Influence of *Styrax camporum* and of Chemical Markers (Egonol and Homoegonol) on DNA Damage Induced by Mutagens with Different Mechanisms of Action

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This study evaluated the influence of *Styrax camporum* stems hydroalcoholic extract (SCHE) and of chemical markers of the genus, egonol (EG) and homoegonol (HE), on DNA damage induced in V79 cells by mutagens with different mechanisms of action. These natural products were combined with different mutagens [methyl methanesulfonate (MMS), hydrogen peroxide (H₂O₂), (S)-(+)–camptothecin (CPT), and etoposide (VP-16)] to evaluate the modulatory effect on DNA damage. The results showed that SCHE was genotoxic at the highest concentration tested (60 µg/mL). Treatment with EG or HE alone induced no genotoxic effect, while genotoxic activity was observed when the two compounds were combined. The SCHE extract was able to reduce the frequency of micronuclei induced by H₂O₂ and VP-16. Similar results were observed when the cell cultures were treated with EG and/or HE plus VP-16. In contrast, the highest concentration (40 µg/mL) SCHE potentiated DNA damage induced by VP-16. Neolignan EG alone or combined with HE also potentiated H₂O₂-induced genotoxicity. However, these natural products did not influence the frequency of DNA damage induced by MMS or CPT. Therefore, the influence of SCHE and of chemical markers (EG and HE) of the genus on the induction of DNA damage depends on the concentration tested and on the mutagen used.

Key words *Styrax camporum*; egonol; homoegonol; genotoxicity; modulatory effect

The family Styracaceae found in various regions of America, Southeast Asia and the Mediterranean is known by the population due to the production of balsamic resins generically called as benzoins.¹ Styrax is one of the most important genera of the family Styracaceae. The genus comprises approximately 130 species and a large volume of information is available.²

*Styrax camporum* is widely found in the Brazilian cerrado and is popularly known as “estoraque-do-campo,” “benjo-eiro,” “cuia-do-brejo,” “canela-poca,” “fruta-de-pomba,” and “pinduíba.”³ Oral administration of the ethanolic extract of *S. camporum* oil,⁴ while HE is a veratryl analog originally isolated from *S. officinalis*.⁶ Furthermore, EG and HE attracted attention of synthetic organic chemists due their activity against tumor cell lines.⁷–¹¹

Previous studies from our research group showed the absence of genotoxic activity of the hydroalcoholic extract of *S. camporum* stems (SCHE) in different tissues examined in Swiss mice; however, the extract reduced genomic and chromosome damage induced by the mutagens methyl methanesulfonate (MMS) and doxorubicin.⁵ These data demonstrated promising chemopreventive activity of SCHE. In this respect, this study aimed to better understand the mechanisms of action involved in the protective effect of the extract in order to establish chemopreventive strategies. Therefore, SCHE was combined with mutagens with different mechanisms of action. Additionally, we investigated the involvement of the chemical markers of the genus *Styrax* (EG and HE) in the effect of SCHE on DNA damage.

MATERIALS AND METHODS

Plant Species, Preparation of the Extract, and Isolation of EG and HE *Styrax camporum* Poil. was collected in May 2012 in Santa Cecília (20°46′12″S and 47°14′24″W), Patrocínio Paulista, São Paulo, Brazil. The specimens were identified by Prof. Dr. Alba Regina Barbosa Araújo, University of Franca, São Paulo, Brazil. A voucher specimen (SPFR 13754) was deposited in the Herbarium of the Department

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Fig. 1. Chemical Structure of the Chemical Markers of the *Styrax* Genus, Egonol (1) and Homoegonol (2)

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of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Brazil. The preparation of the SCHE and the isolation of EG and HE were carried out as described by Oliveira et al.\(^\text{15}\)

**Quantification of EG and HE** A solution of EG and HE (1.0 mg/mL each) was prepared in methanol (1.0 mL). A stock solution (80 µg/mL) was obtained in methanol–water (95 : 5, v/v) and six dilutions of the stock solution (0.50 to 60.00 µg/mL) were injected in triplicate into an HPLC system and used for construction of the calibration curves. SCHE (1.0 mg/mL) was prepared in 1.0 mL methanol–water (95 : 5, v/v).

