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Antioxidant properties of β-carboline alkaloids are related to their antimutagenic and antigenotoxic activities

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The β-carboline alkaloids found in medical plants and in a variety of foods, beverages and cigarette smoke have a range of action in various biological systems. *In vitro* studies have demonstrated that these alkaloids can act as scavengers of reactive oxygen species. In this paper, we report the *in vivo* antioxidative properties of the aromatic (harmane, harmine, harmol) and dihydro-β-carbolines (harmaline and harmalol) studied by using *Saccharomyces cerevisiae* strains proficient and deficient in antioxidant defenses. Their antimutagenic activity was also assayed in *S. cerevisiae* and the antigenotoxicity was tested by the comet assay in V79 cell line, when both eukaryotic systems were exposed to H_2O_2. We show that the alkaloids have a significant protective effect against H_2O_2 and paraquat oxidative agents in yeast cells, and that their ability to scavenge hydroxyl radicals contributes to their antimutagenic and antigenotoxic effects.

Introduction

The β-carbolinic alkaloids are widely distributed, being found in several plant families, such as Apocynaceae, Elaeagnaceae, Leguminosae, Passifloraceae and Zygophyllaceae (1). They are also found in cigarette smoke, overcooked foods and wine (2–5). Certain β-carbolines, such as harman, have been reported as normal constituents of human tissues and body fluids (6). Other β-carbolines, like harmine and harmaline, are responsible for reported hallucinogenic effects of 'ayashuasca', a beverage prepared with *Banisteriopsis caapi* and *Pyschotria viridis*, used for religious purposes in South America and Africa (7,8).

The metabolic pathway leading to the formation of β-carbolines is via Pictet–Spengler condensation between an indolamine (e.g. tryptamine) and aldehydes (e.g. acetaldehyde) (9). The common chemical structure of the alkaloids used in this study comprises one indole nucleus and one six-member pyrrole. According to their oxidation state, these alkaloids can be divided into two groups: dihydro-β-carbolines (harmaline and harmalol) and β-carbolines (harmane, harmine and harmol) (Figure 1).

The β-carbolines have a wide spectrum of action, especially on muscular, cardiovascular and central nervous systems (CNSs), including monoamine oxidase inhibition (10–12), binding to benzodiazepine, serotonin, dopamine and imidazoline receptors (13–17), convulsive or anticonvulsive actions, anxiolytic, tremorogenic and immunomodulatory effects (18–20). They are also DNA intercalating agents (21,22) and inhibit enzymes, e.g. DNA topoisomerases (23). Moreover, toxic and mutagenic effects of these alkaloids have been reported in prokaryotic and eukaryotic cells. These β-carboline alkaloids induce mutagenic effects in various organisms such as *Salmonella typhimurium* (24–26), *Escherichia coli* (25,26), *Saccharomyces cerevisiae* (8), V79 Chinese hamster lung cells (27,28) and human peripheral lymphocytes (29). However, alkaloids decreased the frequency of cell damage when associated with several mutagenic agents (30,31). They were also unable to induce significant genotoxic effects in the same organism where positive results were observed (8,26,28,32).

Some reports indicate that β-carbolines have effective antioxidant properties. In this respect, harmane, harmaline and harmalol showed antioxidant activity by inhibiting lipid peroxidation in microsomal hepatic preparation (9) and by attenuating oxidative damage of hyaluronic acid, cartilage collagen and immunoglobulin G (33,34). In addition, the biological significance of β-carbolines has been related to their neuroprotective actions. Maher and Davis (35) demonstrated that β-carboline alkaloids protect neurons against the excytotoxic effect on dopamine and glutamate. Besides, other studies show that these alkaloids exert a protective effect on oxidative neuronal damage through a scavenging action on reactive oxygen species (ROS) (36–38).

It is very well known that free radicals or ROS are responsible for oxidative stress that can initiate physiopathological processes, as age-related and chronic diseases like diabetes, neurodegenerative and cardiovascular diseases, inflammation, Alzheimer and Parkinson's disease and mainly carcinogenesis, which occurs in a cell or in a tissue when ROS concentration exceeds the antioxidant capability of that cell (39–41). As a consequence, much research has focused on antioxidants and on their action mechanisms. In line with this, several plant extracts or secondary metabolites have been found to show strong antioxidant activity and protection against oxidant-induced damage (42,43).

In view of the fact that ROS are largely involved in DNA damage and mutagenesis, and that β-carboline alkaloids show an antioxidant potential, it was interesting to evaluate its antimutagenic and antigenotoxic effects in different biological models and to determine the concentration threshold of these effects. Therefore, the aim of the present study was to evaluate the antioxidant and antimutagenic/antigenotoxic properties of harmine, harmane, harmol (fully aromatic), harmaline and harmalol (dihydro-β-carboline) alkaloids, and to correlate these biological responses to their chemical structure. We have used

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H_2O_2 and paraquat to induce oxidative damage in *S. cerevisiae* strains defective in several antioxidant defenses. We have also evaluated the mutagenicity and antimutagenicity of the alkaloids using the yeast strain N123, as well as their protective effect against oxidative DNA damage, verified by the comet assay in a culture of permanent lung fibroblast cell line derived from Chinese hamsters.

Materials and methods

Chemicals

The alkaloids harmane (CAS 21655-84-5), harmine (CAS 343-27-1), harmol (CAS 149022-16-2), harmaline (CAS 6027-98-1) and harmalol (CAS 6028-07-5) hydrochlorides, H_2O_2, paraquat (methyl viologen), methyl methanesulfonate (MMS), 4-nitroquinoline-*N*-oxide (4-NQO), hypoxanthine, xanthine oxidase and salicylic acid were obtained from Sigma (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin–ethylenediamine tetraacetic acid (EDTA), L-glutamine, antibiotics and trypan blue were purchased from Gibco BRL (Grand Island, NY, USA). Low-melting point agarose (LMA) and agarose were obtained from Invitrogen (Carlsbad, CA, USA). Yeast extract, bacto-peptone and bacto-agar were obtained from Difco Laboratories (Detroit, MI, USA). All others reagents were of analytical grade.

For treatment of cells, 5 mg/ml stock solutions of the alkaloids were prepared immediately prior to use. Harmine was dissolved in distilled water and harman, harmol, harmaline and harmalol were dissolved in 5% dimethylsulfoxide (DMSO) and distilled water; the final concentration of DMSO in the incubation mixture never exceeds 0.2%. The appropriate concentrations were obtained by dilution of stock solutions in distilled water. The solvent controls included in the genetic tests were found to be negative; 4-NQO, MMS, paraquat and H_2O_2 were used as positive control.

