SPANDIDOS PUBLICATIONS

Antioxidant activity of resveratrol, piceatannol and 3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene in three leukemia cell lines

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Abstract. *trans*-Resveratrol (t-RES) is one of the most relevant and extensively investigated stilbenes with a broad spectrum of biological activities. In contrast to the detailed knowledge of t-RES activities in biological systems, much less is known about the effects of higher hydroxylated stilbenes. Therefore, the aim of this study was to evaluate the protective effects (antioxidant activities) of t-RES and two analogues: the natural metabolite piceatannol (PCA) and the synthesized 3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene (HHS) against H_2O_2-induced DNA damage in leukemic L1210, K562 and HL-60 cells using single-cell gel electrophoresis (SCGE). After 24 h pre-treatment of cells all compounds investigated significantly inhibited the incidence of DNA single strand breaks induced by H_2O_2. The protective effects of PCA and HHS in L1210 cells and of HHS in HL-60 cells were significantly higher compared to the activity of t-RES ($^{+}P<0.05$). In K562 cells the differences of the antioxidant activities of PCA and HHS, and of PCA in HL-60 cells were of much higher significance when compared to t-RES ($^{++}P<0.01$). In conclusion, we can prove that all stilbenes investigated, t-RES, PCA, and HHS, manifested potent antioxidant effects on three leukemic cell lines and the presence of *ortho*-dihydroxy structures enhanced the protective effect against DNA damage caused by $^{\bullet}OH$ radicals.

Introduction

Over the years stilbene-based compounds have attracted the attention of many researchers due to their wide ranging biological activities. One of the most relevant and extensively studied stilbenes is *trans*-resveratrol (3,4',5-trihydroxy-*trans*-stilbene; t-RES) (Fig. 1), a polyphenolic compound found in a wide variety of plants including grapes, peanuts, berries, and pines (1). In these plants, t-RES is synthesized in response to stress conditions such as trauma, UV irradiation, exposure to ozone and fungal infection, and thus can be considered to be a phytoalexin (2,3). t-RES has also been reported to be a phytoestrogen due to its structural similarity to the estrogenic agent diethylstilbestrol (4). It has also been shown to exhibit estrogenic activity in mammals (5,6). t-RES has been reported to have both anti-carcinogenic and cardioprotective activities, which could be attributed to its antioxidant and anti-coagulant properties (7,8). It has been demonstrated to possess chemopreventive and cytostatic properties via the inhibition of tumor initiation, promotion and progression (9). It causes an arrest in the S and G_2 phases of the cell cycle (10) and is capable of inducing differentiation and apoptosis in a multitude of tumor cell lines, such as human leukemic, colonic, breast, prostate and esophageal cells via CD95-dependent or independent mechanisms or through the activation of caspase 3 or cleavage of poly (ADP-ribose) polymerase (11-16). Besides these effects, t-RES has been reported to be effective in inhibiting platelet aggregation and lipid peroxidation, altering the eicosanoid synthesis, modulating lipoprotein metabolism (17-19), and exhibiting vasorelaxing and anti-inflammatory activities (3,20). In different rodent species as well as in humans, t-RES is well absorbed, distributed to various organs, and metabolized to *trans*-resveratrol-3-O-glucuronide and *trans*-resveratrol-3-O-sulfate (21-23).

In contrast to the detailed knowledge of t-RES activities in biological systems, much less is known about the effects of higher hydroxylated stilbenes. t-RES undergoes cytochrome P450 (enzyme CYP1B1) catalyzed hydroxylation to piceatannol (3,3',4',5-tetrahydroxy-*trans*-stilbene; PCA) (Fig. 1) and to two other unidentified mono- and dihydroxy-t-RES analogues (24). PCA differs from t-RES by possessing an additional hydroxyl group and has been isolated, together with t-RES, from grapes and wine (25). Like t-RES, PCA

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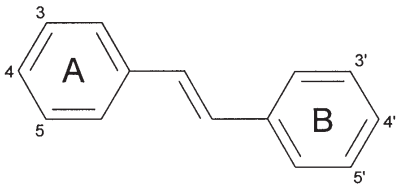
Key words: *trans*-resveratrol, piceatannol, structure-activity relationship, chemopreventive activity, antioxidant activity



Compounds	*3*	*4*	*5*	*3'*	*4'*	*5'*
trans-resveratrol	OH		OH		OH	
piceatannol	OH		OH	OH	OH	
3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene	OH	OH	OH	OH	OH	OH

Figure 1. Chemical structures of *trans*-resveratrol, piceatannol and 3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene.

displays cytotoxic activity in acute leukemia and lymphoma cells and anti-proliferative activity in colorectal cancer cell lines (26).

