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BRIEF REPORT

Study of the cytotoxic activity of *Styrax camporum* extract and its chemical markers, egonol and homoegonol

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Abstract The benzofuran lignans egonol and homoegonol are found in all species of the genus *Styrax*. Since natural products are important sources of new anticancer drugs, this study evaluated the cytotoxic activity of a hydroalcoholic extract of the stems of *S. camporum* (SCHE) and their chemical markers, egonol (EG) and homoegonol (HE), against different tumor cell lines (B16F10, MCF-7, HeLa, HepG2, and MO59J). A normal human cell line (GM07492A) was included. Cytotoxic activity was evaluated at different treatment times (24, 48 and 72 h) using the XTT assay. More effective results were observed after 72 h of treatment. The lowest IC_{50} values were found for the HepG2 cell line, ranging from 11.2 to 55.0 μg/mL. The combination of EG and HE exerted higher cytotoxic activity than SCHE or treatment with either lignan alone, with the lowest IC_{50} (13.31 μg/mL) being observed for the MCF-7 line. Furthermore, treatment with these lignans was significantly more cytotoxic for some tumor cell lines compared to the normal cell line, GM07492A, indicating selectivity. These results suggest that these lignans may be used to treat cancer without affecting normal cells.

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Introduction

The cancer remains a major public health issue as more than one million people are diagnosed with cancer each year. Natural products constitute the major sources of chemical diversity, in purified or structurally identified form, and many drugs used for therapeutic applications are complex natural products or their derivatives (Salvador et al. 2013).

The plant species *Styrax camporum* Pohl (Styracaceae), popularly known as "estoraque do campo" or "cuia de brejo", is found in the states of São Paulo and Minas Gerais, Brazil, and is used in folk medicine to treat ulcers (Lorenzi 1982). Previous phytochemical studies have isolated egonol (EG; Fig. 1a) and homoegonol (HE; Fig. 1b), benzofuran neolignans used as phytochemical markers for the quality control of extracts of the genus *Styrax* (Moraes et al. 2011). Important biological activities are described in the literature on hydroalcoholic extract of *S. camporum* stems such as antiulcer (Bacchi and Sertié 1994; Bacchi et al. 1995) and antiparasitic activities (Braguini et al. 2012) as well as chemical genus markers egonol and homoegonol such as antifungal, antibacterial (Pauletti et al. 2000), anti-complement (Min et al. 2004) and cytotoxic activities (Li et al. 2005).

The development of chemoresistance, toxicity and side effects requires the identification of relatively non-toxic drugs or natural products to wage a more humane war against cancer (Pan and Ho 2008). However, the success of cancer chemotherapy depends on the development of drugs that selectively destroy tumor cells, or at least limit their proliferation without causing severe side effects (Nussbaumer et al. 2011).

Considering the need for new selective molecules for cancer therapy, the present study investigated the cytotoxic activity of a hydroalcoholic extract of *S. camporum* (SCHE) and its chemical markers, EG and HE, against different tumor cell lines.

Materials and methods

Plant material

Styrax camporum Pohl and the fruits of *S. ferrugineus* were collected in May and October 2012 in the Santa Cecilia garden (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. Voucher specimens (SPFR 13754 and SPFR 12169, respectively) were deposited in the Herbarium of the Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo.

Preparation of *S. camporum* crude hydroalcoholic extract

About 790 g of *S. camporum* Pohl stems were dried in a hot air oven at 40 °C. The material was ground in a blender and the powder was immersed in a solution of EtOH–H_2O (7:3 v/v) for 24 h at room temperature and then filtered three times through filter paper. This solution was concentrated at a temperature of less than 40 °C under vacuum to remove the solvent, yielding 95 g of a crude extract.

Isolation of egonol and homoegonol

The fruits of *S. ferrugineus* (94.7 g) were submitted to extraction with MeOH, yielding 8.6 g of a crude extract. Approximately 3.5 g of this extract was subjected to column chromatography on silica gel (70–230 mesh American Society for Testing and Materials (ASTM), 60 Å, São Paulo, Brazil, Sigma-Aldrich). The mobile phase consisting of *n*-hexane:EtOAc was used in increasing order of polarity: *n*-hexane (500 mL), *n*-hexane:EtOAc (9:1, 500 mL; 8:2, 500 mL; 7:3, 500 mL; 6:4, 500 mL; 5:5, 500 mL; 4:6, 500 mL; 2:8, 500 mL; v/v), EtOAc (500 mL), and EtOAc:MeOH (9:1, v/v; 500 mL). Sixty-six subfractions were thus obtained. Egonol was isolated from subfractions 32 to 36 and HE from subfractions 19 to 30. These subfractions were purified by Preparative thin-layer chromatography (TLC preparative) using dichloromethane (DCM)-MeOH (97:3, v/v) as the mobile phase, analyzed by HPLC–UV in comparison to authentic samples, and also identified by nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Culture conditions of the cell lines