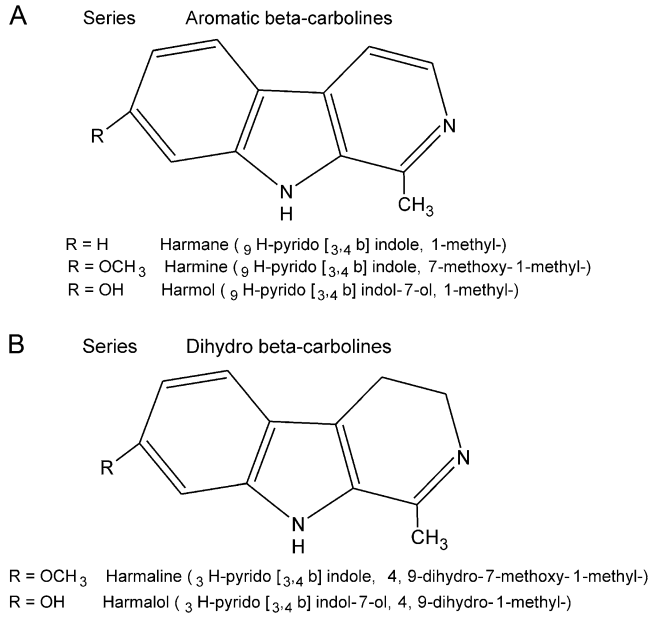

Fig. 1. Chemical structure of the β-carboline alkaloids. (A) Aromatic β-carboline. (B) dihydro-β-carbolines.

Medium and strains of S. cerevisiae

The relevant genotypes of *S. cerevisiae* strains used in this study are listed in Table I. Media, solutions and buffers were prepared as previously described (44). The complete medium (YPD) containing 0.5% yeast extract, 2% peptone and 2% glucose was used for routine growth. For plates, the medium was solidified with 2% bacto-agar. The minimal medium (MM) contained 0.67% yeast nitrogen base without amino acids, 2% glucose and 2% bacto-agar. The synthetic complete medium (SC) was MM supplemented with 2 mg adenine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg uracil and 2 mg tryptophan per 100 ml MM. For mutagenesis, plates were supplemented with 60 μg/ml canavanine (SC + can).

We chose to work in the stationary phase of growth because this resembles most cells of multicellular organisms in important aspects: (i) most energy comes from mitochondrial respiration, (ii) the cells have left the active cell cycle and have entered the G_0 phase and (iii) damage accumulates over time (45,46). The herbicide paraquat, a redox cycling compound, was used to increase the intracellular flux of superoxide anion ($O_2^{\cdot-}$). The appropriate concentrations of H_2O_2 and paraquat were determined by survival assay, according to the differential sensitivity of each strain. Sub-lethal concentration of the oxidants was used for all subsequent experiments.

Survival assays in S. cerevisiae strains

Stationary phase cultures of EG103 [wild type (WT)] and mutant isogenic strains, as well as YPH98 (WT) and the isogenic mutant strain, were obtained by inoculation of an isolated colony into liquid YPD. To evaluate sensitivity to β-carboline alkaloids, cultures were exposed to concentrations varying from 25 to 150 μg/ml and incubated under growth conditions for 1 h in phosphate-buffered saline (PBS) at 30°C. Cells were appropriately diluted and plated in triplicate on solid YPD (2–3 days, 30°C) after colony-forming units were counted.

To verify the antioxidant activity of the alkaloids, cells were pre-treated in PBS with non-cytotoxic concentrations of alkaloids and incubated for 1 h at 30°C. Cells were then washed and treated with paraquat or H_2O_2 in PBS for another hour. For survival determination, suitable aliquots were plated in triplicate on solid YPD. Plates were incubated at 30°C for 2–3 days before counting the colonies. All tests were repeated at least 3-fold, and plating was carried out in triplicate for each dose.

Detection of forward mutation and potential antimutagenic activity in S. cerevisiae

Saccharomyces cerevisiae N123 strain was used for assaying alkaloid mutagenicity as well as the protective effect of the alkaloids against H_2O_2-induced mutagenesis. This strain was chosen because it is very responsive to H_2O_2-induced mutagenesis due to its low glutathione content (47). A suspension of 2×10^8 cells/ml in the stationary phase, grown in YPD (2% glucose), was incubated for 1 h at 30°C with various concentrations of alkaloids in PBS. Survival was determined on SC (2–5 days, 30°C), and mutation induction (CAN revertants) on appropriate supplementation media (4–5 days, 30°C). Forward mutation was measured with the canavanine resistance assay (CAN1-can1) after induction with different treatments. This assay uses a phenotypic marker, canavanine sensitivity, since WT yeast strains express the arginine transporter Can1p, which also imports canavanine from the environment and leads to cell death (48). Thus, mutagen-induced alterations in the *CAN1* gene that impair Can1p functionality can increase cell survival in the presence of canavanine, when compared to a non-mutagenic cell sample.

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Enzymatic defense lacking	Source
EG103 (SOD-WT)	*MATα leu2Δ0 his3-Δ1 trp1-289 ura3-52*	None	E. Gralla
EG118 (*sod1Δ*)	Like EG103, except *sod1*::*URA3*	Cu–Zn SOD (cytosolic)	E. Gralla
EG110 (*sod2Δ*)	Like EG103, except *sod2*::*TRP1*	MnSOD (mitochondrial)	E. Gralla
EG133 (*sod1Δ sod2Δ*)	Like EG103, except *sod1*::*URA3* e *sod2*::*TRP1*	All SOD	E. Gralla
EG223 (*ctt1Δ*)	Like EG103, except *ctt1*::*TRP1*	Cytosolic catalase	E. Gralla
EG213 (*sod1Δ ctt1Δ*)	Like EG103, except *sod1*::*URA3* e *ctt1*::*TRP1*	Cu–Zn SOD and cytosolic catalase	E. Gralla
YPH98 (WT)	*MATa ade2-101 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52*	None	P. Hieter
yap1Δ	Like YPH98 except *yap1*::*URA3*	yAP-1 transcription factor	M. Grey
N123	*MATa his1-7*	None, but exhibits low glutathione content	J. Henriques

For antimutagenic evaluation, the procedure was as follows: cells were submitted to pre-treatment with non-cytotoxic concentrations of alkaloids and incubated for 1 h with shaking at 30°C. Cells were then washed and H_2O_2 was added. The mixture was further incubated at 30°C for 1 h. After treatment, appropriate dilutions of cells were plated onto SC plates to determine cell survival, and 100 µl aliquots of cell suspension (2×10^8 cells/ml) were plated onto SC media supplemented with 60 µg/ml canavanine. Plates were incubated in the dark at 30°C for 3–5 days before counting the survivors and revertant colonies. All mutagenicity assays were repeated at least three times, and plating for each dose was conducted in triplicate.

