al., 2010) to evaluate if GBE may prevent DNA damage in addition to or as a consequence of its antioxidant activity. Yeast spheroplasts were pre-treated with various GBE dilutions in S buffer in order to maintain osmotic protection, subsequently exposed to 10 mM H_2O_2 and analyzed for DNA damage. Deionized water, S buffer or GBE/2 (GBE diluted 2 fold) did not cause DNA damage (Fig. 3A). Hydrogen peroxide dramatically increased comet tail length, which was not alleviated by prior incubation with S buffer (Fig. 3A H_2O_2, S buffer + H_2O_2). When yeast spheroplasts were pre-treated with GBE before exposure to H_2O_2 (Fig. 3A, GBE/2-100 + H_2O_2), a statistically significant dose-dependent decrease in comet tail length was observed, when compared to controls (Fig. 3A, S buffer + H_2O_2). These results are in accordance with a previous report (Wei et al., 2000), describing that the standardized *G. biloba* leaf extract, EGb 761, attenuates oxidative DNA damage induced by H_2O_2 in rat cereberal granular cells. Interestingly, incubation with GBE/2 without subsequent H_2O_2 treatment decreased comet tail length when compared with incubation with S buffer or deionized H_2O alone. This suggests that the GBE may decrease background DNA damage caused by endogenous sources in a given yeast population. Finally, although differences are not statistically significant, GBE seems to have some protection effect on DNA even at 500 and 1,000 fold dilutions, only being similar to control at 10,000 fold (supplementary file 1).

Results obtained in pre-incubation experiments suggest that GBE induces adaptation of cells against oxidative DNA damage. As we show in Fig. 2, intracellular oxidation is decreased when cells are treated with GBE, therefore, a direct antioxidant activity on oxidants would prevent DNA damage. To test this, we performed the experiments with co-incubation to allow contact of GBE with H_2O_2 in the presence of yeast cells. Results of control experiments and of GBE activity assays (Fig. 3B) were comparable, although with slightly less protection in the presence of GBE compared to pre-treatment experiments (Fig. 3A). This suggests that GBE has a direct inactivating effect on H_2O_2, possibly by scavenging ROS.

We also tested the activity of the standardized EGb 761 extract with the comet assay, which is made from *G. biloba* leaves and has known relative composition of flavone glycosides, terpene trilactones and ginkgolic acids, the main unique compounds of this species. This extract is frequently used in experiments related with *G. biloba* effects and is commercialized as a medicinal product worldwide. The assays were performed under the same conditions as mentioned for the pre-treatment assays of our extract. Results obtained were similar to results obtained with our extract (not shown), which suggests that the active compounds with antigenotoxic activity are present in both preparations.

3.4. GBE improves the DNA repair ability in yeast cells

To investigate the possibility of an activating effect on DNA damage repair mechanisms by GBE, we decided to assess DNA repair kinetics using a modification of the comet assay optimized for yeast cells (Azevedo et al., 2010). DNA repair experiments consisted in provoking DNA damage in yeast spheroplasts with 10 mM H_2O_2 for 20 min and then allowing DNA repair with or without GBE diluted two fold in S buffer. DNA repair was measured as the decrease in comet tail length observed over time. Comet tails length

decreased during the 20 min incubation both, with or without GBE (Fig. 4A). However, in GBE-treated cells, comet tails decreased more rapidly and were not detectable after 10 min incubation. This result supports the involvement of GBE not only in DNA damage prevention, but also in DNA repair mechanisms.

