Ethanolic extract of *Casearia sylvestris* and its clerodane diterpen (caseargrewiin F) protect against DNA damage at low concentrations and cause DNA damage at high concentrations in mice’s blood cells

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*Casearia sylvestris* is used in Brazil as a popular medicine to treat ulcer, inflammation and tumour. Caseargrewiin F is a clerodane diterpene isolated from the ethanolic leaf extract of *C. sylvestris*. The aim of the study was to assess the capacity of the ethanolic extract of *C. sylvestris* leaves and caseargrewiin F to protect DNA and verify if both the compounds cause some DNA damage, using the micronucleus (MN) test and comet assay in mice. Balb/C mice were treated with the extract [3.13, 6.25, 12.5, 25, 50 and 75 mg/kg body weight (b.w.)] and caseargrewiin F (0.16, 0.32, 0.63, 1.3, 2.5 and 3.8 mg/kg b.w.) for 14 days. On day 15, DNA damage was induced by intra-peritoneal (i.p.) injection of cyclophosphamide (CP) (i.p.) at 50 mg/kg b.w. after the MN test and comet assay were performed. A protective effect of ethanolic extract was observed in MN test (6.25 and 12.5 mg/kg b.w.) and the comet assay (3.13 and 6.25, 12.5 and 25 mg/kg b.w.). Caseargrewiin F showed protective effect at 0.63, 1.3 and 2.5 mg/kg b.w. only in comet assay. We also tested the ability of compounds of *C. sylvestris* to induce MN and to increase the comet assay tail moment. The experimental design was similar to the DNA protection assay except that in test groups we omitted the CP challenge. We observed increased damage at 50 and 75 mg/kg b.w. of ethanolic extract of *C. sylvestris* and caseargrewiin F at 3.18 mg/kg b.w. in both the MN test and comet assay. We conclude that ethanolic extract of *C. sylvestris* and caseargrewiin F can protect cells against DNA damage induced by CP at low concentrations, but at high concentrations these compounds also induce DNA damage.

Introduction

Medicinal herbs have long been used in medical treatment and are still the sole remedy against several diseases in many parts of the world (1). Also, many natural products present in fruits, beverages and vegetables may be regarded as protective or even anti-proliferation agents for several types of human cancer (2).

Some Brazilian natural products, including those that are isolated from *Casearia sylvestris*, are used in folk medicine, despite their potential toxicity. *C. sylvestris* Swartz (Salicaceae) (3) is a tree widely distributed in Brazil, from Amapá in the north down to Rio Grande do Sul and it is found in a variety of ecosystems from the savannah-like the Cerrado to the tropical Atlantic Rain forest and the equatorial Amazon forest. Moreover, it has also been found in Mexico and the Antilles (4).

*Casearia sylvestris* is commonly known as ‘guacatonga’ and used widely for its anti-inflammatory and anti-ulcerative activities (4–6) as well as its cytotoxic activity against malignant tumour cells (7). In addition, the bark of *C. sylvestris* has been used against fever and for treatment of herpes virus and diarrhoea (8). Phytochemical investigations revealed that the major compounds isolated from *C. sylvestris* exhibited both cytotoxic and anti-fungal activities (7).

Caseargrewiin F is a clerodane diterpene, found in plants of the *Casearia* genus, described as a colourless and amorphous solid (9). While caseargrewiin F was not shown to be cytotoxic to human epidermoid carcinoma in the mouth, it exhibited cytotoxicity against human breast cancer cells and human small cell lung cancer. In the present study, caseargrewiin F was isolated from the leaves of *C. sylvestris*.

In order to complement the studies of biological activity of compounds isolated from *C. sylvestris*, the aim of the present study was to determine whether the ethanolic extract of leaves of *C. sylvestris* and caseargrewiin F protect cells against DNA-induced damage or induce DNA damage by theirselves, using the micronucleus (MN) test and comet assay in Balb-C mice.