Comet assay using V79 cells

Chinese hamster lung fibroblasts (V79 cells) were cultivated under standard condition in DMEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics (49). Cells were maintained in tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, and were harvested by treatment with 0.15% trypsin and 0.08% EDTA in PBS. Cells (2×10^5) were seeded into each flask and cultured 1 day prior to treatment. Alkaloids were added to FBS-free medium to achieve the different designed concentrations, and the cells were treated for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Oxidative challenge with 0.1 mM H_2O_2 was carried out for 0.5 h in the dark in FBS-free medium. The culture flasks were protected from direct light during treatment with the alkaloids and H_2O_2.

The alkaline comet assay was performed as described by Singh *et al.* (50) with minor modifications (51). At the end of treatment, cells were washed with ice-cold PBS and trypsinized with 100 µl trypsin (0.15%). Immediately thereafter, 20 µl of cell suspension ($\sim 10^6$ cells/ml) were dissolved in 0.75% LMA and spread on normal agarose point (1%) pre-coated microscope slides. Cells were ice-cold lysed (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0, with freshly added 1% Triton X-100 and 10% DMSO) for at least 1 h at 4°C in order to remove cellular proteins and membranes, leaving the DNA as 'nucleoids'. Thereafter, slides were placed in a horizontal electrophoresis box, containing freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH ~13.0) for 20 min at 4°C in order to allow DNA unwinding and expression of alkali-labile sites. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 20 min to electrophorese DNA. All the steps above were performed under yellow light or in the dark in order to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver nitrate as described by Nadin *et al.* (52) and analyzed using an optic microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed per group. Cells were also scored visually into five classes, according to tail size (from undamaged, 0, to maximally damaged, 4).

International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method (53). It has a high correlation with computer-based image analysis. The damage index (DI) is based on the length of migration and on the amount of DNA in the tail and is considered a sensitive measure of DNA and of damage frequency (DF), as the proportion of cells that show tails after electrophoresis. Image length (IL) or migration length gives information only about the size of DNA fragments, and is largely dependent upon electrophoresis conditions (i.e. voltage and duration). Thus, DI and DF are emphasized in our analyses. The other parameter, IL, though considered in the analysis, was used only as a complementary DNA damage parameter. DI was thus assigned to each comet according to its class, and ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (54). The DF (%) was calculated as the number of cells with tails versus those without (0–100%). Results are presented as means and ranges of four independent experiments. The solvent was used as negative control; MMS (4×10^{-5} M) and H_2O_2 (0.1 mM) were used as positive control.

Hipoxanthine/xanthine oxidase assay

The method employed to assay the hydroxyl radical-scavenging ability of alkaloids was based on the method of Owen *et al.* (55). Briefly, alkaloids were dissolved in the assay buffer [hypoxanthine, Fe(III), EDTA and salicylic acid] at a concentration of 2.0 mg/ml and diluted appropriately (in triplicate) in assay buffer to a final volume of 1.0 ml giving a range of 0.5–1.5 mg/ml. A 5-µl aliquot of xanthine oxidase dissolved in 3.2 M $(NH_4)_2SO_4$ was added to initiate the reaction. The sample tubes were incubated at 37°C for 3 h, at which time the reaction was complete. A 30-µl aliquot of the reaction mixture was analyzed by high-pressure liquid chromatography (HPLC) using chromatographic conditions as previously described (56). Chromatographic analysis was done using a gradient based on methanol–water–acetic acid with a µBondaPak C18 reverse phase column (Waters) and detection at 325 nm. The HPLC equipment had a 2695 separation module (Waters) and UV detector 2487 (Waters). The hydroxylation of salicylic acid and hypoxanthine was monitored at $A = 325$ and $A = 278$ nm, respectively. The amount of dihydroxyphenol, 2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid (DHBAs), produced by the reaction of salicylic acid with produced hydroxyl radicals ($OH^{\cdot}$) was determined from standard curves of the respective dihydroxyphenols.

Statistics

Statistical analyses of the data were performed using one-way analysis of variance (ANOVA)–Tukey's multiple comparison test. *P*-values under 0.05 were considered significant. Data were expressed as means ± SDs values.

Results

Protective effects of β-carbonilic alkaloids in S. cerevisiae strains

WT cells and isogenic mutant strains of *S. cerevisiae* lacking antioxidant defenses (Table I) were treated with several concentrations of the alkaloids for 1 h during the stationary phase. All strains showed practically the same sensitivity for β-carbolines to that observed for the WT cells (Figure 2). Our findings showed that harmane, harmine and harmol decrease viability but, in a significant way, only in concentrations up to 150 µg/ml, whereas harmaline and harmalol do not induce significant effects in any of the concentrations employed. In this manner, we chose non-cytotoxic alkaloid concentrations (ranging from 25 to 100 µg/ml) to follow experiments in order to verify the protecting activity against oxidants in the same strains.

To verify an intracellular protective effect of the alkaloids, i.e. a possible role of β-carboline alkaloids in cell oxidative stress, yeast cells were pre-treated with non-cytotoxic concentration of harmane, harmine, harmol, harmaline or harmalol, and then further exposed to sub-lethal concentrations of either H_2O_2 or paraquat. A statistically significant survival was observed as a consequence of antioxidant effect of the alkaloids.

The aromatic β-carboline harmane significantly enhanced the survival of all yeast cells, with EG103 background at 50 and 100 µg/ml after treatment with H_2O_2 (Figure 3A), showing a clear antioxidant protective effect. However, after treatment with paraquat, this effect was less significant, being more effective at 50 µg/ml in these strains (Figure 4A). Although harmane did not protect YPH98 WT against any of the two oxidative agents, an important antioxidant effect was observed against H_2O_2 for *yap1Δ*, as verified by the increase in survival after oxidative challenge and shown in Figure 3A.

Figure 3B shows that harmine at 50 µg/ml was able to protect *sod1Δ*, *sod2Δ* and *ctt1Δ* single mutants against H_2O_2 cytotoxicity. In addition, this activity was more effective in the *sod1Δctt1Δ* double mutant. However, this alkaloid did not protect any yeast strain against the deleterious effects of paraquat (Figure 4B).

After treatment with H_2O_2, the harmol antioxidant activity was only observed for the strains deficient in both superoxide dismutases (SODs) (single and double mutants) and in the transcription factor-deficient mutant *yap1* (Figure 3C). However, harmol protected EG103 WT as well as *sod1Δ*, *ctt1Δ* single and *sod1Δsod2Δ*, *sod1Δctt1Δ* double mutants against treatment with paraquat (Figure 4C).

Harmaline (Figures 3D and 4D) showed a significant protection against H_2O_2 and paraquat, respectively, although this effect is more prominent in the single *sod*-deficient mutants.