It has been found that the biological activity of t-RES and its analogues significantly depends on the structural determinants, which are i) the number and position of hydroxyl groups (8,27), ii) intramolecular hydrogen bonding (28-30), iii) stereoisomery (31) and iv) double bond (31). The observation that *trans*-stilbene compounds possessing a 4'-hydroxyl group (27,31,32), double bond (31) and bearing *ortho*-diphenoxyl or *para*-diphenoxyl functionalities (8,27) possess remarkably higher chemopreventive activity than t-RES gave us useful information for further investigations (33).

The aim of this study was to evaluate the antioxidant activity of t-RES and its two analogues: the natural compound PCA and a synthesized hydroxylated analogue of t-RES 3,3',4,4',5,5'-hexahydroxy-*trans*-stilbene (HHS) (Fig. 1) by measuring their DNA protective effects against H_2O_2-induced DNA strand breaks using single-cell gel electrophoresis (SCGE, comet assay). Because all of the compounds investigated have been shown to possess anti-leukemic activity, we tested their ability to inhibit the production of $^{\bullet}OH$ radicals in three leukemic cell lines: the murine leukemia cell line L1210, the human myelogenous leukemia cell line K562 and the human promyelocytic leukemia cell line HL-60. The cytotoxic effects of the compounds tested were determined by trypan blue exclusion technique.

Materials and methods

Cell cultures. The murine leukemia cell line (L1210), the human myelogenous leukemia cell line (K562) and the human promyelocytic leukemia cell line (HL-60) were purchased from the American Type Culture Collection (Manassas, VA, USA). L1210 and K562 cells were kept in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 μg/ml), and streptomycin (100 μg/ml). HL-60 cells were kept in RPMI-1640 medium moreover supplemented with amphotericin B (12.5 μg/ml). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. All *in vitro* experiments were performed during the exponential phase of cell growth.

Chemicals. t-RES, PCA, ethidium bromide (EtBr), Triton X-100 and agarose electrophoresis reagent were obtained from Sigma-Aldrich Chemie, Steinheim, Germany. Na_2EDTA, NaOH, H_2O_2, and NaCl were purchased from Lachema Brno, Czech Republic. Agarose II, RPMI-1640 medium without L-glutamine, and $NaHCO_3$ were bought from Amresco-Biotechnology Grade, USA. Tris(hydroxymethyl)-aminomethane (Tris) was obtained from Serva Feinbiochemica, Heidelberg, Germany. Trypan blue solution (TB) was obtained from Fluka Chemie AG, Switzerland. Phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free) was obtained from Sebak GmbH, Germany. FCS was purchased from Grand Island Biological Co., Grand Island, NY, USA. Amphotericin B, penicillin and streptomycin were obtained from PAN Biotech, GmbH, Germany. HHS was synthesized using standard chemical methodologies with purity within $\pm$0.4% of the theoretical values as described previously (26).

Cytotoxicity assay. Cells were incubated with different concentrations of stilbenes for 24 h at 37˚C in an incubator in the dark together with untreated control samples. Samples were then centrifuged at 1000 x g for 5 min at 25˚C. After pretreatment, cells were washed with PBS and again centrifuged at 1000 x g for 5 min at 25˚C. Cells were stained with TB (0.4%) and the number of viable cells (%) was determined. Data are means of three independently performed experiments.

Single cell gel electrophoresis (comet assay). Comet assay is based on the ability of DNA strand breaks to migrate in a weak electric field in the direction of the anode, giving the nucleolus the appearance of the tail of a comet when visualized by fluorescence microscopy. The procedure of Singh *et al* (34) was used with minor modifications suggested by Slamenová *et al* (35) and Gábelová *et al* (36).

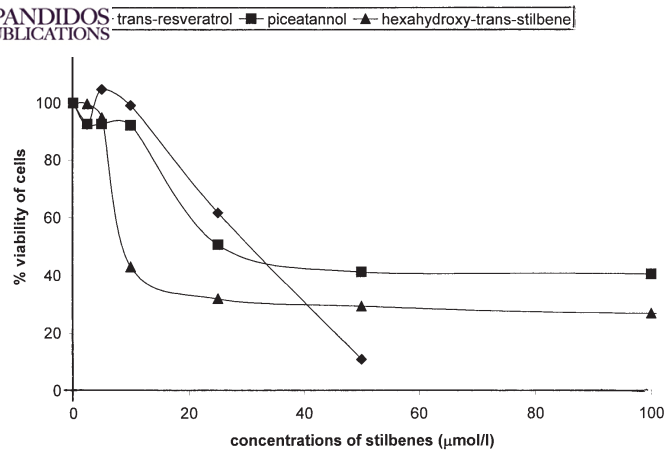


Figure 2. Percentage of viable cells after 24 h pre-treatment of L1210 cells with stilbenes evaluated by the TB exclusion technique. Data are means of three or more determinations. Standard deviations were within 5%.