Materials and methods

Plant material and preparation of extract

Leaves of *C. sylvestris* Swartz (Salicaceae) were collected at the Parque Estadual Carlos Botelho (São Miguel Arcanjo, São Paulo, Brazil) in March and July 2004. Voucher specimens are deposited at the Herbarium ‘Maria Eneida P. Kaufmann’ (Instituto Botânico do Estado de São Paulo, São Paulo, Brazil) with the reference numbers AG504, AG505, AG506, AG513 and AG519. The plant material was identified by Dr Ines Cordeiro (Instituto de Botância do Estado de São Paulo, São Paulo, Brazil). Dried and powdered leaves of *C. sylvestris* (20.5 kg) were extracted with ethanol (ca. 200 litre) in a stainless steel extractor with solvent re-circulation for ca. 24 h at 40°C. The crude extract was concentrated under reduced pressure (rotaveaporator) and dried in a desiccator over silica gel under reduced pressure to yield 1.5 kg of dry residue.

Identification of compounds in the extract

The identification of compounds in ethanolic extract of *C. sylvestris* was carried out in Núcleo de Bioensaios, Biossíntese e Ecoligologia de Produtos Naturais, Chemistry Institute, São Paulo State University, by Andre Gonzaga dos Santos.

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High performance liquid chromatography-diode array detector analysis A 9.90 mg of dried extract was submitted to solid phase extraction (SPE) (C-18 Merck®; 2 × 1 cm × 40–63 μm) and eluted with methanol (98:02, v/v, 5.0 ml). The sample was dried in a desiccator over silica gel under reduced pressure, dissolved in 4.0 ml of methanol and filtered through 0.22 μm membrane filter (PVDF Millipore®) into a high performance liquid chromatography (HPLC) vial. The reference standard used in this work—caseargrewiin F and casearin U—was purified and identified in the laboratories of Núcleo de Bioensaios, Biossintese e Ecosistemas de Produtos Naturais, Chemistry Institute, São Paulo State University, Araraquara, Brazil; for details see Santos et al. (10). The standard solutions were prepared by weighing an appropriate amount of caseargrewiin F into a volumetric flask. After diluting with methanol, the standard solutions (0.0079, 0.0199, 0.0635, 0.2540 × 0.5080 mg/ml) were filtered through 0.22 μm membrane filter (PVDF Millipore®) into a HPLC vial before injections. Analytical reversed-phase HPLC was performed using a Varian® ProStar system (comprising a model 240 pump, a 410 autosampler and a 330 photodiode array detector), with control and data handling managed by Star® Chromatography software. Analysis was conducted using a Phenomenex® Gemini C-18 column (250 × 4.6 mm, 5 μm) eluted with a gradient of acetonitrile: methanol: water (44:22:34 to 47:53:00, v/v/v, in 42 min) and acetonitrile: methanol (53:47, v/v, in 5 min); flow rate 0.8 ml/min; detection range 210–400 nm; injection volume 20 μl. Analysis was realized in triplicate.

Gas chromatography and gas chromatography-mass spectrometry analysis A 5.0 mg of dried extract was submitted to SPE (2 × 1 cm) from silica gel (60–200 μm; Merck®; activated charcoal (Synth®) (1:1, w/w) eluted with hexane: ethyl acetate (95:05, v/v, 5.0 ml). The sample was dried and diluted in hexane to afford 1.0 mg/ml solution. Gas chromatography (GC) analysis was performed on a Shimadzu QP2010 gas chromatograph with flame ionization detector using a DB-5 column (30 m × 0.25 mm × 0.25 μm). The carrier gas was He at a flow rate of 1.33 ml/min, split mode with ratio of 1:20 and injection volume of 1 μl. Injector and detector temperatures were 240 and 250°C, respectively. The oven temperature was programmed from 60°C, with an increase of 3°C/min, to 240°C. Gas chromatography-mass spectrometry analysis was conducted using the same system and conditions interfaced to a mass spectrometer instrument equipped with an ion trap detector operating in electron impact mode at 70 eV. The compounds were identified by their retention index, calculated by linear interpolation relative to retention times of a series of n-alkanes (11) and by comparison of their mass spectra with those of Wiley 7.0 library and of the literature (11). Compound concentrations were calculated from the GC peak areas.

1H nuclear magnetic resonance analysis Dried extract was diluted in CDC13 (50.0 mg/ml) and nuclear magnetic resonance (NMR) spectra were obtained using a Varian INOVA® 500.11 T instrument with CHCl3 as internal standard.

Ethanolic extract emulsions The ethanolic extract of C. sylvestris was dissolved in Tween 20 (20%). Balb-C male mice were treated with 3.13, 6.25, 12.5, 25, 50 and 75 mg/kg. The concentrations of the test were chosen based on the high solubility of the ethanolic extract in the Tween 20 (20%).