Dihydro-β-carboline harmalol demonstrated the strongest antioxidant effect (Figures 3E and 4E). This alkaloid significantly enhanced the survival of yeast cells at 50 and 100 µg/ml for EG103 WT and its *sod* isogenic mutant strains (*sod1Δ*, *sod2Δ* and *sod1Δsod2Δ*) in the pre-treatment assay, using

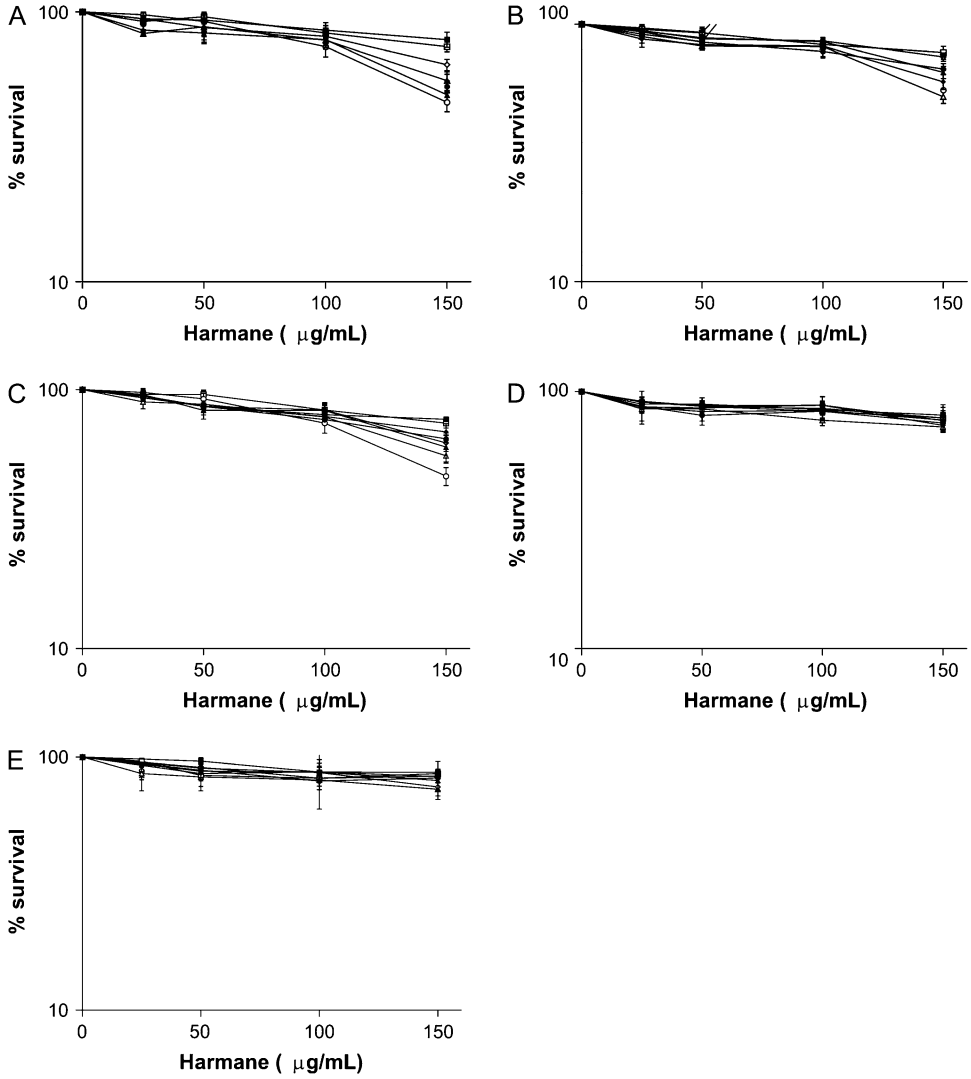

Fig. 2. Sensitivity of cells in the stationary growth phase to harmane (A), harmine (B), harmol (C), harmaline (D) and harmalol (E). EG103 (WT) (filled square) and its isogenic derivative strains: *sod1Δ* (filled diamond), *sod2Δ* (filled circle), *sod1Δsod2Δ* (filled triangle), *ctt1Δ* (open diamond), *sod1Δctt1Δ* (open circle), YPH98 (WT) (open square) and isogenic strain *yap1* (open triangle). Cells were treated for 1 h at 30°C.

H_2O_2 (Figure 3E) and paraquat (Figure 4E) as oxidants. Similar results were obtained for the mutant lacking the transcription factor Yap1p (*yap1Δ*).

Induction of forward mutation and antimutagenic effects in S. cerevisiae

The β-carboline alkaloids did not induce mutagenic effect in stationary growth phase in *S. cerevisiae* N123 strain (Table II). Once again, we chose a non-cytotoxic alkaloid concentration (10–50 μg/ml) to follow experiments in order to verify the protective effects of β-carbolinic alkaloids against the H_2O_2-induced forward mutagenesis in N123 yeast strain. Table III shows that all β-carbolines inhibited the mutagenic action of H_2O_2, mainly by an increase in cell survival. Dihydro-β-carboline harmalol had the most prominent effect, increasing the survival during H_2O_2 treatment and simultaneously decreasing induced mutation in yeast.

Comet assay

The effects of all alkaloids on DI and DF, as measured by DNA damage in V79 cells, according to the comet assay, are shown in the presence (Table IV) and absence (Table V) of H_2O_2. We evaluated the genotoxic effect in this cell line as well as its antigenotoxic properties. Table IV shows that aromatic β-carbolines alkaloids (harmane, harmine and harmol) induced DNA damage, as verified by DI and DF increase at the highest concentration employed (40 μg/ml). On the other hand, dihydro-β-carbolines (harmaline and harmalol) did not generate significant DNA damage at the concentration range evaluated.

H_2O_2-induced DNA damage was used to check any possible antigenotoxic effect of these alkaloids. As expected, exposure of V79 cells to H_2O_2 resulted in a significant increase in DNA damage parameters DI and DF (Table V). β-Carboline pretreatment at lower concentrations significantly inhibited the DNA damage induced by this agent, reducing the DI and DF. Harmane, harmine and harmol showed a significant decrease in the DI and DF at lower concentrations (10 and 20 μg/ml), in comparison to the DNA-damaging effects of H_2O_2 (Table V). On the other hand, harmalol and harmaline clearly demonstrated a significant reduction in DI and DF in a large concentration range (10–40 μg/ml). This decrease of damage score does not occur in a dose-dependent manner and is similar for