3.5. GBE does not improve the DNA repair ability in a cdc9 mutant strain

The comet tail shortening over time observed in Fig. 4A, was interpreted as DNA repair, although a non-specific process such as DNA degradation could not be excluded. In order to confirm that the observed DNA damage repair depended on DNA repair mechanisms, we hypothesized that a mutant strain deficient in DNA damage repair would not display the decrease in comet tail length observed in Fig. 4A. We chose to test DNA repair in a conditional temperature-sensitive mutant affected in the essential gene *CDC9* (Makovets et al., 2004). *CDC9* encodes a ligase involved in the final ligation steps of replication (Johnston, 1983), NER and BER (Wu et al., 1999). Mutants affected in this gene are deficient in ligation of Okazaki fragments in replication and in the ligation of the newly synthesized strands in the final steps of NER and BER. As shown in Fig. 4B, both yeast mutant and the parental strain displayed similar increase in DNA repair kinetics in presence of GBE at the permissive temperature of 23ºC. However, at 37ºC, the restrictive temperature (Fig. 4C), unlike the parental strain, GBE did not improve efficiency of DNA repair in the *cdc9* mutant. This insensitivity of the *cdc9* strain is expected since, upon oxidative damage, the lack of the ligation step will keep chromosomal DNA uncoiled, which will migrate in the electrophoresis as damaged DNA. These results (Fig. 4C) indicate that GBE is not efficient in DNA protection from oxidative damage in the absence of active Cdc9p and strongly suggest an involvement of GBE in DNA repair mechanisms.

4. Discussion

In this work we have investigated the protective effects of *G. biloba* leaf extract on genomic DNA upon oxidative shock. This extract improves yeast cells viability under oxidative stress, acts as antioxidant, has antigenotoxic effects and improves the DNA damage repair. We have used *S. cerevisiae* as experimental model to take advantage of the simplicity of manipulation and of the mutant strains and genetic tools available. These tools open the possibility for molecular biology approaches in the investigation of mechanisms of action of toxicants and protective compounds. The use of a conditional mutant strain affected in the essential gene *CDC9*, allowed us to obtain evidence supporting an induction of DNA damage repair pathways of yeast cells by GBE. Unlike the antioxidant activity of *G. biloba* extracts, the antigenotoxic activity has previously not been significantly investigated. Here, we provide the first evidence of this property.

In previous reports on the biological effect of *G. biloba* extracts, diverse activities that can be explained by the antioxidant properties of GBE are described. These include improvement of memory, antiasthma activity, increase of cerebral blood flow and circulation and beneficial effects in patients of Alzheimer's disease (Wei et al., 2000; Altiok et al., 2006; Thiagarajan et al., 2002; Stromgaard and Nakanishi, 2004). The antioxidant effect of GBE we report in this study (Fig. 2) is in accordance with reports in the literature (Silva et al., 2005; Wei et al., 2000; Tendi et al., 2002; Altiok et al., 2006; Thiagarajan et al., 2002; Stromgaard and Nakanishi, 2004; Liu et al., 2006; Yeh et al., 2009). In addition, our results show an increased viability in cells pre-treated with GBE (Fig. 1), probably caused by the antioxidant action of the extract. The mechanism of antioxidant action of GBE can be a direct inactivating effect on H_2O_2 by scavenging hydroxyl radicals or an activation of the cellular oxidative stress response, which would allow increased viability and decreased DNA damage under oxidative

stress. As we show in Fig. 2B, the antioxidant activity of GBE is present even without externally imposed oxidative shock. This suggests that there are compounds in the GBE that can scavenge directly endogenous ROS and/or efficiently recycle endogenous scavenger cellular proteins like glutathione, thioredoxin, superoxide dismutase, catalase, and/or induce the pentose phosphate pathway activity in regenerating NADPH.

A possible repercussion of an antioxidant activity is the protection of DNA against the strong reactivity of ROS. In our experiments of antigenotoxic activity of GBE, we have observed a statistically significant correlation between GBE dilutions and comets tail length in pre-treatment and simultaneous treatment experiments. Hence, this dose/response effect can be attributed to chemical components of the extract, which would have a scavenging effect on H_2O_2 and/or an induction of oxidative stress response and/or induction of DNA damage repair. Concurrent antioxidant and antigenotoxic properties have been reported for other plant extracts and phytochemicals. Digallic acid from *Pistacia lentiscus* (Bhouri et al., 2010), *Phyllanthus amarus* extract (Karuna et al., 2009), alizarin from *Rubia cordifolia* (Yen et al., 2000; Kaur et al., 2010), resveratrol (Quincozes-Santos et al., 2010), extra-virgin olive oil (Owen et al., 2000; Anter et al., 2010), black tea and green tea (Sinha et al., 2010) and biflorin from *Capraria biflora* (Vasconcellos et al., 2010) were all reported to have these properties against toxicity of H_2O_2. It is generally accepted that antioxidant and antigenotoxic activities found in plant extracts are from phenolic compounds as is suggested by the above mentioned phytochemicals and from further studies with different phenolic compounds like quercetin, epicatechin, luteolin, kaempferol, apigenin, bisabolol and protocatechuic acid (Najafzadeh et al., 2009; Rusak et al., 2010; Anter et al., 2011).

