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In Vitro and In Vivo Antitumor Effects of the Essential Oil from the Leaves of Guatteria friesiana

Abstract

Guatteria friesiana (W.A. Rodrigues) Erkens & Maas (synonym Guatteriopsis friesiana W.A. Rodrigues), popularly known as “envireira”, is a medicinal plant found in the Brazilian and Colombian Amazon basin that is used in traditional medicine for various purposes. Recent studies on this species have demonstrated antimicrobial activity. In this study, the antitumor activity of the essential oil from the leaves of G. friesiana (EOGF) and its main components (α-, β-, and γ-eudesmol) were determined using experimental models. In the in vitro study, EOGF and its components α-, β-, and γ-eudesmol displayed cytotoxicity against tumor cell lines, showing IC₅₀ values in the range of 1.7 to 9.4 µg/mL in the HCT-8 and HL-60 cell lines for EOGF, 5.7 to 19.4 µg/mL in the HL-60 and MDA-MB-435 cell lines for α-eudesmol, 24.1 to > 25 µg/mL in the SF-295 and MDA-MB-435 cell lines for β-eudesmol, and 7.1 to 20.6 µg/mL in the SF-295 and MDA-MB-435 cell lines for γ-eudesmol, respectively. In the in vivo study, the antitumor effect of EOGF was evaluated in mice inoculated with sarcoma 180 tumor cells. Tumor growth inhibition rates were 43.4–54.2% and 6.6–42.8% for the EOGF treatment by intraperitoneal (50 and 100 mg/kg/day) and oral (100 and 200 mg/kg/day) administration, respectively. The treatment with EOGF did not significantly affect body mass, macroscopy of the organs, or blood leukocyte counts. Based on these results, we can conclude that EOGF possesses significant antitumor activity and has only low systemic toxicity. These effects could be assigned to its components α-, β-, and γ-eudesmol.

Introduction

The screening of plant products has led to the discovery of a number of growth-inhibiting compounds that have proved to be clinically useful in cancer chemotherapy [1]. These include the vinca alkaloids (e.g., vinblastine and vincristine), taxanes (e.g., paclitaxel and docetaxel), and camptothecins (e.g., topotecan and irinotecan). Vinblastine is an alkaldoid derived from the periwinkle plant Vinca rosea, and paclitaxel is an alkaloid ester derived from the Pacific yew (Taxus brevifolia) and the European yew (Taxus baccata). Their mechanism of action involves disturbance of tubulin polymerization, which disrupts the assembly of microtubules, an important part of the cytoskeleton and the mitotic spindle [2]. The camptothecins are natural products derived from the Camptotheca acuminata tree originally found in China. They inhibit the activity of topoisomerase I, which is the key enzyme responsible for cutting and reconnecting single DNA strands [3].

Guatteria friesiana (W.A. Rodrigues) Erkens & Maas (synonym Guatteriopsis friesiana W.A. Rodrigues, family Annonaceae), popularly known as “envireira”, is a medicinal plant found in the Brazilian and Colombian Amazon basin that is used in traditional medicine for various purposes [4–6]. The phytochemical and antibacterial studies on the methanolic extract of the stem and the essential oil from the leaves of G. friesiana have been reported [7–8]. In particular, Costa et al. [7] described the isolation and identification of the essential oil from the leaf of G. friesiana. The leaf oil of G. friesiana contained predominantly α-, β-, and γ-eudesmol. Eudesmols are sesquiterpenoid alcohols that present multiple pharmacological effects. α- and β-Eudesmol showed in vitro antitypanosomal activity against Trypanosoma brucei [9]. α-Eudesmol is able to block voltage-gated Ca(2+) channels and can be useful in neurogenic inflammation and brain injury [10–12]. β-Eudesmol has an anti-inflammatory effect on mast cell-mediated inflammatory response [13]. In this
Material and Methods

Plant material

The leaves of *G. friesiana* were collected at the Federal University of Amazonas (UFAM), Manaus, AM, Brazil, in January 2008. Random collections of leaves were taken regardless of the age or plant and combined into one sample. The plant sample was identified by Annonaceae specialist Dr. Antonio Carlos Webber from the Federal University of Amazonas. A voucher specimen of *G. friesiana* (no. 7341) was deposited in the Herbarium of the Department of Biology, UFAM, Manaus, Amazonas, Brazil.