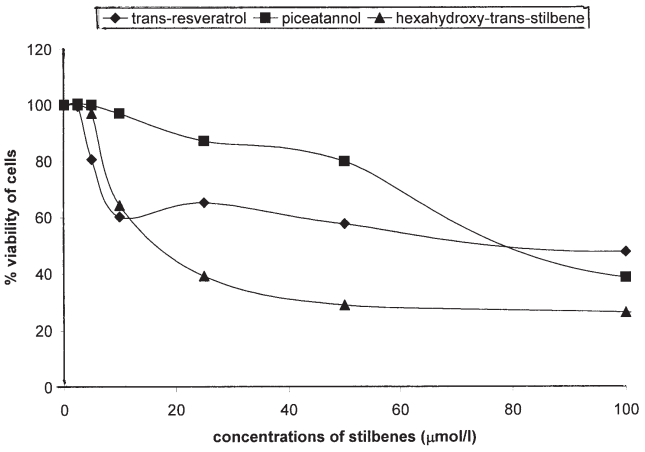


Figure 3. Percentage of viable cells after 24 h pre-treatment of K562 cells with stilbenes evaluated by the TB exclusion technique. Data are means of three or more determinations. Standard deviations were within 5%.

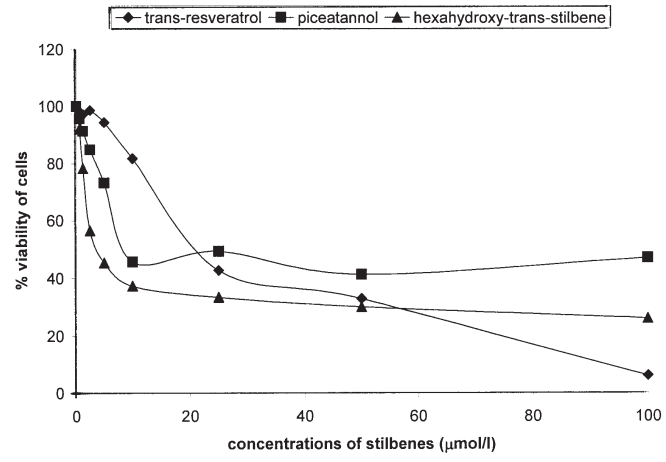


Figure 4. Percentage of viable cells after 24 h pre-treatment of HL-60 cells with stilbenes evaluated by the TB exclusion technique. Data are means of three or more determinations. Standard deviations were within 5%.

L1210, K562, and HL-60 cells were incubated with different concentrations of stilbenes (0.625-5 μmol/l) for 24 h at 37˚C in an incubator in the dark together with untreated control samples. A base layer of 100 μl of 1% NMP (normal melting point) agarose in PBS buffer was placed on microscope slides. Treated as well as control cells ($2x10^4$) were suspended in 0.75 % agarose II (low-melting point) and spread on the base layer. Triplicate slides were prepared per sample. Slides were treated with 50 μl of 100 μmol/l H_2O_2 for 5 min on ice in the dark and were washed twice with PBS. All slides were placed in lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, 1% Triton X-100, pH 10) for 1 h at 4˚C to remove cellular proteins. The slides were then transferred to an electrophoresis box containing an alkaline solution at pH 13 (300 mmol/l NaOH, 1 mmol/l Na_2EDTA). The slides were kept in this solution for 40 min unwinding time at 4˚C. Current of 25 V (300 mA) was applied for 30 min. The slides were removed, neutralized with Tris (0.4 mmol/l, pH 7.5), and stained with 20 μl EtBr (5 μg/ml). EtBr-stained nucleoids were evaluated with a Zeiss Jenalumar fluorescence microscope. For each sample, 100 comets were scored by computerized image analysis (Komet 5.0, Kinetic Imaging, Ltd., Liverpool, UK) for determination of DNA in the tail, linearly related to the frequency of DNA strand breaks (37). Data are means of three independently performed experiments.