Purification and structure determination of caseargrewiin F Dried and powdered leaves of C. sylvestris were extracted by steeping in ethanol. The dried extract obtained was separated by SPE on silica gel: activated charcoal and fraction SPE2 was separated further by normal-phase low-pressure column chromatography (CC) on silica gel. Fractions CC15-19 were purified by preparative reversed-phase (C18) HPLC to yield caseargrewiin F (850.0 mg). The structure of the compound was determined from spectrometric data (nuclear magnetic resonance, ultraviolet, infrared and mass spectrometry) and by comparison with spectral information available in the literature. For details see Santos et al. (10). The chromatographic purity of the caseargrewiin F in the assays used was 98.6% (HPLC-UV detected at 235 nm).

Caseargrewiin F emulsions Caseargrewiin F (Figure 1) was emulsified in 6% Tween 80 for both assays in mice. The Balb-C male mice were treated with 0.16, 0.32, 0.63, 1.3, 2.5 and 2.5 mg of caseargrewiin F per kg body weight (b.w.). These concentrations were based on ethanolic extract from C. sylvestris in which doses of 3.13, 6.25, 12.5, 25, 50 and 75 mg/kg b.w. were used; since caseargrewiin F represents 4% of the ethanolic extract, we selected doses that are ~4% of the ethanolic extract doses.

Animals Six-week-old Balb-C male mice were maintained under controlled conditions of temperature (22 ± 2°C) and humidity (55 ± 10% room humidity), in a 12-h light/dark cycle. Before the experiments, the animals were acclimatized for 1 week, during which they had free access to a commercial diet (Purina®) and water. The study was conducted with approval of Brazilian Ethics Committee to Experimentation Animals (process CEP/PFC/CAr nº 01/2006).

Mutagenic chemical—Positive control Cyclophosphamide (CP, Sigma–Aldrich Co., St Louis, MO, Lot. No. 036K1225, purity 99%) was used as mutagenic agent in experiments. CP was diluted in 0.9% NaCl just before use and the animals were exposed to the chemical by intra-peritoneal (i.p.) injection of 50 mg/kg b.w. in 0.3 ml.

Mutagenicity—MN test Experimental design The assays were performed using eight animals/group. Group 1 (negative control) mice received only drinking water (0.6 ml per day by gavage) for 2 weeks before treatment with 0.9% NaCl by i.p. injection of 0.3 ml. Group 2 (positive control) also received drinking water for 2 weeks and was treated on day 15 with CP (50 mg/kg b.w., i.p.). Group 3 was treated with Tween 20 (20%) or Tween 80 (6%), 0.6 ml by gavage, for 2 weeks and on day 15 the mice were injected i.p. with 0.3 ml 0.9% NaCl. Groups 4–9 were treated for 2 weeks by gavage (0.6 ml per day per animal) with various concentrations of ethanolic extract (3.13, 6.25, 12.5, 25, 50 and 75 mg/kg b.w.) or caseargrewiin F (0.16, 0.32, 0.63, 1.3, 2.5 and 3.8 mg/kg b.w.). These groups also received treatment with 0.3 ml 0.9% NaCl by i.p. on day 15. Bone marrow from each animal was collected 24 h after the last administration (12).

The reference standard used in this work was the marrow MN test, which is based on the alkaline version of the comet assay (single cell gel electrophoresis) performed as described by Singh (14). Briefly, 20 μl of blood was taken, homogenized with low-melting point agarose, spread on microscope slide pre-coated with normal melting point agarose and covered with a coverslip. After 5 min at 4°C, the coverslip was removed from slides and they were immersed in cold lysing solution (2.4 M NaCl; 100 mM ethylenediaminetetraacetic acid (EDTA); 10 mM Tris, 10% dimethylsulfoxide and 1% Triton-X, pH 10) for 24 h. After lysis, the slides were placed in an electrophoresis chamber, covered with electrophoresis buffer (300 mM NaOH plus 1 mM EDTA, pH >13) and left for 20 min for the DNA to unwind. The electrophoresis was run for 20 min (25 V and 300 mA), after which the slides were submerged for 15 min in neutralization buffer (300 mM NaOH plus 1 mM EDTA, pH <7.5) and fixed in 96% ethanol for 5 min. Slides were prepared in duplicate with ethidium bromide and 50 cells were screened per sample in a fluorescent microscope (ZEISS®, Germany) equipped with an excitation filter of 515–560 nm, a barrier filter of 590 nm and a ×40 objective.
DNA damage was assessed by an image analysis system (TriTek CometScore™ 1.5, 2006) and the percentage of DNA in tail was obtained.