SCHE, EG and HE were analyzed by HPLC using the Shimadzu LC-20AD system (Kyoto, Japan) equipped with a DGU-20A5 degasser, an SPD-20A series photodiode array detector, a CBM-20A communication bus module, an SIL-20A-HT autosampler with a 20-µL loop, and LCsolution software. Chromatographic separation of the samples was accomplished on a Phenomenex Onyx monolithic C18 column (100×4.60 mm) equipped with a pre-column. The mobile phase consisted of methanol–water–acetic acid (57 : 42.9 : 0.1, v/v/v) and was eluted at a flow rate of 0.8 mL/min. The detector wavelength was set at 311 nm and the UV spectra were recorded between 200 and 400 nm. The photodiode array detector was used to identify EG and HE and to verify the purity of these patterns in the extract from the areas of the corresponding peaks.

**Cell Culture and Conditions** For the experiments, Chinese hamster lung fibroblasts (V79 cells) were maintained in culture flasks (25 cm\(^2\), Corning, U.S.A.) in HAM-F10+Dulbecco’s modified Eagle’s medium (DMEM) medium (Sigma-Aldrich, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Nutricell), 1.2 g/mL sodium bicarbonate (Sigma-Aldrich), 0.1 g/mL streptomycin (Sigma-Aldrich), and 0.06 g/mL penicillin (Sigma-Aldrich) at 37°C in a biological oxygen demand (BOD)-type chamber. Under these conditions, the average cell cycle time was 12 h and the cell line was used after the 4th passage.

**Colony-Forming Assay** Colony formation of V79 cells was used to evaluate the cytotoxicity of SCHE according to the protocol proposed by Franken et al.\(^\text{16}\). The colonies formed were counted under a magnifying glass to determine the survival fraction (%).\(^\text{17}\)

**DNA Damage-Inducing Agents** Four DNA damage-inducing agents with different mechanisms of action were used to evaluate the mutagenic potency of SCHE and the marker compounds:

- Methyl methanesulfonate (MMS; Sigma-Aldrich) dissolved in phosphate buffer (PBS): 22 µg/mL for the comet assay and 44 µg/mL for the micronucleus test. MMS is a direct mutagen that reacts with DNA and induces oxidative DNA damage, including breaks and base modifications.\(^\text{18}\)
- Hydrogen peroxide (H\(_2\)O\(_2\); 30%; Sigma-Aldrich) dissolved in distilled water: 3.4 µg/mL. H\(_2\)O\(_2\) is a mediator of oxidative stress that generates hydroxyl radicals and induces oxidative DNA damage, including breaks and base modifications.\(^\text{19}\)
- Camptothecin (CPT; Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich): 43 µg/mL. CPT acts by inhibiting topoisomerase I, an enzyme necessary for DNA replication.\(^\text{20}\)
- Etoposide (VP-16; Sigma-Aldrich) dissolved in DMSO: 1 µg/mL. VP-16 is an anticancer agent that inhibits topoisomerase II.\(^\text{21}\)

**Experimental Design** For the assessment of genotoxicity by the comet and micronucleus assays, the concentrations of SCHE (5, 10, 20, 40, 60 µg/mL) were chosen based on the results obtained in the clonogenic efficiency assay using cytotoxicity as a criterion. The concentrations of EG and HE were selected based on the amount of these chemical markers in SCHE, which account for 0.66 and 0.043%, respectively. Therefore, the treatments were 0.26 µg/mL EG, 0.017 µg/mL HE, and EG combined with HE. SCHE was dissolved in distilled water, while EG and HE were dissolved in DMSO (2.7 µg/mL, 1%). The experiments were conducted in triplicate.

The influence of the natural products on DNA damage was analyzed by the micronucleus test. The cell cultures were treated with SCHE (5, 10, 20, 40 µg/mL), EG and/or HE combined with each mutagen (MMS, H\(_2\)O\(_2\), CPT, and VP-16).

**Comet Assay** A total of 300 000 cells were seeded into tissue culture flasks (110×16 mm) and incubated for 25 h in 2.5 mL complete HAM-F10/DMEM medium. After this period, the cells were washed with PBS and treated for 3 h as described above in culture medium without FBS. At the end of the treatment, the cells were washed with PBS and trypsinized with 200 µL trypsin. After 3 min, the cells were gently resuspended in complete medium and 20 µL of the cell suspension was immediately used for the test.