Statistical analysis. The significance of differences between the samples was evaluated by the Student's t-test in all assays used. Statistically significant decreases of DNA strand breaks by H_2O_2 or stilbenes were as follows: $^{+}P<0.05$, $^{++}P<0.01$, and $^{+++}P<0.001$.

Results

Cytotoxicity of stilbenes. The cytotoxicity of the compounds studied was evaluated by the TB exclusion technique after 24 h pre-treatment of cells. The SCGE concentrations of the examined compounds were consecutively selected according to the cytotoxicity of cells appointed by the TB exclusion technique. The viability of cells should not be lower than 80%. Incubation of murine leukemia cells L1210 in RPMI-1640 medium containing different concentrations of t-RES (0-50 μmol/l), PCA (0-500 μmol/l), and HHS (0-500 μmol/l) caused cytotoxicity at concentrations lower than 20 μmol/l (Fig. 2). An even greater cytotoxic effect was observed in K562 cells pre-treated with t-RES and HHS (both 0-500 μmol/l) (Fig. 3). Their cytotoxic effect was below 10 μmol/l. On the other hand, incubation of K562 cells with PCA (0-500 μmol/l) induced lower cytotoxic activity (50 μmol/l). HL-60 cells were more sensitive to toxic effects of the investigated stilbenes when compared to L1210 and K562 cells. Incubation of HL-60 cells with t-RES, PCA, and HHS (all 0-100 μmol/l) caused cytotoxicity at concentrations below 10 μmol/l. Results are depicted in Fig. 4.