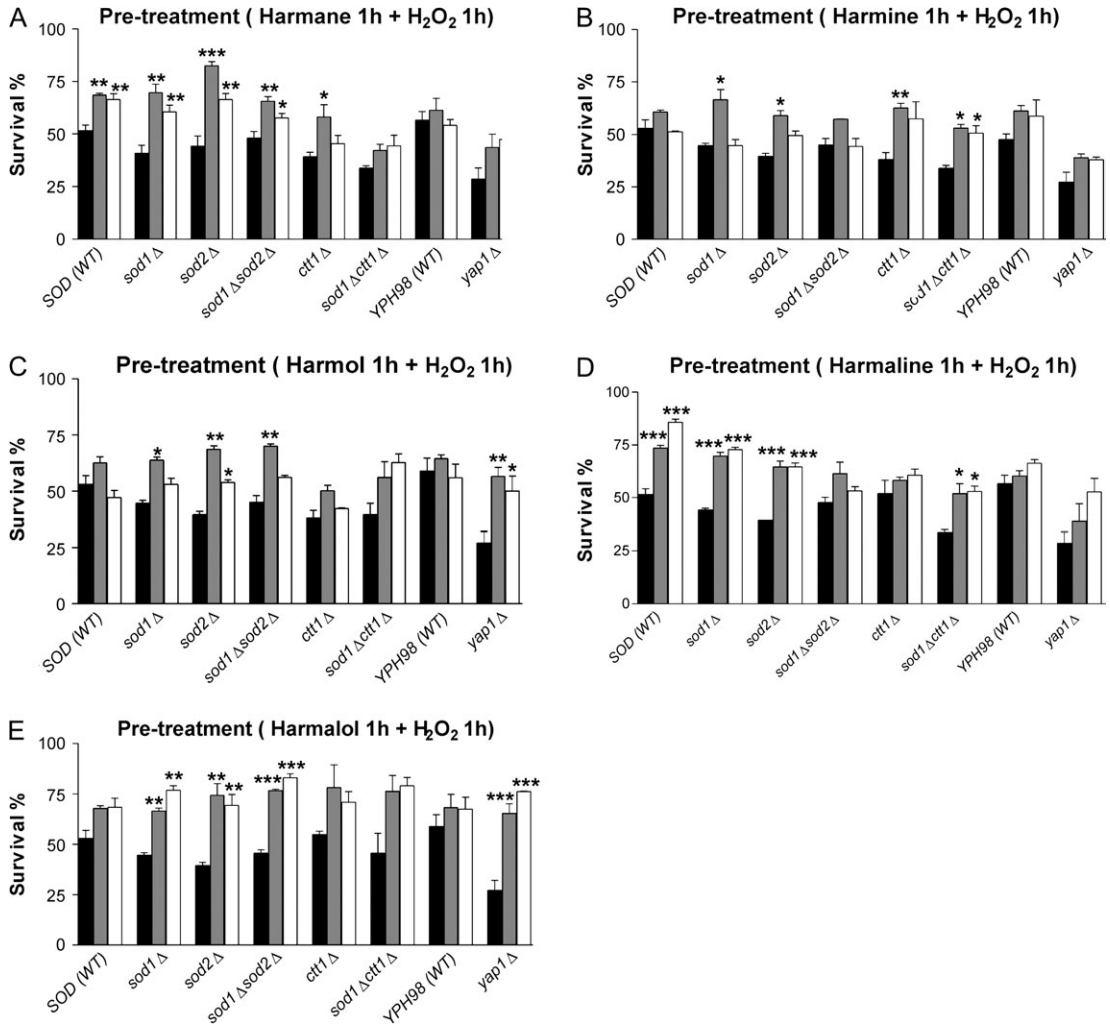

Fig. 3. Effect of pre-treatments with β-carbolines on survival after treatment with oxidant H_2O_2 in EG103 (WT) and mutant isogenic strains and YPH98 (WT) and mutant isogenic strains. (A) H_2O_2 (5 mM) (black bars), 50 μg/ml harmane + H_2O_2 (5 mM) (gray bars) and 100 μg/ml harmane + H_2O_2 (5 mM) (white bars). (B) H_2O_2 (5 mM) (black bars), 50 μg/ml harmine + H_2O_2 (5 mM) (gray bars) and 100 μg/ml harmine + H_2O_2 (5 mM) (white bars). (C) H_2O_2 (5 mM) (black bars), 50 μg/ml harmol + H_2O_2 (5 mM) (gray bars) and 100 μg/ml harmol + H_2O_2 (5 mM) (white bars). (D) H_2O_2 (5 mM) (black bars), 50 μg/ml harmaline + H_2O_2 (5 mM) (gray bars) and 100 μg/ml harmaline + H_2O_2 (5 mM) (white bars). (E) H_2O_2 (5 mM) (black bars), 50 μg/ml harmalol + H_2O_2 (5 mM) (gray bars) and 100 μg/ml harmalol + H_2O_2 (5 mM) (white bars). Percentage survival is expressed relative to the untreated control culture (100%). Values shown are the mean at least three determinations. Data significant in relation to oxidant-treated samples at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$/one-way ANOVA–Tukey's multiple comparison test.

all concentrations used. This response is also very interesting since it shows that a significant antigenotoxic effect can be reached by using low concentrations of the alkaloids. The frequency of damage class was different for each alkaloid for each dose.

In vitro antioxidant capacity of β-*carboline alkaloids*

The antioxidant capacity of β-carboline alkaloids was determined by monitoring the production of hydroxyl benzoic acids (DHBA) due to the attack of ROS on salicylic acid in the hypoxanthine–xanthine oxidase assay. The reduction of total oxidation products as a function of the concentration of alkaloids added to the assay is shown in Figure 5. All β-carboline alkaloids demonstrated a significant antioxidant capacity in a dose-dependent manner. Harmane, harmalol and harmaline had a more pronounced activity, reducing the formation of both DHBA species to 2.7, 7.15 and 8.72%, respectively, in the highest concentration used (1.5 mg/ml), whereas harmol (38.2%) and harmine (42.2%) showed moderate activity in the reduction of DHBA. In this manner, β-carbolines showed a significant antioxidant capacity in a dose-dependent manner at high concentrations due the compounds' hydroxyl radical-scavenging ability.

Discussion

β-Carboline alkaloids are active constituents of hallucinogenic plants used in South American Indian cultures (7) and have been identified in plants that have a long tradition in ethnopharmacology. Pharmacological investigations on the alkaloids have demonstrated interesting biological activities, including the inhibition of monoaminoxidase, binding to a wide range of CNS receptors and anxiolytic and tremorogenic effects (11–20). Furthermore, *in vitro* studies show antioxidative and neuroprotective actions of the β-carboline alkaloids (9,33–38). In this manner, our interest was placed on the evaluation of the antioxidant and antimutagenic/antigenotoxic effects of these molecules on yeast defective in antioxidant defenses and in