The following cancer cell lines were used in the present study after the 4th passage: murine melanoma (B16F10) kindly provided by the Department of Biochemistry (Faculty of Medicine, University of São Paulo), human breast adenocarcinoma (MCF-7), human hepatocellular liver carcinoma (HepG2) (courtesy of Mutagenesis Laboratory, Department of Biological Sciences, Universidade Estadual Paulista), human cervical adenocarcinoma (HeLa), and human glioblastoma (MO59J) obtained from the Cell Bank of the Federal University of Rio de Janeiro. A normal human cell line (lung fibroblasts, GM07492A) (courtesy of Mutagenesis Laboratory of the University of São Paulo) was included to evaluate the

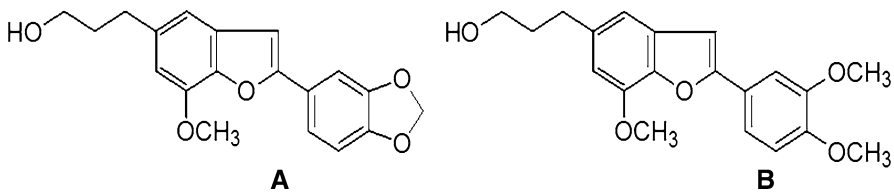

Fig. 1 Chemical structure of egonol (**A**) and homoegonol (**B**)

possible selective activity of the natural products tested. The different cell lines were maintained as monolayers in plastic culture flasks (25 cm^2) containing HAM-F10 plus DMEM (1:1; Sigma-Aldrich) for B16F10, MCF-7, HeLa, MO59J and GM07492A or only DMEM for HepG2, both supplemented with 10 % fetal bovine serum (Nutricell, Campinas, Brazil), 2.38 mg/mL Hepes (Sigma-Aldrich) and antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich). The cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere.

Cytotoxicity testing

The cytotoxic effects of the crude extract and isolated compounds was determined by monitoring the growth of untreated and treated cells using the Cell Proliferation Kit (an XTT-based colorimetric assay, Roche, Mannheim, Germany) after 24, 48 and 72 h of incubation. SCHE was directly diluted in complete culture medium at concentrations of 2.44–5000 μg/mL, while EG and HE were dissolved in dimethylsulfoxide (0.02 μg/mL; Sigma-Aldrich) and complete medium to obtain concentrations ranging from 0.78 to 1250 μg/mL. For the purpose of comparison, the chemotherapeutic agents doxorubicin (Zodiac, São Paulo, Brazil), (S)-(+)-camptothecin (Sigma-Aldrich) and etoposide (Sigma-Aldrich) were examined under the same experimental conditions. For the experiments, 10^4 cells were plated onto 96-well microplates. Each well received 100 μL HAM-F10/DMEM or DMEM medium containing the different concentrations of SCHE, EG and HE and the cells were cultured in a 5 % CO_2 atmosphere for 24, 48 and 72 h at 37 °C. After incubation, the culture medium was removed and the cells were washed with 100 μL phosphate-buffered saline and exposed to 100 μL HAM-F10 culture medium without phenol red. At designated time points, the tetrazolium salt (sodium 3'-[1-phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT, Roche Life Science, Indianapolis, IN, USA) was added to each well (25 μL) and the microplates were incubated for 17 h at 37 °C to allow the formation of an orange formazan dye product by metabolically active cells. Absorbance was read spectrophotometrically in an ELISA reader (Asys UVM 340/Microwin 2000 (Biochrom, Holliston, MA, USA)) at a wavelength of 450 nm and at a reference wavelength of 620 nm. The experiments were performed in triplicate.

Calculation of the selectivity index

The selectivity index was calculated by dividing the IC_{50} value of the isolated compound obtained for GM07492A cells by the IC_{50} value obtained for the cancer cell line.

Statistical analysis

Cytotoxicity was assessed using the IC_{50} value (50 % cell growth inhibition) as a response parameter, which was calculated with the GraphPad Prism program by plotting cell survival against the respective concentrations of the natural products tested. One-way ANOVA was used for the comparison of means ($P < 0.05$).