The procedures for the comet assay described by Singh et al.\(^\text{22}\) were adopted, with minor modifications as described in detail by Speit and Hartmann\(^\text{23}\) and Munari et al.\(^\text{17}\). The slides were stained with 40 µL ethidium bromide (20 µg/mL). The nuclei were visualized under a Axios Imager fluorescence microscope (Carl Zeiss, Germany) using a 40× objective and the images were captured with image analysis software (Comet imager V.2.0.0). DNA damage was quantified using 50 nucleoids per repetition, for a total of 150 nucleoids per treatment, and is reported as % DNA in the comet tail.

Cell viability in the different treatments was evaluated by the Trypan blue exclusion method\(^\text{23}\) using the same cell suspension as obtained in the comet assay. Two hundred cells were analyzed per culture (600 cells/treatment).

**Micronucleus Test** For the micronucleus test, 50 0000 cells were seeded into a culture flask (25 cm\(^2\)) containing 5 mL HAM-F10+DMEM medium and incubated for 25 h. After this period, the cells were washed with PBS and treated for three hours according to the treatment groups as described above in culture medium without fetal bovine serum. The cells were then washed with PBS, complete culture medium containing cytochalasin-B (Sigma-Aldrich; 3 µg/mL) was added, and the mixture was incubated for 17 h. The cells were harvested and fixed as described by Furtado et al.\(^\text{25}\). The criterion established by Fenech\(^\text{26}\) was used for the analysis of micronuclei in which 3000 binucleated cells were scored per treatment, for a total of 1000 cells/treatment/repetition.

The nuclear division index (NDI) was determined for 1500 cells analyzed per treatment (500 cells/repetition). Cells with well-preserved cytoplasm that contained 1–4 nuclei were scored and the NDI was calculated according to Eastmond and Tucker\(^\text{27}\) using the following formula:
NDI = \frac{M_1 + 2M_2 + 3M_3 + 4M_4}{N}

where \( M_1 \sim M_4 \) are cells with 1, 2, 3 and 4 nuclei, respectively, and \( N \) is the total number of viable cells.

Additionally, the cytotoxicity index (CI) was calculated according to the method proposed by Kirsch-Volders et al.\(^{28}\):

\[
CI = 100 \left( \frac{NDIT - 1}{NDIC - 1} \right)
\]

where NDIT is the NDI for the treatments and NDIC is the NDI of the negative control.

**Percentage of DNA Damage Reduction** The percentage of DNA damage reduction in the treatments with the natural products combined with the mutagens was calculated using the following formula\(^{29}\):

\[
\% \text{ Reduction} = \frac{A - B}{A - C} \times 100
\]

where \( A \) corresponds to the damage obtained for the treatment with the different positive controls; \( B \) corresponds to the combination of the natural products and the different positive controls, and \( C \) corresponds to the negative control.

**Statistical Analysis** The data were analyzed statistically by ANOVA with the calculation of respective \( p \) values. In cases in which \( p<0.05 \), treatment means were compared by the Tukey’s test and the least significant difference was calculated for 0.05.

**RESULTS**

**Isolation and Quantification of EG and HE** The isocratic elution mode, with the mobile phase consisting of methanol–acetic acid–water (57:42.9:0.1) eluted at a flow rate of 0.8 mL/min, provided adequate resolution and peak purity. Peak purity was determined with a photodiode array detector using the LCsolution software (Fig. 2). This chromatographic condition thus permitted the quantification of EG and HE in SCHE.

The regression equations obtained from the calibration curves were \( y = 71450x + 96380 \) and \( y = 90910x + 122700 \), with correlation coefficients \( r \) of 0.9887 and 0.9882 for EG and HE, respectively, and a coefficient of variation <2% for analysis in triplicate. The standards were quantified in the SCHE corresponds to EG: 6.645±0.015 \( \mu \)g/mL and HE: 0.432±0.002 \( \mu \)g/mL, which represent 0.66 and 0.043% of the SCHE (7:3, v/v). Their presence was confirmed based on retention times (\( t_R1 \) 13.40 min and \( t_R2 \) 25.05 min) and UV-Vis spectra with \( \lambda_{max} \) around 311 nm (Fig. 2).