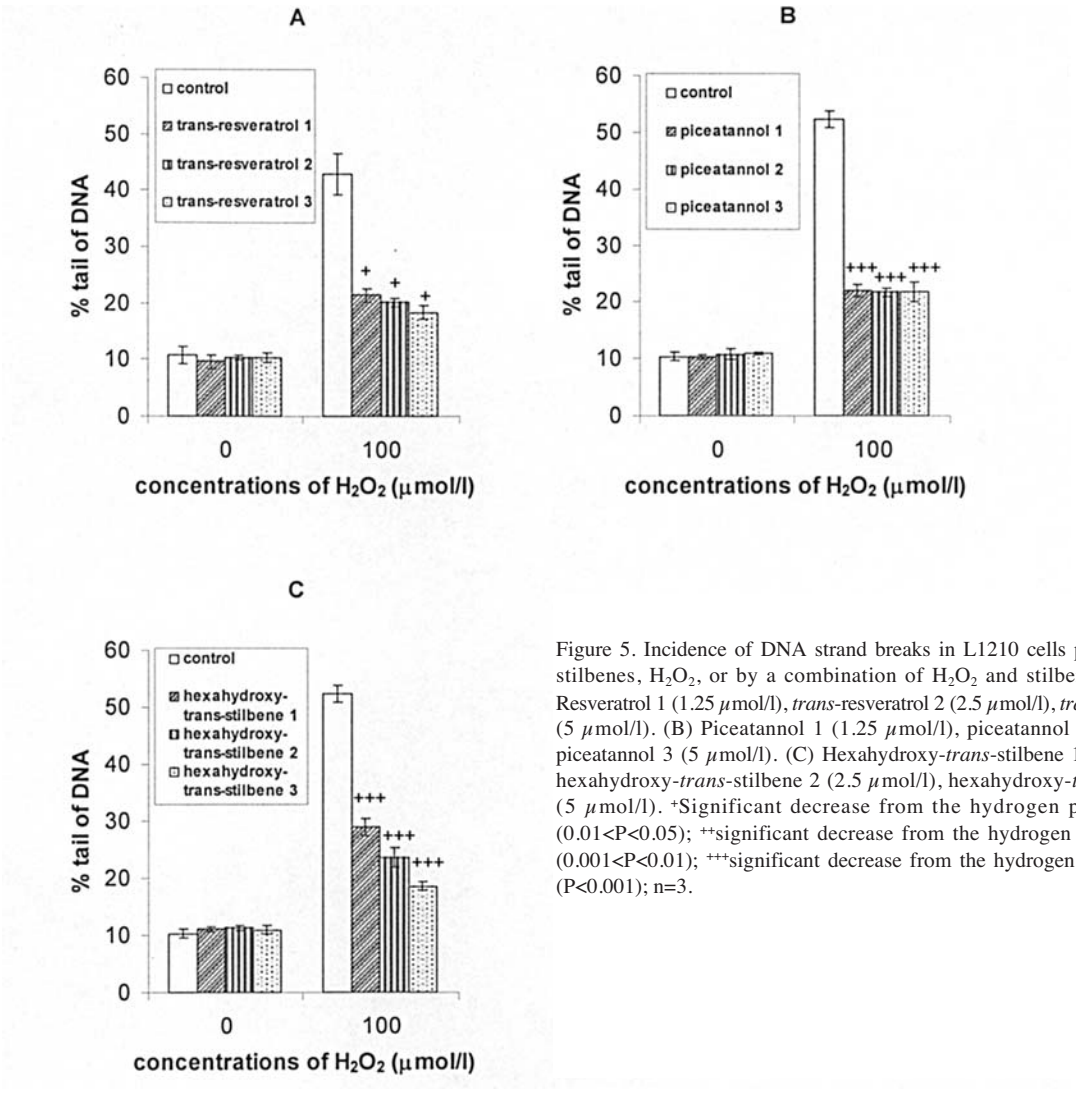


Figure 5. Incidence of DNA strand breaks in L1210 cells pre-treated with stilbenes, H_2O_2, or by a combination of H_2O_2 and stilbenes. (A) *trans*-Resveratrol 1 (1.25 μmol/l), *trans*-resveratrol 2 (2.5 μmol/l), *trans*-resveratrol 3 (5 μmol/l). (B) Piceatannol 1 (1.25 μmol/l), piceatannol 2 (2.5 μmol/l), piceatannol 3 (5 μmol/l). (C) Hexahydroxy-*trans*-stilbene 1 (1.25 μmol/l), hexahydroxy-*trans*-stilbene 2 (2.5 μmol/l), hexahydroxy-*trans*-stilbene 3 (5 μmol/l). $^{+}$Significant decrease from the hydrogen peroxide value ($0.01<P<0.05$); $^{++}$significant decrease from the hydrogen peroxide value ($0.001<P<0.01$); $^{+++}$significant decrease from the hydrogen peroxide value ($P<0.001$); n=3.

Effects of stilbenes on the level of single DNA strand breaks induced by H_2O_2. We measured the level of single DNA strand breaks in H_2O_2-treated L1210, K562 and HL-60 cells in order to test the antioxidant effects of the investigated compounds. The optimal concentration of H_2O_2 in our experiments was 100 μmol/l and the damage induced by H_2O_2 was regarded as the positive controls. Fig. 5 shows protective effects of 24 h pre-treatment of L1210 cells with t-RES, PCA, and HHS (all 1.25-5 μmol/l) in combination with a short-term treatment of cells with H_2O_2. All three stilbenes significantly inhibited the incidence of DNA single strand breaks induced by H_2O_2. Fig. 6 presents the level of H_2O_2-induced DNA strand breaks in K562 cells pre-incubated with t-RES, PCA, and HHS (all 1.25-5 μmol/l). A significant decrease of DNA lesions induced by H_2O_2 was observed in comparison with the control samples. Fig. 7 presents protective activity of 24 h pre-treatment of HL-60 cells with t-RES, PCA, and HHS (all 0.625-2.5 μmol/l) in combination with a short-term treatment of cells with H_2O_2. All compounds investigated significantly decreased the level of DNA single strand breaks.

The protective effects of PCA and HHS in L1210 cells and of HHS in HL-60 cells were significantly higher compared to the activity of t-RES ($^{+}P<0.05$). In K562 cells the differences of the antioxidant activities of PCA and HHS, and of PCA in HL-60 cells were of much higher significance when compared to t-RES ($^{++}P<0.01$).

Discussion

Recent decades have seen a tremendous increase in the interest in biological properties of natural products by the means of identification of novel small compounds that could have beneficial effects in clinical medicine. Many investigators are still trying to elucidate the mechanisms of action relating to the free radical scavenging effects of polyphenols. However, inconsistent results have been obtained regarding their antioxidant and reactive oxygen species (ROS) scavenging properties (38). The uncontrolled production of ROS contributes to the pathogenesis of cardiovascular disease and cancer, and inflammatory cells infiltrated in the atheroma plaque or tumor are the major source of ROS and eicosanoids (39). H_2O_2, a well-defined and investigated oxidative agent, does not react with DNA. It is envisaged that biological membrane-crossing H_2O_2 penetrates the cell nucleus and reacts with ions of iron or copper to form $^{\bullet}OH$ radicals. The attack of $^{\bullet}OH$ radicals on DNA results in breaks of nucleotide sugar moieties and a terminal sugar