In accordance with the antioxidant activity in the absence of H_2O_2 treatment (Fig. 2B), pre-treatment with GBE decreased tail length in non-shocked cells (Fig. 3A), suggesting that GBE can protect DNA from endogenous damaging agents. The present report is one of few on

GBE antigenotoxic activity and, to our knowledge, the first time GBE is reported to be involved in DNA damage repair induction. We have included replicas with a standardized *G. biloba* leaf extract named EGb 761 (Schwabe Pharmaceuticals) with similar antigenotoxic activity, suggesting that the active compounds are present in both extracts. Therefore, our extract can be used for further studies aiming at the identification of the antigenotoxic compounds of GBE.

The determination of the mechanism of action of the active compounds can be studied by exploring the amenability of the genetic system of *S. cerevisiae*. The results obtained with the conditional *CDC9* mutant are the first evidence linking GBE activity to specific cellular pathways, potentially providing important insights on its mechanism of action.

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Legends to figures

Fig. 1. GBE increases viability of *S. cerevisiae* cells under oxidative stress. Yeast cells were incubated with S buffer (circles); S buffer containing 5mM H_2O_2 (squares); GBE diluted 6 fold in S buffer for 20min, washed and suspended in S buffer containing 5mM H_2O_2 (triangles); or GBE diluted 6 fold in S buffer (diamonds). At different time points, a 100μL aliquot was collected, diluted serially to 10^{-4} and plated on solid YPD medium. Colonies were counted after 48h incubation at 30ºC. In each time point survival was calculated as the percentage of colonies, assuming 100% at 0min. One representative experiment is shown from three independent experiments.