**Antimutagenicity—MN test**

**Experimental design** In Group 1 (negative control), mice received only drinking water (0.6 ml per day by gavage) for 2 weeks before treatment with 0.9% NaCl by i.p. injection. Group 2 (positive control) also received drinking water for 2 weeks and were treated on day 15 with CP (50 mg/kg b.w., i.p.). Group 3 was treated with 0.6 ml Tween 20 (20%) or Tween 80 (6%) by gavage, for 2 weeks, and on day 15 received CP (50 mg/kg b.w., i.p.); the aim of this group is to ensure that Tween has no influence in possible DNA protective effect. Groups 4–9 were treated by gavage (0.6 ml per day per animal) with solutions of ethanolic extract of *C. sylvestris* (3.13, 6.25, 12.5, 25, 50 and 75 mg/kg b.w.) and caseargrewiin F (0.16, 0.32, 0.63, 1.3, 2.5 and 3.8 mg/kg b.w.) for 2 weeks and, on day 15, CP (50 mg/kg b.w., i.p) was administered. Bone marrow was taken 24 h after the injection (12). The MN test was performed as described previously for the mutagenicity assays.

**Antigenotoxicity—comet assay**

The experimental design for the Comet assay was the same as for antimutagenicity MN test and the comet assay for antigenotoxicity was carried out as described for the genotoxicity assay. Recently, Collins et al. (15) described that when comet assay is performed in *in vivo* experiments low concentrations of genotoxic agents are unlikely to induce significant cytotoxicity, they also affirm that a low comet assay DNA damage would be a better indicator that cells are alive than common cytotoxicity tests. In this context, to the comet assay we did not performed cytotoxicity test once at low concentrations, the compounds were not genotoxic and consequently did not present cytotoxicity that could influence in antigenotoxicity assay.

**Statistical analysis**

For a statistical interpretation, results were analyzed with Sigma Stat 3.5, Systat, CA. The Shapiro–Wilk test was used to test data for normal distribution, in all assays performed in the study. Since the results did not show a normal distribution, non-parametric tests were applied. In mutagenicity/genotoxicity assays, the Mann–Whitney *U* test was used to compare DNA damage with the negative control. In the MN tests, the PCE/NEC ratio was evaluated with the Kruskal–Wallis test. In antimutagenicity/antigenotoxicity assays, Mann–Whitney *U* test was used to compare all groups tested with the Tween 20 (20%) plus CP and positive control. When the test group. Consequently, there is no difference between vehicle control this solution was administered before the CP to ensure that it has no influence on the antimutagenic effect of the Tween 20 (20%) plus CP group, to assess the decrease in DNA damage. To test whether any groups demonstrated significant reduction in DNA damage to basal levels, those groups were also compared with negative control by the Mann–Whitney *U* test.

**Results**

**Identification of compounds in the extract**

The 1H NMR spectrum from extract showed signals with absorptions in δ 1.98–2.09, 2.20–2.40, 3.43, 5.07–5.45, 6.25–6.75, similar to those described for casearins and other clerodane diterpenes isolated from *C. sylvestris* (10,16–18). Both casearin U and caseargrewiin F were identified in the extract. However, casearin U was not assayed in the present study. Quantitative High performance liquid chromatography-diode array detector analysis demonstrated that the content of caseargrewiin F in the extract was 40.0 mg/g. Additionally, the chromatogram (Figure 2) presented 29 peaks with UV spectrum similar to observed for casearins and caseargrewiin F (λ<sub>max</sub> = 231–236 nm). The results of chemical analysis suggest the presence of these clerodane diterpenes in the extract.

**Mutagenicity and genotoxicity—ethanolic extract of *C. sylvestris***

The results of the mutagenicity test in mouse bone marrow (MN test) and the genotoxicity test in mouse peripheral blood cells (comet assay) for ethanolic extract of *C. sylvestris* are shown in Table I. Mutagenicity and genotoxicity were observed at 50 and 75 mg/kg (both *P* < 0.01). The ratio between PCEs and NCEs did not show statistically significant variation, indicating that there was no cytotoxic effect on mouse bone marrow cells.