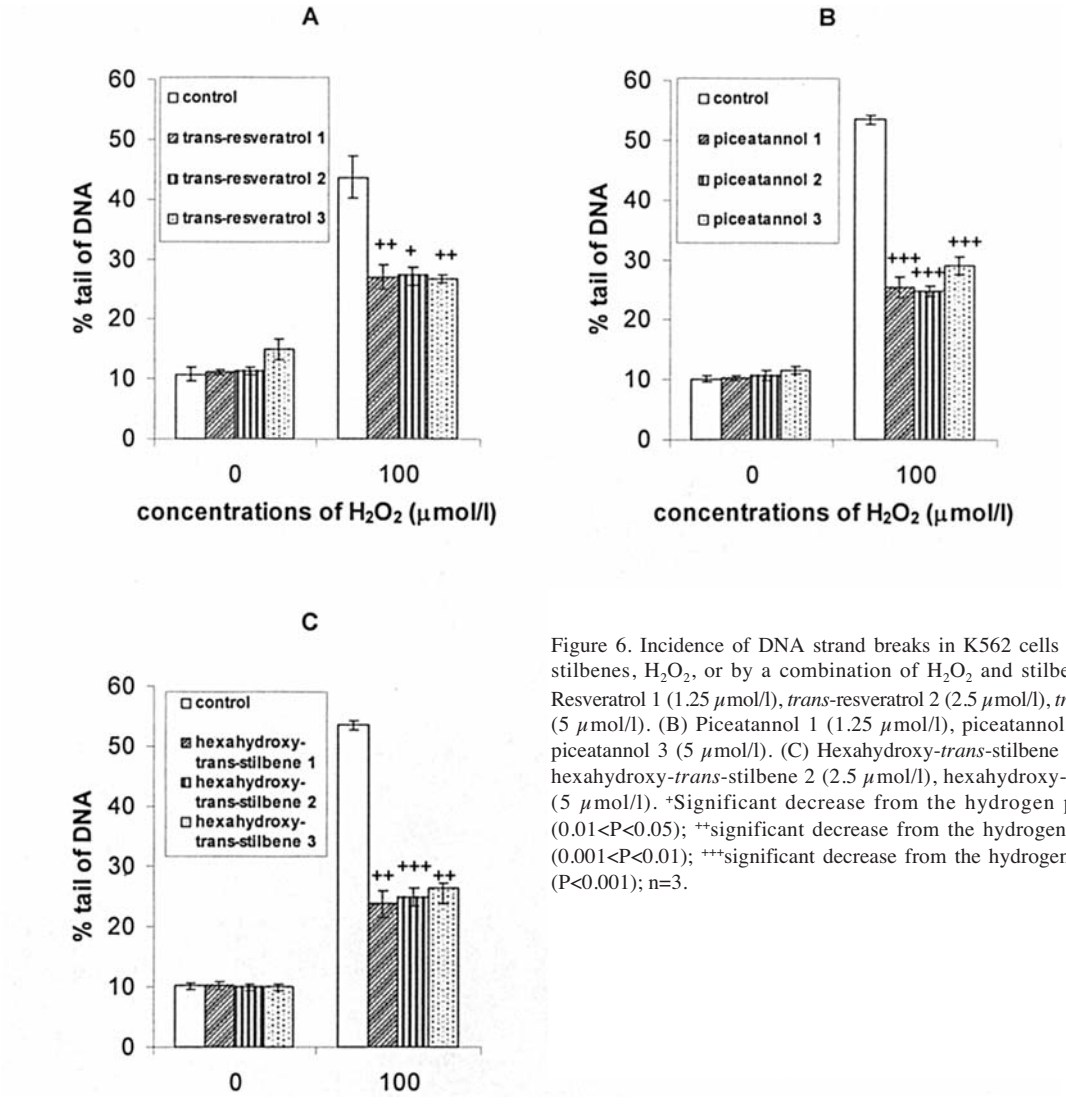


Figure 6. Incidence of DNA strand breaks in K562 cells pre-treated with stilbenes, H_2O_2, or by a combination of H_2O_2 and stilbenes. (A) *trans*-Resveratrol 1 (1.25 μmol/l), *trans*-resveratrol 2 (2.5 μmol/l), *trans*-resveratrol 3 (5 μmol/l). (B) Piceatannol 1 (1.25 μmol/l), piceatannol 2 (2.5 μmol/l), piceatannol 3 (5 μmol/l). (C) Hexahydroxy-*trans*-stilbene 1 (1.25 μmol/l), hexahydroxy-*trans*-stilbene 2 (2.5 μmol/l), hexahydroxy-*trans*-stilbene 3 (5 μmol/l). $^{+}$Significant decrease from the hydrogen peroxide value ($0.01<P<0.05$); $^{++}$significant decrease from the hydrogen peroxide value ($0.001<P<0.01$); $^{+++}$significant decrease from the hydrogen peroxide value ($P<0.001$); n=3.