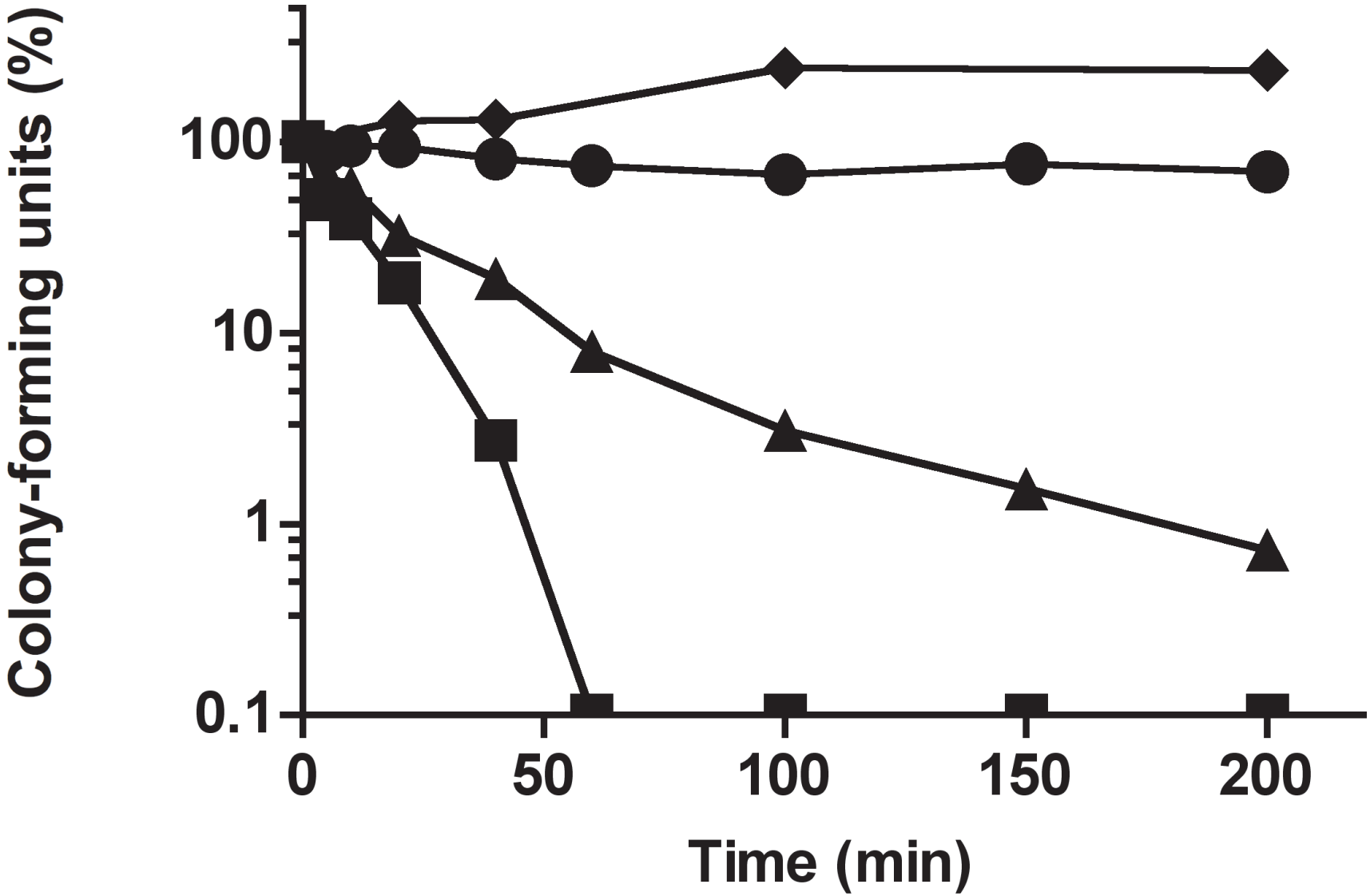
Fig. 2. GBE decreases intracellular oxidation in *S. cerevisiae* cells. Yeast cells were loaded with 50µM H_2DCFDA for 60min in the dark. After washing with PBS, cells were analysed by flow cytometry for fluorescence of the oxidised form of H_2DCF (A); or incubated with 10mM H_2O_2 for 20min, washed with PBS and analysed by flow cytometry (B); or incubated with GBE diluted two fold in PBS for 20min, washed with PBS, incubated with 10mM H_2O_2 for 20min, washed with PBS and analysed by flow cytometry (C). Inset: yeast cells were loaded with 50µM H_2DCFDA for 60min in the dark, washed with PBS and analysed by flow cytometry (shaded) or incubated with GBE diluted two fold in PBS for 20min prior to flow cytometry analysis (unshaded). Data are from one representative experiment from at least three independent experiments.

Fig. 3. GBE protects DNA of *S. cerevisiae* cells from oxidative damage by H_2O_2. A: yeast spheroplasts were incubated with GBE (diluted 2, 4, 20 or 100 fold in S buffer, respectively, GBE/2+H_2O_2, GBE/4+H_2O_2, GBE/20+H_2O_2 and GBE/100+H_2O_2), or S buffer (S buffer+H_2O_2), for 20min, washed with S buffer, and incubated with 10mM H_2O_2 for 20min. Positive control, (H_2O_2) represents treatment exclusively with 10mM H_2O_2; and negative controls (GBE/2, S buffer and H_2O) represent treatment exclusively with GBE diluted two fold in S buffer, S buffer and H_2O, respectively. DNA damage was analyzed with the comet assay method (see Materials and Methods). Mean ± SD values are from at least three independent experiments (*** represents $p < 0.001$). B: yeast spheroplasts were incubated with GBE (diluted 4, 20 or 100 fold in S buffer, respectively, GBE/4+H_2O_2, GBE/20+H_2O_2 and GBE/100+H_2O_2), or S buffer (S buffer+H_2O_2), simultaneously with 10mM H_2O_2 for 20min. Positive control, (H_2O_2) represents treatment exclusively with 10mM H_2O_2; and negative controls (GBE/4, S buffer and H_2O) represent treatment exclusively with GBE diluted four fold in S buffer, S buffer and H_2O, respectively. DNA damage was analyzed with

the comet assay method (see Materials and Methods). Mean ± SD values are from three independent experiments (*** represents $p < 0.001$ and * represents $p < 0.05$).