Results

The results obtained are summarized in Table 1. As can be seen in the table, the natural products exerted a time-dependent cytotoxic effect against most of the cell lines tested. For all cell lines, the lowest IC_{50} values were observed at 72 h of treatment with SCHE, EG and/or HE.

The lowest IC_{50} values were observed when the cells were treated simultaneously with EG and HE for most of the cell lines tested. The lower IC_{50} values were obtained for MCF-7 cells (40.9, 23.3 and 13.3 μg/mL at 24, 48 and 72 h of treatment, respectively). The value observed for these cells at 72 h of treatment is similar to that obtained for the doxorubicin (13.4 μg/mL at 72 h of treatment).

Considering the selectivity, most of the tumor cell lines treated with HE exhibited lower IC_{50} values than those observed for the normal cell line, wherein the selectivity index ranged from 1.2 (MCF-7 cells, 24 h of treatment) to 2.8 (HeLa cells, 48 h of treatment).

Although low IC_{50} values were observed for cells treated with EG plus HE, the lowest IC_{50} value was obtained for HepG2 cells treated with EG (11.2 μg/mL at 72 h of treatment), with a selectivity index of 6.3.

Discussion

According to Suffness and Pezzuto (1990), a selectivity index of 2.0 or higher is interesting since this value indicates that the compound is two-fold more

Table 1 IC_{50} values and selectivity index (SI) of *Styrax camporum* hydroalcoholic extract (SCHE) and its chemical markers, egonol (EG) and homoegonol (HE), against different cell lines after 24, 48 and 72 h of treatment

Cell line	Treatment (μg/mL)													
	SCHE		EG		HE		EG + HE		DXR		CPT		VP16	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
24 h														
GM07492A	321.8 ± 26.7	–	184.7 ± 6.7	–	109.5 ± 5.0	–	52.9 ± 8.8[b]	–	0.5 ± 0.2	–	3.2 ± 0.1	–	1.2 ± 0.5	–
B16F10	319.7 ± 6.2	1.0	NE	–	82.3 ± 2.5[a]	1.3	46.3 ± 0.9	1.1	101.6 ± 24.1	–	7.3 ± 1.4	–	325.4 ± 6.7	–
HeLa	854.6 ± 8.9	–	NE	–	127.6 ± 9.3	–	81.7 ± 3.5	–	5.3 ± 1.3	–	36.0 ± 12.4	–	82.6 ± 15.6	–
HepG2	116.4 ± 16.1[a]	2.7	NE	–	67.5 ± 7.3[a]	1.6	73.2 ± 7.6	–	21.9 ± 9.0	–	19.8 ± 0.7	–	225.5 ± 31.8	–
MCF-7	853.4 ± 8.2	–	150.4 ± 8.2	1.2	87.9 ± 4.4	1.2	40.9 ± 6.3[b]	1.3	62.1 ± 2.0	–	11.8 ± 1.9	–	235.3 ± 6.4	–
MO59J	451.0 ± 26.7	–	335.8 ± 9.2	–	347.0 ± 3.7	–	552.0 ± 7.4	–	16.2 ± 2.5	–	11.1 ± 2.7	–	42.9 ± 0.4	–
48 h														
GM07492A	196.5 ± 15.0[c]	–	131.2 ± 3.8[c]	–	106.0 ± 3.9	–	35.0 ± 2.5[b,c]	–	2.2 ± 0.1	–	0.1 ± 0.0[c]	–	4.2 ± 0.6	–
B16F10	273.7 ± 21.1	–	220.4 ± 8.2	–	49.1 ± 3.5[a,c]	2.2	43.9 ± 0.5	–	3.2 ± 1.1[c]	–	0.6 ± 0.1[c]	–	12.4 ± 0.8[c]	–
HeLa	475.4 ± 21.5[c]	–	253.0 ± 5.4	–	74.0 ± 2.9[a,c]	1.4	43.2 ± 2.2[b,c]	–	3.4 ± 0.4	–	0.2 ± 0.0[c]	–	6.1 ± 0.1[c]	–
HepG2	75.7 ± 3.9[a,c]	2.6	273.0 ± 5.3	–	37.6 ± 0.2[a,c]	2.8	65.1 ± 3.0	–	4.1 ± 1.3[c]	–	0.9 ± 0.0[c]	–	31.5 ± 1.9[c]	–
MCF-7	531.9 ± 4.1[c]	–	128.6 ± 4.3	1.0	40.5 ± 2.3[a,c]	2.6	23.3 ± 1.7[a,c]	1.5	4.1 ± 1.3[c]	–	1.0 ± 0.0[c]	–	11.4 ± 1.4[c]	–
MO59J	423.2 ± 3.3	–	308.7 ± 6.3	–	131.5 ± 8.4[c]	–	158.1 ± 6.3[c]	–	3.5 ± 0.6[c]	–	0.9 ± 0.0[c]	–	16.6 ± 1.7[c]	–
72 h														
GM07492A	183.4 ± 9.4[c]	–	70.4 ± 3.2[c,d]	–	71.3 ± 9,7[c,d]	–	21.3 ± 4.9[c,d]	–	0.8 ± 0.0[d]	–	0.2 ± 0.1[c,d]	–	0.3 ± 0.1[c,d]	–
B16F10	226.5 ± 12.6[c,d]	–	171.4 ± 5.4[d]	–	46.1 ± 0.8[a,c]	1.5	35.3 ± 0.4[c,d]	–	2.4 ± 0.5[c]	–	0.4 ± 0.0[c,d]	–	9.4 ± 0.7[c]	–
HeLa	359.9 ± 15.3[c,d]	–	228.5 ± 11.5	–	45.6 ± 0.8[a,c,d]	1.5	38.7 ± 1.1[c]	–	3.3 ± 0.5[c]	–	0.7 ± 0.0[c,d]	–	5.3 ± 0.9[c]	–
HepG2	55.0 ± 3.9[a,c,d]	3.3	11.2 ± 0.4[a,d]	6.3	42.3 ± 2.4[a,c]	1.5	53.3 ± 1.6[c,d]	–	2.2 ± 0.4[c,d]	–	0.1 ± 0.0[a,c,d]	2.0	0.7 ± 0.0[c,d]	–
MCF-7	306.6 ± 1.8[c,d]	–	46.2 ± 6.6[a,c,d]	1.5	40.6 ± 7.6[a,c]	1.7	13.3 ± 0.6[a,b,c,d]	1.6	13.4 ± 1.1[c,d]	–	1.8 ± 0.3[c]	–	4.3 ± 0.5[c,d]	–
MO59J	216.7 ± 18.8[c,d]	–	180.9 ± 1.9[c,d]	–	106.6 ± 16.2[c,d]	–	62.2 ± 4.1[b,c,d]	–	1.8 ± 0.2[c,d]	–	0.1 ± 0.0[a,c,d]	2.0	5.0 ± 0.7[c,d]	–