Isolation of the essential oil and its main components

The process of isolation of the essential oil and its main components (α-, β-, and γ-eudesmol) were performed as described by Costa et al. [7] and Aciole et al. [14]. Samples of leaves (250 g) were dried at room temperature for 5 days and then submitted to hydrodistillation for 4 h in a Clevenger-type apparatus. The oil was dried over anhydrous Na2SO4, and its percentage contents to hydrodistillation for 4 h in a Clevenger-type apparatus. The oil was dried over anhydrous Na2SO4, and its percentage contents were calculated on the basis of the dry weight of plant material. The oils were stored at 4°C until further analysis. The specimen was obtained from a flowering plant.

Chemical composition of the essential oils was obtained by GC/FID and GC/MS analysis, according to Aciole et al. [14]. The analysis of the volatile compounds was performed on a Hewlett Packard 6890 GC system with a fused capillary column (30 m × 0.25 mm × 0.25 µm, SA-5, Crossbond 5% phenyl-95% dimethylpolysiloxane; Sigma–Aldrich) directly coupled to a flame ionization detector. Conditions of injection were modified from Adams [15]: injector temperature 240°C; oven temperature program of 60–300°C at a rate of 3°C/min; split 20:1 during 1.50 min, carrier gas He: 1 mL/min, constant flow; sample volume 1 µL. The GC/MS analyses were performed in the EI mode on a Hewlett Packard 6890 GC system with a fused capillary column (30 m × 0.25 mm × 0.25 µm, HP-5MS, Crossbond 5% phenyl-95% dimethylpolysiloxane) directly coupled to a Hewlett Packard 5973 selective mass detector. The mass spectrometer was operated at 70 eV. The constituents of the essential oils were identified by comparison of their mass spectral pattern and retention indices with those given in the literature [15]. The retention indices were calculated according to van den Dool and Kratz [16]. The leaf oil of *G. friesiana* contained β-eudesmol (52.0%), γ-eudesmol (24.0%), α-eudesmol (15.1%), spathulenol (2.5%), elemol (2.2%), 10-epi-γ-eudesmol (1.3%), hinesol (0.9%), and carisone (0.2%).

The essential oil of *G. friesiana* (300 mg) was fractionated by silica gel 60 (Merck, 0.063–0.200 mm) column chromatography (1.5 × 43.0 cm) using petroleum ether with increasing amounts of CH2Cl2 from (0, 5, 10, 20, 50, and 80%) followed by CH2Cl2 with increasing amounts of EtOAc (0, 5, 10, 20, and 50%) as eluent, according to Costa et al. [7]. Forty-five fractions (30 mL) were obtained. The eluted fractions were evaluated and pooled according to TLC and NMR analysis, resulting in ten groups of fractions (GF1–GF10). Additional chromatographic separation of GF6 (200 mg) was carried out by preparative TLC with 1% AgNO3 doped silica gel, eluted with petroleum ether–EtOAc (80:20, v/v) to provide β-eudesmol (purity 99.8%), γ-eudesmol (purity 98.9%), and α-eudesmol (purity 95.1%) pure enough for NMR spectroscopic identification. β-Eudesmol: amorphous solid (92.5 mg). 1H, 13C NMR spectroscopic and EIMS data: same as the data reported in Kusuma et al. [17] and Raharivelomanana et al. [18], γ-Eudesmol: colorless oil (42.1 mg), 1H and 13C NMR spectroscopic data: same as the data reported in Raharivelomanana et al. [18]. EIMS data: same as the data reported in van Beek et al. [19], α-Eudesmol: amorphous solid (25.7 mg). 1H and 13C NMR spectroscopic data: same as the data reported in Raharivelomanana et al. [18]. EIMS data: same as the data reported in van Beek et al. [19].

Animals

A total of 70 Swiss mice (males, 25–30 g) obtained from the central animal house of the Federal University of Sergipe, Brazil, were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12:12-h light-dark cycle (lights on at 6:00 a.m.). Animals were treated according to the ethical principles for animal experimentation of the SBAC (Brazilian Association of Laboratory Animal Science), Brazil. The Animal Studies Committee of the Federal University of Sergipe approved the experimental protocol (number 60/2010).