residue fragment (40,41). A direct rejoining repairs these single breaks of DNA. In addition to this, ˙OH radicals attack DNA bases and produce thymine glycol, 8-hydroxyguanine or 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Most of these oxidative DNA lesions are repaired by base excision repair. However, some forms of oxidative damage of bases are repaired by systems that apparently recognize elements of DNA helix distortion rather than specific base damage (42).

Several studies have demonstrated that t-RES exerts a powerful antioxidant effect on multiple ROS (such as $O_2^{\cdot-}$ and H_2O_2) production in macrophage cells subjected to lipopolysaccharide (LPS)- or phorbol ester (PMA)-treatment. $O_2^{\cdot-}$ production by LPS- or PMA-treated phagocytic cells occurs via the NADPH oxidase pathway. Thus, it is possible that mechanisms affecting the NADPH oxidase activation are additionally involved in the effects of t-RES on ROS production (43). Maccarrone *et al* (44) studied apoptosis induced in K562 cells by H_2O_2 and other unrelated stimuli. t-RES reversed the elevation of leukotriene B4 and prostaglandin E2 induced by H_2O_2 challenge in K562 cells and also blocked lipoperoxidation induced by H_2O_2 in K562 cell membranes. The observation that t-RES is an efficient oxygen radical scavenger suggests a further mode of action of its anti-carcinogenic activity (45). Cai *et al* (46) found that the anti-oxidant activity of t-RES analogues containing 3,4-dihydroxyl groups was significantly higher than that of t-RES and other analogues. These data were supported by other investigators who also found free radical scavenging activity that was more significant when compared to t-RES in tumor cells (47-49).

The aim of this study was to investigate the protective effects of t-RES and its analogues PCA and HHS towards DNA damage induced by H_2O_2. We found that t-RES exerted a remarkable antioxidant activity against H_2O_2-induced DNA damage in L1210 murine leukemia cells (50.1-57.4% at concentrations of 1.25-5 μmol/l) (Fig. 5). The antioxidant activity of PCA determined by SCGE was even higher when compared to t-RES ($^{+}P<0.05$). Here, the protective activity of PCA was 58.0-58.4% at concentrations between 1.25-5 μmol/l (Fig. 5). The percentage of the protective effects exerted by HHS in L1210 cells was 44.6% at a concentration of 1.25 μmol/l, 54.9% at 2.5 μmol/l and 64.4% at 5 μmol/l, respectively (Fig. 5). In comparison with t-RES, an increase in the protective activities of HHS was observed ($^{+}P<0.05$).