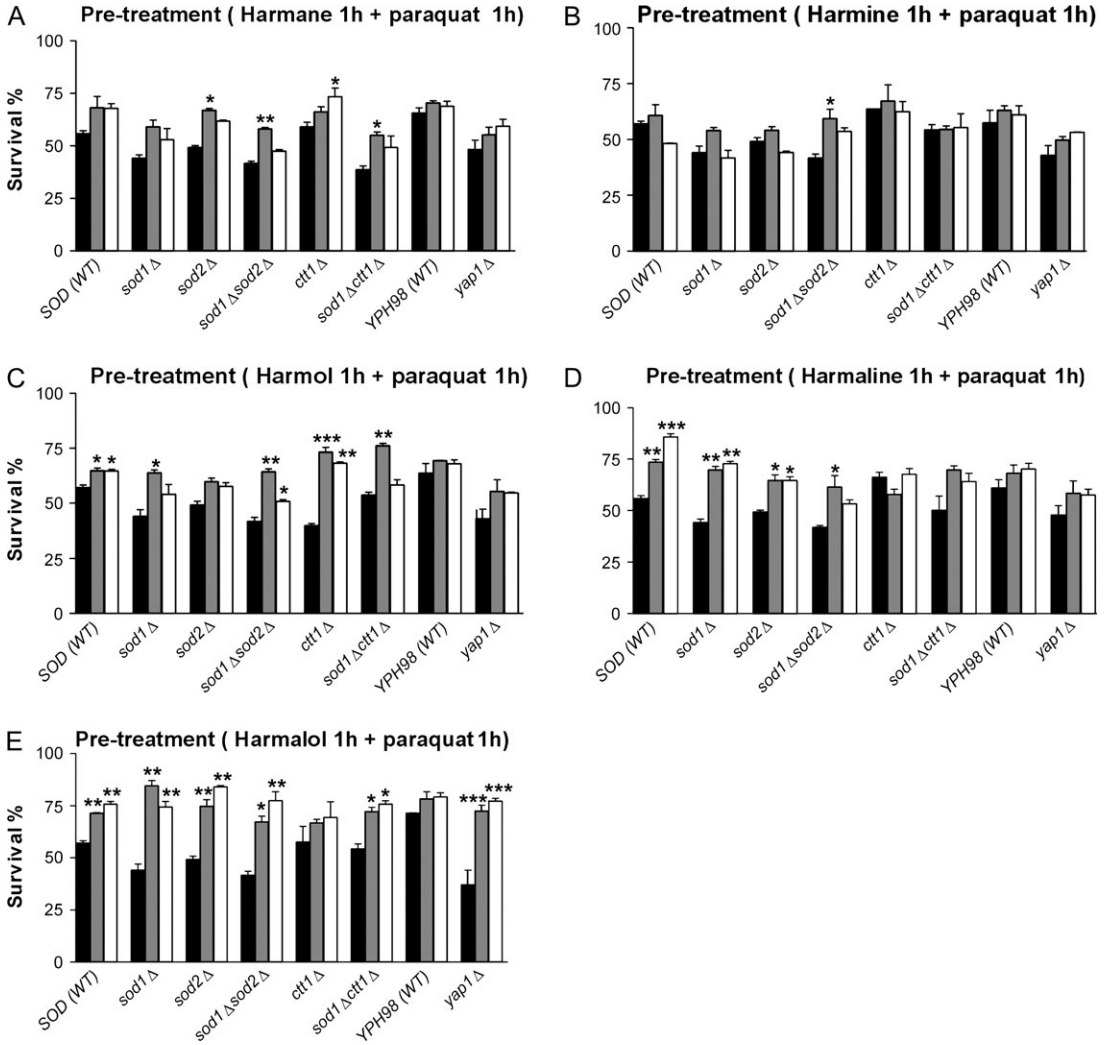

Fig. 4. Effect of pre-treatments with β-carbolines on survival after treatment with oxidant paraquat in EG103 and mutant isogenic strains and YPH98 (WT) and mutant isogenic strains. (A) Paraquat (5 mM) (black bars), 50 μg/ml harmano + paraquat (5 mM) (gray bars) and 100 μg/ml harmano + paraquat (5 mM) (white bars). (B) Paraquat (5 mM) (black bars), 50 μg/ml harmine + paraquat (5 mM) (gray bars) and 100 μg/ml harmine + paraquat (5 mM) (white bars). (C) Paraquat (5 mM) (black bars), 50 μg/ml harmol + paraquat (5 mM) (gray bars) and 100 μg/ml harmol + paraquat (5 mM) (white bars). (D) Paraquat (5 mM) (black bars), 50 μg/ml harmaline + paraquat (5 mM) (gray bars) and 100 μg/ml harmaline + paraquat (5 mM) (white bars). (E) Paraquat (5 mM) (black bars), 50 μg/ml harmalol + paraquat (5 mM) (gray bars) and 100 μg/ml harmalol + paraquat (5 mM) (white bars). Percentage survival is expressed relative to untreated control culture (100%). Values shown are the means of at least three determinations. Data significant in relation to oxidant-treated samples at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$/one-way ANOVA–Tukey's multiple comparison test.

V79 cell line, in the first study to investigate these alkaloids as potential protective agents using H_2O_2 and paraquat as oxidative agents.

In this sense, we demonstrate that β-carboline alkaloids show a protective effect against oxidative agents using yeast and mammalian cells as two eukaryotic model organisms. The antioxidative effect observed for harmane, harmine, harmol, harmaline and harmalol clearly depends on the structure and concentration of the alkaloid.

The yeast *S. cerevisiae* has been a useful model for studies of the eukaryotic response to oxidant challenge (46). In this study, we have used yeast strains null mutant in the cytosolic CuZnSOD gene (*sod1Δ* strains), mitochondrial MnSOD gene (*sod2Δ* strains), cytosolic catalase gene (*ctt1Δ* strain) and double mutants (*sod1Δsod2Δ* and *sod1Δctt1Δ*). Besides, null mutant in yAP-1 transcription factor was also used. The H_2O_2 and paraquat concentrations used in the assays were appropriate for the differential sensitivity of each strain, which is dependent on their genetic background, and also influences the response to the alkaloids treatment. The results in yeast survival tests, employing the EG103 isogenic strains *sod1Δ*, *sod2Δ*, *ctt1Δ*, *sod1Δctt1Δ* and *sod1Δsod2Δ*, pre-treated with these alkaloids, showed a general survival increment after exposure to H_2O_2 (Figure 3) and paraquat (Figure 4). Furthermore, the dihydro-β-carboline alkaloids (harmalol and harmaline) had a higher protective effect as compared to the aromatic β-carbolines (harmane, harmine and harmol). The antioxidant effect was more pronounced for H_2O_2, which may suggest that the alkaloids are acting as scavengers of hydroxyl radicals ($OH^{\cdot}$), generated through the Haber-Weiss–Fenton reaction (57). It is important to take into account that the superoxide anion (generated by paraquat treatment) is known to oxidize exposed (4Fe-4S) clusters in certain enzymes, leading to inactivation of the enzyme and liberation of iron (58,59), which thus becomes available to participate in the Fenton reaction, and consequently yields $OH^{\cdot}$ radicals. In addition, Bayliak *et al.* (60) suggest that SOD play an important role in yeast survival under

Table II. Induction of forward mutation (*can1*) in haploid N123 strain of *Saccharomyces cerevisiae* after β-carbolinic alkaloids treatments in stationary phase in PBS