Values are the mean ± SD, n = 3

Doxorubicin (DXR), (S)-(−)camptothecin (CPT) and etoposide (VP16) were used as positive controls. GM07492A (human lung fibroblasts), B16F10 (murine melanoma), MCF-7 (human breast adenocarcinoma), HeLa (human cervical adenocarcinoma), HepG2 (human hepatocellular carcinoma), and MO59J (human glioblastoma)

NE not effective

[a] Significantly different from the normal cell line (GM07492A) ($P < 0.05$)

[b] Significantly different from treatment with either EG or HE ($P < 0.05$)

[c] Significantly different from the 24-h treatment

[d] Significantly different from the 48-h treatment. The selectivity index is the ratio of the IC_{50} value of the extract or isolated substance obtained for GM07492A cells to the value found for the tumor cell line

cytotoxic against the tumor cell line compared to the normal cell line.

According to the U.S. National Cancer Institute, only extracts with IC_{50} values of <30 μg/mL against experimental tumor cell lines are promising agents for anticancer drug development (Suffness and Pezzuto 1990). The present results showed that EG and HE were more cytotoxic than SCHE against all tumor cell lines tested. The combination of EG and HE showed IC50 values of less than 30 μg/mL for MCF-7 and EG for HepG2. Furthermore, the natural products exhibited high selective indices in some cases. These findings suggest the combination of EG and HE as well as EG alone to be a promising alternative for the development of anticancer drugs.

Literature data have demonstrated the cytotoxic activity of these benzofuran lignans, especially EG. Teles et al. (2005), evaluating the two benzofuran lignans, EG and HE, in cultures of Hep-2 (larynx epidermoid carcinoma), HeLa and C6 (rat glioma) cells, showed a dose-dependent cytotoxic effect of the two lignans against all cell lines tested. The authors observed significant activities ($IC_{50} < 10$ μg/mL) of EG against C6 and Hep-2 cells and of HE against C6 and HeLa cells when compared to the positive control streptonigrin after 24 h of treatment. Reiter et al. (2014), evaluating the cytotoxicity of EG and derivatives against a wild-type human T cell lymphoblast-like cell line (CCRF-CEM) and a human leukemia cell line (CEM/ADR5000), observed considerable cytotoxicity of EG ($IC_{50} = 1.88 \pm 0.41$ μM).