**Comet Assay** The frequency of DNA damage in V79 cells exposed to the different concentrations of SCHE was observed in cultures treated with SCHE concentrations of 2.5, 5, 10, 20 and 40 \( \mu \)g/mL. However, a concentration of 80 \( \mu \)g/mL or higher resulted in a significant reduction in colony formation compared to the negative control. Therefore, concentrations of 5, 10, 20, 40 and 60 \( \mu \)g/mL were chosen for the subsequent studies.

**Colony-Forming Assay** The survival fraction of V79 cells after treatment with the different concentrations of SCHE is shown in Fig. 3. No significant difference in colony formation was observed in cultures treated with SCHE concentrations of 2.5, 5, 10, 20 and 40 \( \mu \)g/mL. However, a concentration of 80 \( \mu \)g/mL or higher resulted in a significant reduction in colony formation when compared to the negative control. Therefore, concentrations of 5, 10, 20, 40 and 60 \( \mu \)g/mL were chosen for the subsequent studies.

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Micronucleus Test  The frequencies of micronuclei in V79 cells after treatment with SCHE and with the chemical markers of the genus (EG and/or HE) are shown in Figs. 4A.2 and B. No significant difference in micronucleus induction was observed between cultures treated with 5, 10, 20 or 40 µg/mL of SCHE compared to the negative control group. On the other hand, a significant increase in chromosome damage was observed at the highest concentration tested (60 µg/mL), revealing a genotoxic effect (Fig. 4A.2). A significant reduction was observed in the NDI at the highest concentration tested (60 µg/mL), with a cytotoxicity index of 27.0%.

The results showed that treatment with EG or HE did not increase the frequency of micronuclei in relation to the negative control group, indicating the lack of genotoxic effects. On the other hand, treatment with EG plus HE significantly increased the frequency of micronuclei compared to the negative control, revealing a genotoxic effect (Fig. 4B).

The Figs. 5 and 6 shows the results of simultaneous treatment with the natural products and mutagens. SCHE was unable to significantly reduce the frequency of micronuclei induced by MMS and CPT (Fig. 5). When combined with H₂O₂, SCHE significantly reduced the frequency of micronuclei at all concentrations tested (5, 10, 20, 40 µg/mL) when compared to treatment with H₂O₂ alone, demonstrating an antigenotoxic effect (Fig. 5). The percentage of DNA damage reduction ranging from 45.9 to 56.7%. Regarding the combination of SCHE with VP-16, the cultures treated with the three lower concentrations (5, 10, 20 µg/mL) exhibited a significant decrease in the frequency of micronuclei with the reduction in chromosome damage ranging from 25.9 to 36.3%. Moreover, treatment with the highest concentration (40 µg/mL) combined with VP-16 exerted a potentiating effect on DNA damage (Fig. 5). In cultures treated with the mutagens H₂O₂ and VP-16 combined with SCHE, a gradual increase in the concentration did not lead to a proportional increase in DNA damage reduction, demonstrating the absence of a dose–response correlation (Fig. 5).

Treatment of the cell cultures with EG and/or HE combined with MMS or CPT did not differ significantly from treatment with MMS or CPT alone. Regarding the combination with H₂O₂, HE was unable to influence the frequency of micronuclei induced by H₂O₂. However, treatment with EG or EG plus
HE resulted in a significant increase in the frequency of micronuclei when compared to treatment with \( \text{H}_2\text{O}_2 \), revealing a potentiating effect on DNA damage. A significant reduction in the frequency of micronuclei was observed in cultures treated with EG and/or HE combined with VP-16 when compared to those treated only with VP-16. These reductions were 43.0, 30.5 and 31.3% for the treatments with EG, HE and EG plus HE, respectively (Fig. 6).

**DISCUSSION**

In this study, SCHE exerted genotoxic effects at the highest concentration evaluated. We also evaluated the benzofuran lignans EG and HE to correlate and explain the results obtained with the genotoxic analysis of SCHE. The results showed that treatment with either EG or HE was not genotoxic. On the other hand, genotoxicity was observed for the combined treatment with EG and HE. Therefore, the genotoxic effect of SCHE may be due, at least in part, to the combined genotoxic activity of EG and HE in the extract.