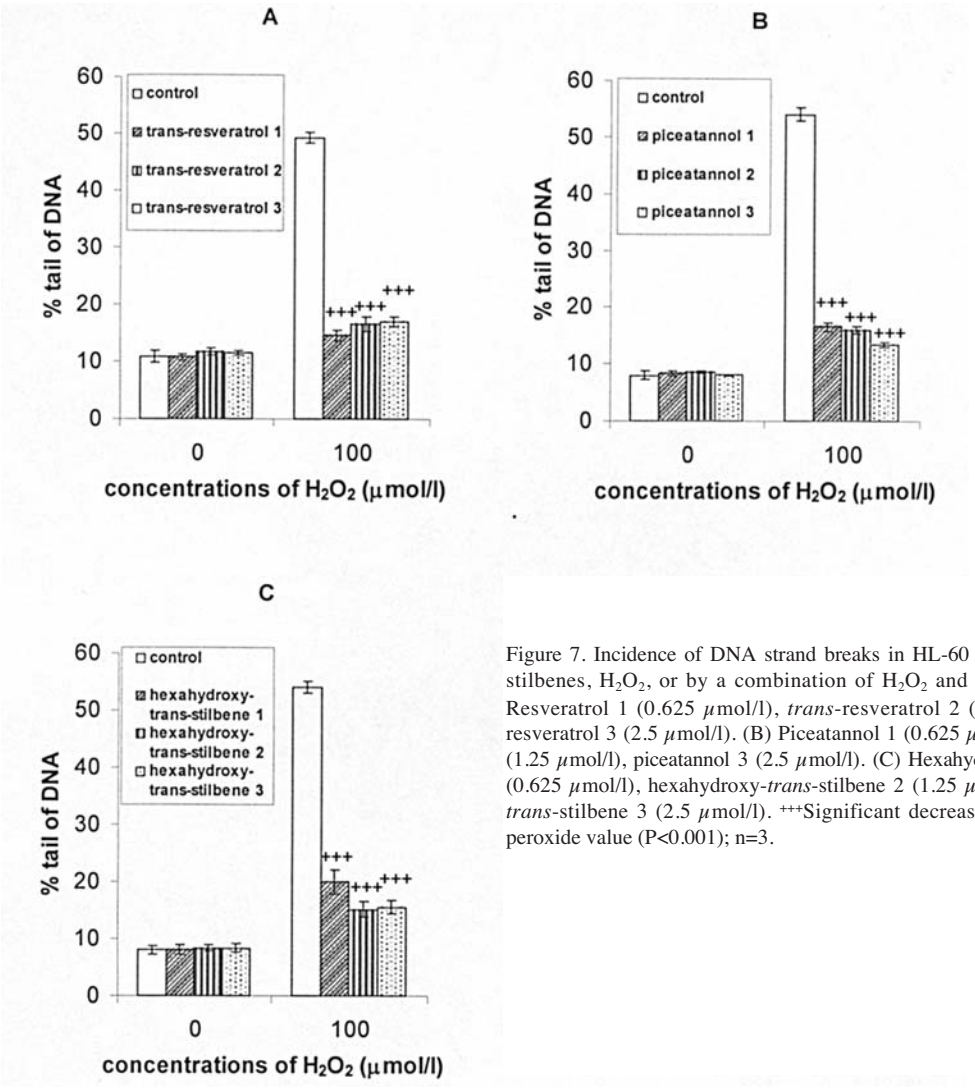


Figure 7. Incidence of DNA strand breaks in HL-60 cells pre-treated with stilbenes, H_2O_2, or by a combination of H_2O_2 and stilbenes. (A) *trans*-Resveratrol 1 (0.625 μmol/l), *trans*-resveratrol 2 (1.25 μmol/l), *trans*-resveratrol 3 (2.5 μmol/l). (B) Piceatannol 1 (0.625 μmol/l), piceatannol 2 (1.25 μmol/l), piceatannol 3 (2.5 μmol/l). (C) Hexahydroxy-*trans*-stilbene 1 (0.625 μmol/l), hexahydroxy-*trans*-stilbene 2 (1.25 μmol/l), hexahydroxy-*trans*-stilbene 3 (2.5 μmol/l). $^{+++}$Significant decrease from the hydrogen peroxide value ($P<0.001$); n=3.

The extent of DNA protection determined for t-RES by SCGE against H_2O_2-induced DNA damage in K562 human myelogenous leukemia cells was 37.2-38.8% at 1.25-5 μmol/l. The percentages of PCA and HHS protective effects in K562 cells were significantly higher ($^{++}P<0.01$) in the same concentration range when compared to t-RES (53.8-45.8%; 55.6-50.7%) (Fig. 6).

Similarly to the results obtained in L1210 and K562 cells, all stilbenes also inhibited the incidence of DNA single strand breaks induced by H_2O_2 in HL-60 cells. The extent of DNA protection determined for t-RES in HL-60 human promyelocytic leukemia cells was 70.4-65.5% at concentrations of 0.625-2.5 μmol/l. The percentages of PCA and HHS protective effects were 69.3-75% and 63.03-71.9%, respectively. Similarly to the results obtained in L1210 and K562 cells, the protective activities of PCA ($^{++}P<0.01$) and of HHS ($^{+}P<0.05$) were significantly higher compared to t-RES. Furthermore, the SCGE concentrations of stilbenes in HL-60 cells were lower (0.625-2.5 μmol/l) when compared to L1210 and K562 cells (1.25-5 μmol/l) according to their cytotoxicity (Figs. 2, 3 and 4).

All investigated stilbenes were shown to be potent antioxidant agents and the presence of *ortho*-dihydroxy structures significantly enhanced the protective effect against DNA damage caused by $^{\cdot}OH$ radicals. Our data are consistent with the findings of Cai *et al* (46) and Murias *et al* (26) who also found that *ortho*-dihydroxyl groups are responsible for the improved antioxidant activity of t-RES. Therefore, further investigations are warranted to explore the mechanisms of action of these promising stilbene derivatives.

Acknowledgements

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