Li et al. (2005) evaluated the cytotoxicity of fractions of an EtOH extract of *S. perkinsiae* and isolated compounds in two breast cancer cell lines (MCF-7 and MDA-MB-231) after 48 h of treatment using a colorimetric chemosensitivity assay with sulforhodamine B and demonstrated the cytotoxic activity of EG at high concentrations ($IC_{50} > 100$ μg/mL). These authors believe that most benzofuran neolignans and nor-lignans with phenolic hydroxy groups possess cytotoxic activity. It has been demonstrated that the phenolic hydroxy groups largely contribute to this biological activity and significant differences in activity have been related to the lignan structure (Hayakawa et al. 2004).

Further biological and phytochemical studies should be conducted to identify the mechanism of action underlying the cytotoxicity of lignans EG and HE.

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Conflict of interest The authors declared that they have no conflicts of interest.

References

Bacchi EM, Sertié JA (1994) Antiulcer action of *Styrax camporum* and *Caesalpinia ferrea* in rats. Planta Med 60:118–120

Bacchi EM, Sertié JA, Villa N, Katz H (1995) Antiulcer action and toxicity of *Styrax camporum* and *Caesalpinia ferrea*. Planta Med 61:204–207

Braguini CG, Bertanha CS, Gonçalves UO, Magalhães LG, Rodrigues V, Melleiro-Gimenez VM, Groppo M, Silva ML, Cunha WR, Januário AH, Pauletti PM (2012) Schistosomicidal evaluation of flavonoids from two species of *Styrax* against *Schistosoma mansoni* adult worms. Pharma Biol 50:925–929

Hayakawa I, Shioya R, Agatsuma T, Furukawa H, Sugano Y (2004) Thienopyridine and benzofuran derivatives as potent anti-tumor agents possessing different structure-activity relationships. Bioorg Med Chem Lett 14:3411–3414

Li QL, Li BG, Qi HY, Gao XP, Zhang GL (2005) Four new benzofurans from seeds of *Styrax perkinsiae*. Planta Med 71:847–851

Lorenzi H (1982) Árvore Brasileiras. Manual de identificação e cultivo de plantas arbóreas nativas do Brasil. Nova Odessa Plantarum, São Paulo

Min BS, Oh SR, Ahn KS, Kim JH, Lee J, Kim DY, Kim EH, Lee HK (2004) Anti-complement activity of norlignans and terpenes from the stem bark of *Styrax japonica*. Planta Med 70:1210–1215

Moraes AC, Bertanha CS, Gimenez VM, Groppo M, Silva ML, Cunha WR, Januário AH, Pauletti PM (2011) Development and validation of a high-performance liquid chromatography method for quantification of egonol and homoegonol in *Styrax* species. Biomed Chromatogr 26:869–874

Nussbaumer S, Bonnabry P, Veuthey JL, Fleury-Souverain S (2011) Analysis of anticancer drugs: a review. Talanta 85:2265–2289

Pan MH, Ho CT (2008) Chemopreventive effects of natural dietary compounds on cancer development. Chem Soc Rev 37:2558–2574

Pauletti PM, Araújo AR, Young MCM, Giesbrecht AM, Bolzani VS (2000) Nor-lignanas from the leaves of *Styrax ferrugineus* (Styracaceae) with antibacterial and antifungical activity. Phytochemistry 5:597–601

Reiter C, Capcl Karagöz A, Fröhlich T, Klein V, Zeino M, Viertel K, Held J, Mordmüller B, Emirdağ Öztürk S, Anıl H, Efferth T, Tsogoeva SB (2014) Synthesis and study of cytotoxic activity of 1,2,4-trioxane-and egonol-derived hybrid molecules against *Plasmodium falciparum* and a

multidrug-resistent human leukemia cells. Eur J Med Chem 75:403–412

Salvador JA, Carvalho JF, Neves MA, Silvestre SM, Leitão AJ, Silva MM, Sá e Melo ML (2013) Anticancer steroids: linking natural and semi-synthetic compounds. Nat Prod Rep 30:324–374

Suffness M, Pezzuto JM (1990) Assays related to cancer drug discovery. In: Hostettmann K (ed) Methods in plant biochemistry: assay for bioactivity. Academic Press, London, pp 71–133

Teles HL, Hemerly JP, Paulettit PM, Pandolfi JR, Araujot AR, Valentini SR, Young MC, Bolzani VS, Silva DH (2005) Cytotoxic lignans from the stems of *Styrax camporum* (Styracaceae). Nat Prod Res 14:319–323