Fig. 4. GBE increases DNA repair ability in *S. cerevisiae* cells upon damage by H_2O_2. A: spheroplasts of yeast strain BY4741 were incubated with 10mM H_2O_2 for 20min, washed with S buffer and incubated with
GBE (diluted two fold in S buffer; squares) or S buffer (circles) until analysis of DNA damage. At each time point spheroplasts were washed with S buffer and DNA damage was analyzed with the comet assay method (see Materials and Methods).B: spheroplasts of yeast parental strain NK1 (circles) and *cdc9* temperature-sensitive mutant NK427 (squares) were incubated with 10mM H_2O_2 for 20min at 23ºC, washed with S buffer and incubated with GBE (diluted two fold in S buffer; empty symbols) or S buffer (filled symbols) at 23ºC. At each time point spheroplasts were washed with S buffer and DNA damage was analyzed with the comet assay method (see Materials and Methods). C: the same as B except for the additional 1h incubation of cells at 37ºC before the experiment and all subsequent incubations at 37ºC instead of 23ºC. All results are the mean of three independent experiments.

Fig. 1



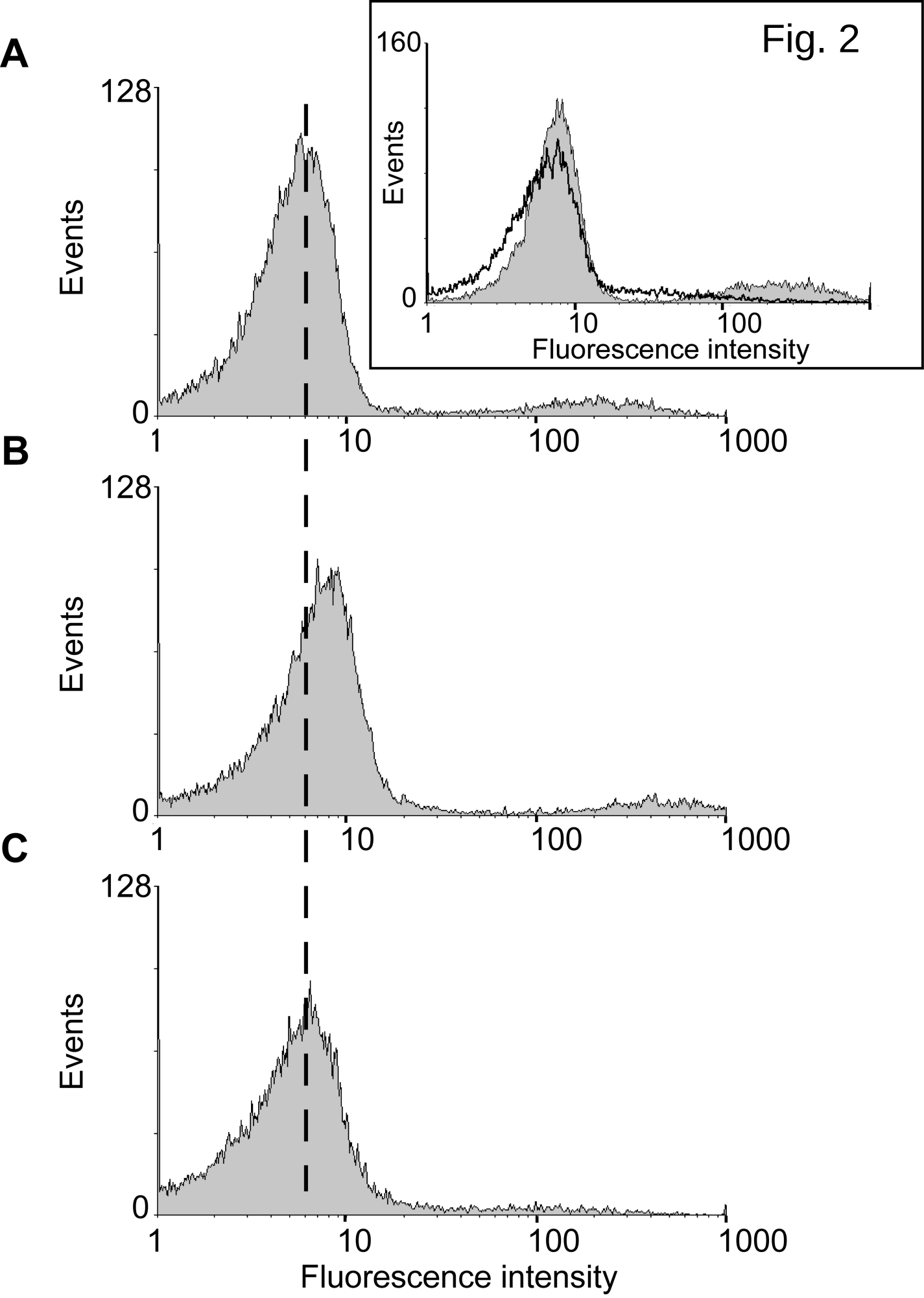
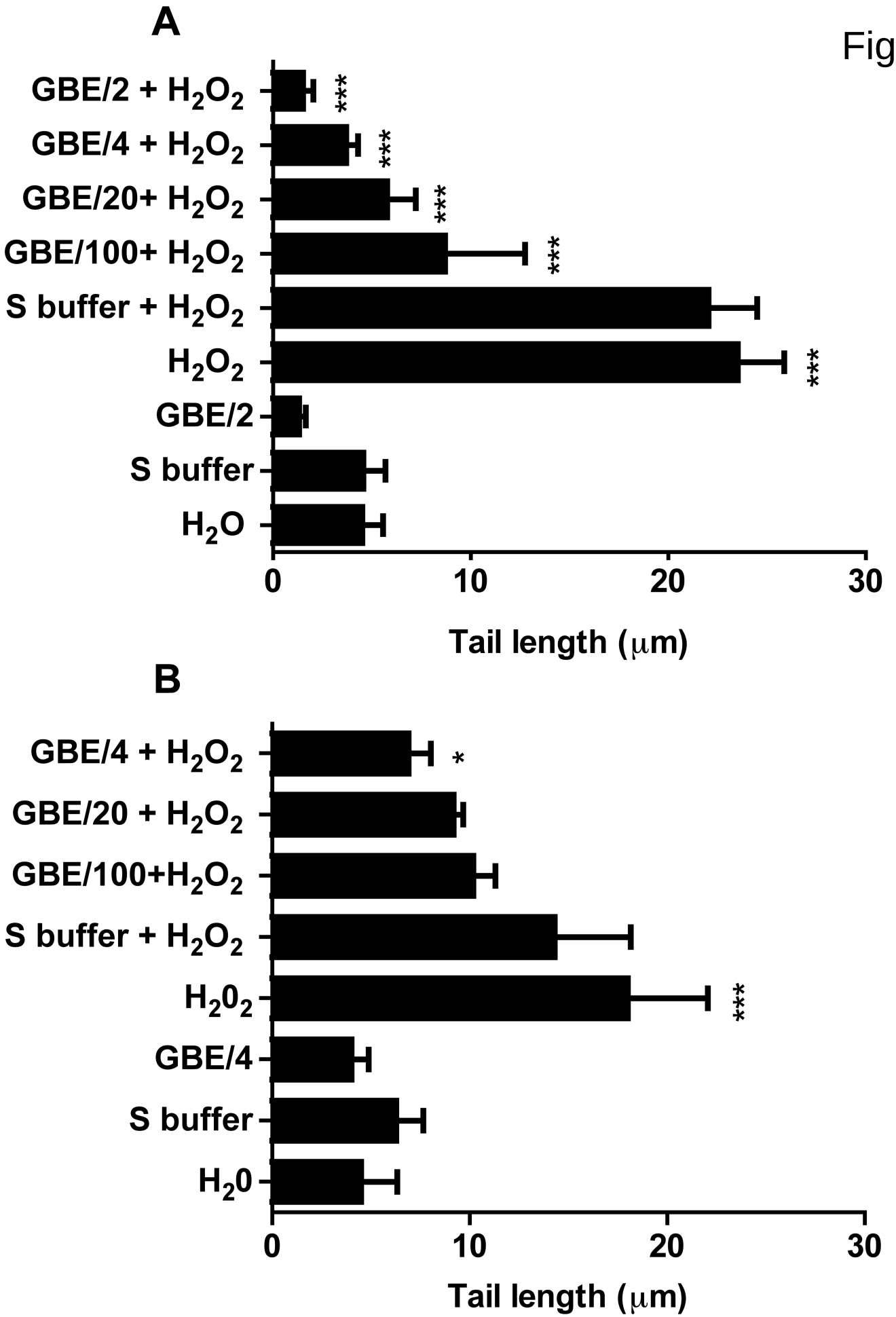