**Antimutagenicity and antigenotoxicity of ethanolic extract of *C. sylvestris***

Table II shows the protective effect of the ethanolic extract from *C. sylvestris* against inducible DNA damage in mouse blood cells through the MN test and comet assay. Since Tween 20 (20%) was used to dissolve the ethanolic extract, in the vehicle control this solution was administered before the CP to ensure that it has no influence on the antimutagenic effect of the test group. Consequently, there is no difference between Tween 20 (20%) plus CP and positive control. When the protective effect was evaluated by the MN test, an antimutagenic effect was observed in mice treated with 6.25 (*P* < 0.01) and 12.5 mg/kg (*P* < 0.05). However, the ethanolic extract of *C. sylvestris* did not reduce the DNA damage to the basal level. In the comet assay, the ethanolic extract from *C. sylvestris* significantly reduced the % DNA in tail at concentrations of 3.13 (*P* < 0.01), 6.25 (*P* < 0.01), 12.5 (*P* < 0.05) and 25 mg/kg (*P* < 0.05).

**Mutagenicity and genotoxicity—caseargrewiin F**

The results of the mutagenicity test in mouse bone marrow (MN test) and the genotoxicity test in mouse peripheral blood cells (comet assay) of ethanolic extract of *C. sylvestris* are shown in Table III. Clastogenicity and primary damage were observed at 3.8 mg/kg b.w. of caseargrewiin F (both *P* < 0.05).

Fig. 2. Results from HPLC-DAD analysis. (A) Chromatogram of extract from the leaves of *Casearia sylvestris*; asterisk (*) indicates peaks with UV spectrum similar to casearins and caseargrewiina F. (B) Caseargrewiin F UV spectrum.
in, respectively, the MN test and comet assay. The parameter used to assess genotoxicity by comet assay was % DNA in tail. The ratio between PCEs and NCEs did not show statistically significant variation, indicating that caseargrewiin F was not cytotoxic to mouse bone marrow cells.

**Antimutagenicity and antigenotoxicity of caseargrewiin F**

The antimutagenesis/antigenotoxicity assays were performed to determine if caseargrewiin F protects DNA from inducible damage (CP, 50 mg/kg b.w.). Table IV shows the protective effect of caseargrewiin F against inducible DNA damage in mouse blood cells through the MN test and comet assay. In the positive control, water was administered before CP, in order to maintain the same conditions as in the test groups (gavage intake) while determining the DNA damage induced by the CP without protection. To verify that Tween 80 has no influence on the antimutagenic effect in the test group, we administered the emulsifying solution before the CP. The negative control was used to check basal levels of DNA damage. The caseargrewiin F did not show a statistically significant protective effect when assessed by the MN test. However, in the comet assay, it significantly reduced the % DNA in tail at concentrations of 0.63 ($P < 0.01$), 1.3 ($P < 0.01$) and 2.5 ($P < 0.01$) mg/kg b.w.

**Discussion**

Previously, natural products have been used in popular medicine to treat several diseases without any knowledge about how harmful these compounds may be to human health. Recently, several studies have been conducted to evaluate the mutagenicity and genotoxicity of these natural products (19–21).

In the present study, distinct assays were performed to reveal whether the ethanolic extracts of *C. sylvestris* and caseargrewiin F were mutagenic and or genotoxic. The results presented here demonstrated that the extract was genotoxic and mutagenic at highest concentrations in comet assay and MN test (50 and 75 mg/kg b.w.). Caseargrewiin F was both mutagenic and
genotoxic at the highest dose of 3.8 mg/kg b.w. A similar result was obtained in a recent study that describes mutagenic and genotoxic activity at high concentrations of another clerodane diterpene assessed by the MN test and comet assay (22). Considering that comet assay detects genomic lesions that are susceptible to DNA repair while MN test reveals more drastically lesions in chromosome level (23), it is important to realize that the two highest concentrations of ethanolic extract of *C. sylvestris* and at the high concentration caseargrewiin F induced unreparable DNA damage. A mutagenic effect of the ethanolic extract of *C. sylvestris* and caseargrewiin F could represent a potential antitumour property, as previous works on *C. sylvestris* demonstrated strong antitumour activity against Sarcoma 180 ascites in mice (17) and its casearins (clerodane diterpenes) exhibited a strong cytotoxic activity against V-79 tumour cells (18).