SCHE, EG or HE did not influence the chromosomal damage induced by MMS. This mutagen is a classic \( \text{SN}_2 \) type agent, which shows high affinity for purine causing \( \text{N}^2 \)-alkylation.\(^{18,30}\) Additionally, MMS is known to facilitate the formation of adducts, such as \( \text{N}_7 \)-methylguanine (\( \text{N}_7\text{MeG} \)), \( \text{N}_7 \)-methylguanine (\( \text{N}_7\text{MeG} \)) and \( \text{N}_7 \)-methyladenine (\( \text{N}_7\text{MeA} \)), and crosslinks expressed as base substitution mutations.\(^{31}\) These adducts are unable to block replication, but may produce apurinic sites that can cause consequent double strand breaks.\(^{18,30}\) The \( \text{N} \)-methylation adducts can be repaired by base excision repair, which is considered the main defense mechanism against \( \text{SN}_2 \) agents.\(^{32}\) The lack of effect of SCHE, EG and HE on the genotoxicity induced by MMS suggests that these compounds are not targets for MMS alkylation, probably because of their chemical characteristics, and consequently are not able to compete with DNA. Furthermore, SCHE, EG and HE were unable to act on DNA repair or to recruit enzymes involved in this process.

The results obtained also showed that SCHE acted as a chemopreventive agent when combined with \( \text{H}_2\text{O}_2 \). Since \( \text{H}_2\text{O}_2 \) induces oxidative DNA damage, the protective effect observed may be attributed to the antioxidant activity of chemical constituents of the extract. The genotoxic effect of reactive oxygen species has been documented in \( V79 \) cells, causing DNA-strand breakage and base modification.\(^{23}\) Hydrogen peroxide is converted to the hydroxyl radical that causes oxidative damage close to the site of its formation.\(^{24}\) It has been postulated that \( \text{H}_2\text{O}_2 \) can also damage genomic DNA indirectly by inducing enzymatic digestion of DNA by higher-order chromatin degradation in oligomers.\(^{19,35}\)

Oxidative damage is known to affect macromolecules such as proteins, DNA and lipids. DNA base alterations, strand breakage and mutations are problems that are usually associated with free radical attacks on DNA. This damage can be prevented, reduced and even reversed with antioxidant supplementation.\(^{29}\) In the study of Braguini et al.,\(^{32}\) chemical characterization of \( S. \text{camporum} \) fractions led to the isolation and structural identification, for the first time, of flavonoids, including quercetin and kaempferol. These flavonoids are known for their well-established relationship between chemical structure and antioxidant activity and for their ability to inhibit cyclooxygenase and lipoxygenase enzymes.\(^{37}\) Flavonoids are also considerable natural antimutagens.\(^{38}\)

According to the literature, the protective activity of plant extracts can also be attributed to the presence of lignans, which are able to scavenge free radicals, protecting and stabilizing the genome.\(^ {39}\) Resende et al.\(^ {40}\) evaluated the genotoxic and chemopreventive activity of (−)-hinokinin, a substance derived from dibenzyltubrolactone lignan (−)-cubebin, in \( V79 \) cells. These authors postulated the antioxidant activity as a chemopreventive mechanism presented by (−)-hinokinin.

Although an antioxidant mechanism may be proposed for phenolic lignans, HE did not reduce the frequency of micronuclei induced by \( \text{H}_2\text{O}_2 \). Moreover, EG and EG plus HE potentiated the genotoxic effect of \( \text{H}_2\text{O}_2 \). These data permit us to conclude that EG and HE are not involved in the antigenotoxic activity of SCHE.