Fig. 2
A
B
C
Events
128
0
1
10
100
1000
160
Fluorescence intensity

Fig. 3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GBE/20+ H2O2
GBE/100+ H2O2
S buffer + H2O2
H2O2
GBE/2
S buffer
H2O
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0
10
20
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Tail length (μm)
B
GBE/4 + H2O2
GBE/20 + H2O2
GBE/100+H2O2
S buffer + H2O2
H202
GBE/4
S buffer
H20
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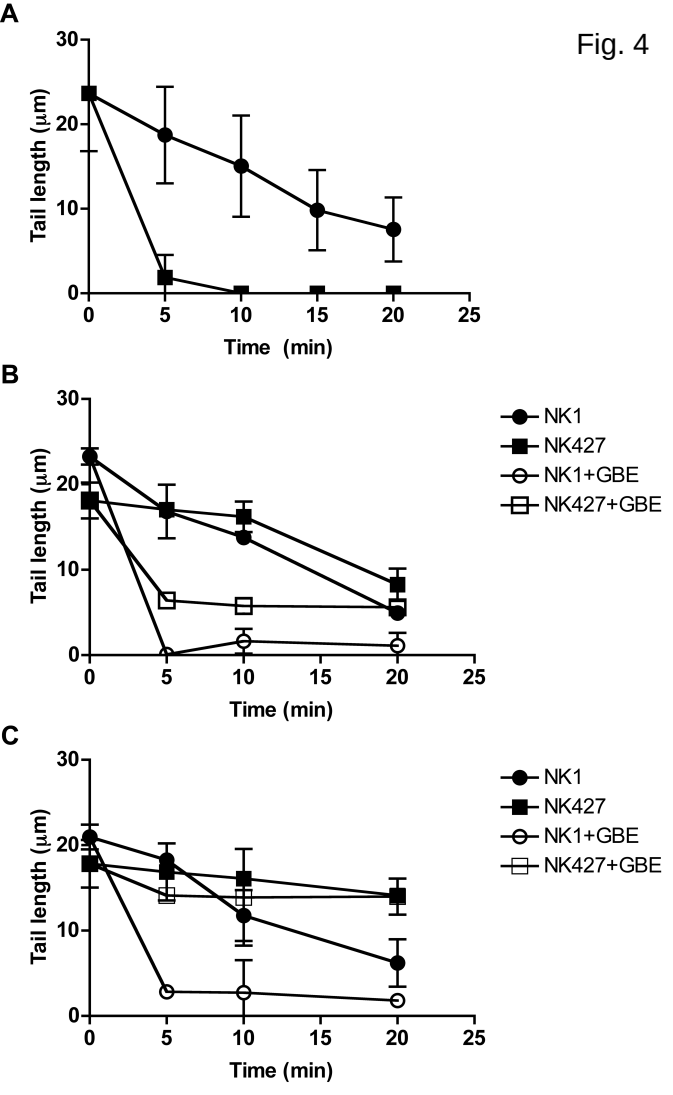
Tail length (μm)

Fig. 4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20
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5
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15
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25
Time (min)
B
Tail length (μm)
30
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20
25
Time (min)
NK1
NK427
NK1+GBE
NK427+GBE
C
Tail length (μm)
30
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25
Time (min)
NK1
NK427
NK1+GBE
NK427+GBE