Interestingly, in the comet assay, the ethanolic extract of *C. sylvestris* was more efficient at preventing primary DNA damage at low concentrations (3.13 and 6.25 mg/kg b.w.). The molecules with non-mutagenic and/or antimutagenic properties could have antioxidant properties and, sometimes, may activate DNA repair pathways (24). They may act directly on the mutagenic process, by increasing the fidelity in DNA replication (desmutagens) or by stimulating DNA damage error-free repair (bio-antimutagenesis) (25). Additionally, ethanolic extract of *C. sylvestris* did not presented a dose-dependent response in both comet assay and MN test, and this result can be affected by various associated factors such as dependence of the effect of the substance on dose, the complexity of the test organism and the influence of simultaneous metabolic routes and enzymatic pathways (26). Caseargrewiin F was not antimutagenic when assessed by the MN test, but showed an antigentoxic dose-dependent response in the comet assay over the range of 0.63–2.5 mg/kg b.w. (Table IV).

In accordance with the present work, previous study has demonstrated that essential oil of *C. sylvestris* has clastogenicity and anticlastogenicity in HTC cell line (27). Monoterpenes and sesquiterpenes were identified in the essential oil (28) whereas in the present study, sesquiterpenes and clerodane diterpenes were identified in the ethanolic extract. Among 15 sesquiterpenes identified in the ethanolic extract, 13 were previous identified in essential oil (11,28). The mutagenic and genotoxic activity as well as the protective effect of the ethanolic extract of *C. sylvestris* could be related to the sesquiterpenes. Additionally, there are recent studies that describe sesquiterpenes as chemopreventive (27,29). However, we cannot exclude the potential biological effects of the clerodane diterpenes since caseargrewiin F protected DNA from primary damage. Moreover, the use of mice instead of cell lines to assess protective effect of ethanolic extract could better verify absorption and metabolism of *C. sylvestris* in a complex organism.

Recently, natural products have been described to protect DNA from damage and cause DNA damage (30,31) as observed in the ethanolic extract of *C. sylvestris* and caseargrewiin F. Similar results were obtained in earlier studies and described these products as Janus compounds, referring to the mythological Roman god Janus, who had two faces in the same: one looking forward and the other backward (32). Comet assay also revealed that *C. sylvestris* exhibited chemoprotective effects, but was not able to reduce the levels of DNA damage to negative controls values. Other studies suggested similar effects. Santos and Takahashi (33) demonstrated that selenomethionine, a selenium-derived antioxidant compound, was both genotoxic and antigenotoxic and reduced the levels of doxorubicin-induced DNA damage, but not to values as low as the negative control.

Cytotoxic events could lead to overestimation of the chemoprotective effects observed here. Thus, it is also important to consider that *C. sylvestris* and caseargrewiin F did not exhibit cytotoxic effects alone neither in combination with the CP as observed in the PCE/NCE values. Likewise, the chemoprotective effects can be considered only in the concentrations that did not exhibit genotoxic effects, a recommendation of the publication of Zeiger (34). Thus, our results demonstrate that the benefits of using ethanolic extract of *C. sylvestris* and caseargrewiin F as DNA protective compounds could be respectively safe at doses <50 mg/kg b.w. and 3.8 mg/kg b.w.

We conclude that the ethanolic extract of *C. sylvestris* and caseargrewiin F protects DNA against damage in low concentrations, which was carefully estimated by using MN test and comet assay. Keeping in mind that the same ethanolic extract and caseargrewiin F also can cause DNA damage in high concentrations.

**Funding**

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2005/58472-9) to A.M.d.O.; Biota-FAPESP (2003/02176-7) to V.S.B; Bioprospecta (2004/07932-7) to D.H.S.S.; Conselho Nacional de Desenvolvimento a Pesquisa (305615/2006-8) to C.P.S.

**Acknowledgements**

We wish to thank Maximino Antonio Boschi, Marcos Aparecido Dangona and Maria Izabel Feliciano for the excellent technical assistance.

Conflict of interest statement: None declared.

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