SCHE, EG or HE did not influence the genotoxicity in-

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**Fig. 6. Frequency of MN in V79 Cells after Treatment with EG, HE, and the Combination of EG with HE (EG+HE) Combined with MMS, \( \text{H}_2\text{O}_2 \), CPT and VP16 and Their Respective Controls**

SCHE, EG, homoeogonol; DMSO, dimethylsulfoxide (0.02 µg/mL, solvent control); MMS, methyl methanesulfonate (44 µg/mL). \( \text{H}_2\text{O}_2 \), hydrogen peroxide (3.4 µg/mL); CPT, (5+−)-camptothecin (43 µg/mL); VP16, etoposide (1 µg/mL). The values are the mean±S.D. The concentrations of EG and HE tested were 0.26 and 0.017 µg/mL, respectively. The combination corresponds to the same concentrations (0.26 EG and 0.017 HE µg/mL) in the same cultures. A total of 3000 binucleated cells were analyzed per treatment group. *Significantly different from the \( \text{H}_2\text{O}_2 \) control group (\( p<0.05 \)), ** Significantly different from the VP-16 control group (\( p<0.05 \)).
duced by CPT. This mutagen acts by binding to and stabilizing the covalent complex formed between topoisomerase I and DNA, preventing DNA religation cleaved, causing irreversible breaks during DNA replication. 43) The lack of effect of the natural products tested in this study suggests that they do not act at the topoisomerase I level.

Our results showed that SCHE acts as a chemopreventive agent at lower concentrations and potentiates DNA damage at higher concentrations when combined with VP-16. The latter acts on topoisomerase II, which uses ATP to cross a double helix of DNA, and causes double-strand breaks through changes in DNA topology. Two classes of inhibitors that interfere with topoisomerase II have been described: topoisomerase II poisons (etoposide, teniposide, doxorubicin), which stabilize the cleavable complex formed between DNA and topoisomerase II, generating high levels of enzyme-mediated breakage, and consequently become potent cellular toxins, and topoisomerase II catalytic inhibitors, which block the enzymatic activity of DNA without stabilizing the topoisomerase II complex or inducing DNA-strand breaks. 42) Thus, our results allow us to infer that the mechanism of action of SCHE is directly related to the modulation of topoisomerase II activity. The chemopreventive effect found for the combination of EG and HE with VP-16 supports the conclusion that these lignans may be involved for the modulation of topoisomerase II activity displayed by SCHE.

The complexity of the interactions between topoisomerase II and intercalating agents is demonstrated by the observation that these agents can antagonize or enhance the formation of double-strand breaks 43) depending on their concentration and experimental design. 44) The formation of double-strand breaks is enhanced when an intercalating agent modifies the chromatin structure in such a way that it facilitates rather than antagonizes the ability of a toxic agent to form the cleavable complex with topoisomerase II. 45) Our results suggest that the chemopreventive effect at lower concentrations may be related to the fact that SCHE acts directly on the cleavable complex prevented the action of VP-16 on the complex or destabilized it. Furthermore, in the highest concentration evaluated, SCHE may be acting as an inhibitor of topoisomerase II, facilitating stabilization of the complex, preventing religation of the cleaved strands of DNA, or as a catalytic inhibitor of topoisomerase II, blocking enzymes that reconnect the DNA strand. Therefore, the inhibitors of topoisomerase II can have a synergistic and antagonistic effect, depending on the concentration and experimental design.

The chemopreventive activity of SCHE, EG and HE was not dose dependent. According to Knasmüller et al., 46) dose–response evaluations are complicated by the fact that many chemopreventive agents may act simultaneously on different levels of protection and by the erratic absorption of these natural products by the cell membrane, which leads to erratic bioavailability of the substance within the cell. The lack of a dose–response relationship may therefore be attributed to the activation of different mechanisms depending on the dose tested.

Under the experimental conditions used, the present study showed that SCHE was genotoxic at the highest concentration tested. SCHE was effective in reducing chromosome damage induced by H2O2 and VP-16 at lower concentrations and potentiated this damage at higher concentrations. The similar results obtained for EG and HE suggest that these lignans are responsible, at least in part, for the genotoxicity of SCHE and its influence on the genotoxicity induced by VP-16. However, the chemopreventive effect of SCHE on chromosome damage induced by H2O2 is associated with the presence of other chemical constituents in the extract. The chemopreventive effect found for the combination of these natural products with VP-16 is related to the modulation of topoisomerase II activity. On the other hand, the protective effect of SCHE when combined with H2O2 may be attributed to the antioxidant activity of chemical constituents of the extract.

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Conflict of Interest
The authors declare no conflict of interest.

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