Agent	Treatment (μg/ml)	Survival (%)	Can/10^7 survivors[a]
NC[b]	0	100 (237)[c]	1.05 ± 0.57[d]
4NQO[e]	0.5	45.14 (107)***	30.28 ± 3.43***
Harmane	10	98.78 (234)	2.54 ± 0.02
	25	91.71 (217)	1.98 ± 1.03
	50	87.23 (207)	2.55 ± 0.42
Harmine	10	96.55 (228)	1.71 ± 0.45
	25	93.13 (221)	1.39 ± 0.52
	50	90.72 (215)	2.77 ± 0.36
Harmol	10	90.71 (215)	1.90 ± 0.59
	25	88.30 (209)	2.08 ± 0.42
	50	86.56 (205)	2.23 ± 0.43
Harmaline	10	96.63 (229)	1.55 ± 0.29
	25	95.47 (226)	1.65 ± 0.98
	50	93.96 (222)	2.03 ± 0.30
Harmalol	10	92.26 (219)	1.25 ± 0.75
	25	91.49 (217)	1.97 ± 0.28
	50	89.23 (211)	2.03 ± 0.24

[a]Locus-specific revertants.
[b]Negative control (solvent).
[c]Number of colonies.
[d]Mean and standard deviation per three independent experiments.
[e]Positive control.
Data significant in relation to negative control group (solvent) at ***$P < 0.001$/one-way ANOVA–Tukey's multiple comparison test.

Table III. Effects of β-carbolinic alkaloids on induced mutagenicity by H_2O_2 in haploid N123 strain of *Saccharomyces cerevisiae* in the stationary phase in PBS

Agent	Treatment	Survival (%)	Can/10^7 survivors[a]
NC[b]	0	100 (247)[c]	1.02 ± 0.12[d]
H_2O_2[e]	4 mM	42.10 (104)	19.29 ± 2.08
Harmane	10 μg/ml + H_2O_2	74.89 (185)	5.67 ± 0.80***
	25 μg/ml + H_2O_2	69.73 (172)	6.99 ± 0.10**
	50 μg/ml + H_2O_2	67.20 (166)	9.64 ± 1.81**
Harmine	10 μg/ml + H_2O_2	70.85 (175)	5.95 ± 0.38***
	25 μg/ml + H_2O_2	60.42 (149)	7.49 ± 1.11**
	50 μg/ml + H_2O_2	55.46 (137)	13.5 ± 1.57*
Harmol	10 μg/ml + H_2O_2	79.77 (197)	4.46 ± 0.57***
	25 μg/ml + H_2O_2	72.82 (180)	4.93 ± 0.41***
	50 μg/ml + H_2O_2	61.34 (152)	10.92 ± 2.26*
Harmaline	10 μg/ml + H_2O_2	65.47 (162)	4.44 ± 0.22***
	25 μg/ml + H_2O_2	67.26 (166)	6.68 ± 1.66**
	50 μg/ml + H_2O_2	73.99 (183)	7.59 ± 3.15**
Harmalol	10 μg/ml + H_2O_2	68.70 (170)	8.88 ± 1.30**
	25 μg/ml + H_2O_2	74.89 (185)	4.38 ± 0.32***
	50 μg/ml + H_2O_2	71.25 (176)	1.98 ± 0.19***

[a]Locus-specific revertants.
[b]Negative control (solvent).
[c]Number of colonies.
[d]Mean and standard deviation per three independent experiments.
[e]Positive control (H_2O_2).
Data significant in relation to positive control group at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$/one-way–ANOVA Tukey's multiple comparison test.

Table IV. Effects of β-carbolines alkaloids in V79 cells exposed for 2 h and evaluated by comet assay

Substance	Treatment	DI[a]	DF (%)[a]
NC[b]	0	46.00 ± 4.00	49.00 ± 2.00
MMS[c]	4.0×10^{-5} M	227.00 ± 4.35***	86.00 ± 9.84***
Harmane	10 μg/ml	80.00 ± 8.93	41.33 ± 3.05
	20 μg/ml	84.33 ± 3.79	58.00 ± 3.00
	40 μg/ml	182.71 ± 6.19**	69.66 ± 1.57*
Harmine	10 μg/ml	74.00 ± 16.28	54.33 ± 4.61
	20 μg/ml	81.01 ± 0.73	65.66 ± 6.42
	40 μg/ml	113.2 ± 4.58*	71.64 ± 1.82*
Harmol	10 μg/ml	82.68 ± 5.85	54.33 ± 3.53
	20 μg/ml	80.02 ± 13.89	56.00 ± 2.00
	40 μg/ml	103.33 ± 7.57*	58.30 ± 14.97
	80 μg/ml	150.00 ± 12.76***	72.00 ± 5.29**
Harmaline	10 μg/ml	49.66 ± 20.42	35.66 ± 10.96
	20 μg/ml	66.34 ± 11.93	50.60 ± 7.8
	40 μg/ml	80.67 ± 10.42	52.62 ± 4.72
	80 μg/ml	79.00 ± 13.51	54.00 ± 7.20
Harmalol	10 μg/ml	55.38 ± 6.65	48.66 ± 10.96
	20 μg/ml	58.66 ± 4.60	50.00 ± 4.35
	40 μg/ml	63.05 ± 2.83	52.66 ± 10.59
	80 μg/ml	62.33 ± 15.53	53.65 ± 8.62

[a]Means values and standard deviation obtained from average of 100 cells per experiment—total of four experiments per dose for each substance.
[b]Negative control (solvent).
[c]Positive control.
Data significant in relation to negative control (solvent) groups at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$/one-way ANOVA–Tukey's multiple comparison test.

Table V. Effect of β-carboline alkaloids in V79 cells exposed for 2 h plus oxidant H_2O_2 for 0.5 h and evaluated by comet assay

Substance	Treatment	DI[a]	DF (%)[a]
NC[b]	0	46.35 ± 4.83	22.45 ± 6.11
H_2O_2[c]	100 μM	219.66 ± 36.08	86.36 ± 6.35
Harmane	10 μg/ml + H_2O_2	92.33 ± 13.79***	41.33 ± 3.05***
	20 μg/ml + H_2O_2	122.00 ± 6.00***	58.00 ± 3.00**
Harmine	10 μg/ml + H_2O_2	106.00 ± 21.96***	55.66 ± 6.42**
	20 μg/ml + H_2O_2	123.33 ± 24.58*	67.33 ± 2.30
Harmol	10 μg/ml + H_2O_2	92.66 ± 11.60***	44.33 ± 2.51***
	20 μg/ml + H_2O_2	108.66 ± 19.03***	56.33 ± 2.00**
Harmaline	10 μg/ml + H_2O_2	106.33 ± 11.93***	60.00 ± 7.80***
	20 μg/ml + H_2O_2	107.66 ± 7.57***	50.33 ± 15.71***
	40 μg/ml + H_2O_2	106.00 ± 1.73***	54.33 ± 4.61***
Harmalol	10 μg/ml + H_2O_2	86.33 ± 12.85***	50.00 ± 11.36***
	20 μg/ml + H_2O_2	112.33 ± 8.38***	49.66 ± 4.93***
	40 μg/ml + H_2O_2	115.33 ± 17.00***	54.66 ± 3.51***

[a]Mean values and standard deviation obtained from average of 100 cells per experiment—total of four experiments for each substance.
[b]Negative control (solvent).
[c]Positive control (H_2O_2).
Data significant in relation to positive control (oxidant) group at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$/one-way ANOVA–Tukey's multiple comparison test.

oxidative stress induced by H_2O_2, and it has been shown that there is a strong relationship between catalase and SOD activities under different experimental conditions. Therefore, the lack of SOD as well as of catalase activities imputes sensitivity to H_2O_2. Thus, we believe that the protective effect against paraquat toxicity can be due to the direct action against H_2O_2 or against $OH^{\cdot}$ radical generated through the Haber-Weiss–Fenton reaction, since there are evidences demonstrating that these alkaloids are not active against superoxide anions *in vitro* when tested using SOD-inhibitable reduction ferricytochrome c (36).

It is also important to note the antioxidant response observed in the *yap1* mutants for most alkaloid treatments, especially for the dihydro-β-carbolines (Figures 3D, 3E, 4D and 4E). Yap1 is a key regulator of oxidative stress tolerance in *S. cerevisiae*, and has been shown to regulate a broad set of genes in response to oxidative stress, including *TRX2* (thioredoxin), *TRR1*

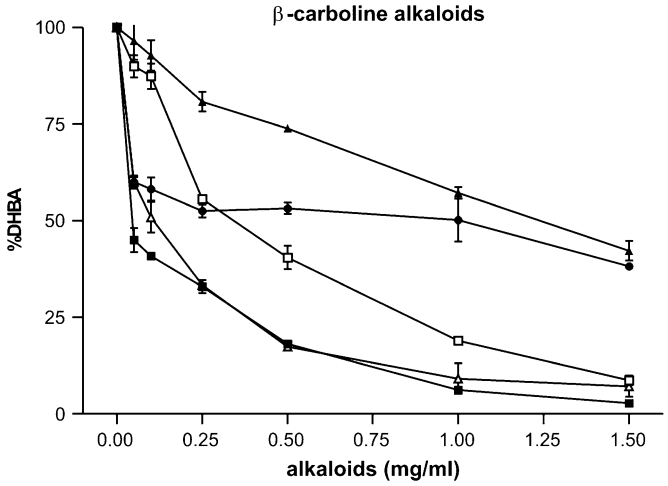

Fig. 5. Inhibition of the generation of reactive oxygen species by β-carboline alkaloids in hypoxanthine–xanthine oxidase systems. Solvent (hexane) (filled square), harmane (filled triangle), harmine (filled circle), harmol (open square), harmalol (open circle) and harmaline (open triangle).

(thioredoxin reductase), *GLR1* (glutathione reductase) and *GSH1* (γ-glutamylcysteinesynthase) (61,62). Consequently, *yap1Δ* mutants are sensitive to oxidative stress (62). Hence, only potent antioxidants are able to protect this strain in this model. Our results thus demonstrate a putative direct action of the alkaloids as ROS scavengers, rather than an induction of other antioxidant defenses that would lead to an adaptive response in yeast.

The capacity of β-carbolines to scavenge hydroxyl radicals was confirmed in the results of the *in vitro* hypoxanthine–xanthine oxidase assay (Figure 5). In agreement with our findings, Lee *et al*. (36) have shown that β-carbolines (harmalol, harmaline and harmine) caused the decomposition of hydroxyl radicals, assayed by inhibition of 2-deoxy-D-ribose degradation, and that the dihydro-β-carbolines harmalol and harmaline produce the most effective activity.

ROS-induced DNA damage may cause mutations resulting in neurodegenerative disease, cancer and ageing (41,63–65). Oxidative lesions in DNA include base modifications, sugar damage, strand breaks and abasic sites. H_2O_2 causes strand breaks and base damage in DNA by a mechanism that requires transition metal ions (65,66). Metals, such as copper and iron, which are present in biological systems, react with H_2O_2 via Fenton reactions to produce hydroxyl radicals ($OH^{\cdot}$) and then induce DNA strand breakage (67). The hydroxyl radical is extremely short lived, and reacts rapidly with almost any cellular biomolecule, including DNA (68). β-Carboline alkaloids, in a general way, showed strong activity against H_2O_2-induced oxidative DNA damage in the antimutagenic assay using *S. cerevisiae* N123 strain (Table III) and in the antigenotoxic assay using mammalian V79 cells (Table V). In this respect, the dihydro-β-carbolines, once more, showed a more pronounced effect than aromatic β-carbolines. However, at the highest concentration, the fully aromatic β-carbolines, such as harmane, harmine and harmol (Figure 1), were able to induce DNA strand breaks in V79 cells (Table IV). As reported in other studies, the degree of aromaticity and planarity of these compounds can be related on their ability to interact with DNA and therefore to induce DNA damage (26,28).

It may therefore be suggested that the activities reported here probably result from a structure-dependent scavenger action of the alkaloids against ROS, especially hydroxyl radicals, in the following decreasing order: harmalol > harmaline > harmol > harmane > harmine.

Indole precursors of the β-carbolines, tryptophan and tryptamines, are known to have antioxidative activities, possibly by scavenging reactive oxygen radicals and forming a stable indole radical at the pyrrole ring (69–71). Tse *et al*. (9) suggest that the indole nucleus of the β-carbolines can have similar antioxidant properties. This could explain the strong *in vitro* antioxidant activity observed by harmane (in the xanthine oxidase assay), which does not possess a ring substitution. However, since this alkaloid can intercalate in DNA (26,28,72,73), this could also be responsible for the reduced antioxidant activity observed in yeast cells, as well as for the reduced antimutagenic and antigenotoxic activities in our experiments.

Our results reinforce what was described by Tse *et al*. (9): the β-carboline antioxidative actions are dependent on chemical structure. Dehydrogenation of the pyridyl ring (e.g. harmalol to harmol, harmaline to harmine) resulted in a considerable decrease in antioxidant efficacies. The replacement of the hydroxyl group by a methoxyl group also decreases the antioxidant effect (e.g. harmalol to harmaline, harmol to harmine). Of all β-carbolines studied, harmalol was found to have the highest scavenger action.

In summary, our findings indicate that the β-carboline alkaloids have a significant antioxidative effect in yeast and that their hydroxyl radical-scavenging property appears to contribute to their antimutagenic and antigenotoxic effects, observed in yeast and mammalian cells, respectively. Since no other data regarding the *in vivo* effects of harmane, harmine, harmol, harmaline and harmalol are available, further studies should be conducted to define the antioxidant properties of these β-carbolines.

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