Cells

The cytotoxicity assay was performed using HL-60 (leukemia), MDA-MB-435 (melanoma), SF-295 (brain), and HCT-8 (colon) human cancer cell lines; all obtained from the National Cancer Institute, Bethesda, MD, USA. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin, and incubated at 37°C in a 5% CO2 atmosphere.

Sarcoma 180 tumor cells, which had been maintained in the peritoneal cavity of Swiss mice, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará.
Determination of the effect of the essential oil from the leaves of G. friesiana and its main components on tumor cells in culture

Tumor cell growth was determined by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product [20]. For all experiments, cells were seeded in 96-well plates (10 4 cells/well) for adherent cells or 0.5 × 10 5 cells/well for suspension cells in 100 µL of medium. After 24 h, the drugs (0.78 to 50 µg/mL) were dissolved in dimethyl sulfoxide (DMSO) and added to each well (using the HTS – high-throughput screening – Biomek 3000: Beckman Coulter, Inc.) and the cells were incubated for 72 h. 5-Fluorouracil (5-FU, purity > 99%; Sigma Chemical Co.) was used as the positive control. At the end of incubation, the plates were centrifuged, and the medium was replaced by fresh medium (150 µL) containing 0.5 mg/mL MTT. Three hours later, the formazan product was dissolved in 150 µL DMSO, and the absorbance was measured using a mult plate reader (DTX 880 Multimode Detector; Beckman Coulter, Inc.). The drug effect was expressed as the percentage of control absorbance of reduced dye at 595 nm.

Determination of the effect of the essential oil from the leaves of G. friesiana on the growth of solid tumor in vivo

Ten-day-old sarcoma 180 ascites tumor cells (2 × 10 6 cells per 500 µL) were implanted subcutaneously into the left hind groin of mice [21–23]. The EOGF was dissolved in 10% DMSO and given to mice intraperitoneally (i.p.) or orally (p.o.) to the stomach (gaavage) once a day for 7 consecutive days. At the beginning of the experiment, the mice were divided into seven groups of ten animals each as follows: Group 1: animals treated by i.p. injection of vehicle 10% DMSO; Group 2: animals treated by i.p. injection of 5-FU (25 mg/kg/day); Group 3: animals treated by i.p. injection of EOGF (50 mg/kg/day); Group 4: animals treated by i.p. injection of EOGF (100 mg/kg/day); Group 5: animals treated by p.o. administration of vehicle 10% DMSO; Group 6: animals treated by p.o. administration of EOGF (100 mg/kg/day); Group 7: animals treated by p.o. administration of EOGF (200 mg/kg/day). The dosages were determined based on previous articles. The treatments were started one day after tumor cell injection. On day 8, peripheral blood samples of the mice were collected, and the animals were sacrificed. The tumors, livers, spleens, and kidneys were excised and weighed. Percent inhibition (%) was calculated as the average tumor weight of the negative control and B is that of the treated group.

Statistical analysis

Data are presented as mean ± SEM or IC 50 values and their 95% confidence intervals (CI 95%) obtained by nonlinear regression. The differences between experimental groups were compared using ANOVA (analysis of variance) followed by the Student-Newman-Keuls test (p < 0.05). All statistical analyses were performed using the GraphPad program Intuitive Software for Science.

Results

Several tumor cell lines were treated with increasing concentrations of EOGF and its main components (α-, β-, and γ-eudesmol) for 72 h and analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium hydrobromide (MTT) assay. The obtained IC 50 values are shown in Table 1. EOGF and its components α-, β- and γ-eudesmol displayed cytotoxicity against all tumor cell lines tested, showing IC 50 values in the range of 1.7 to 9.4 µg/mL in the HCT-8 and HL-60 cell lines for the EOGF, 5.7 to 19.4 µg/mL in the HL-60 and MDA-MB-435 cell lines for α-eudesmol, 24.1 to > 25 µg/mL in the SF-295 and MDA-MB-435 cell lines for β-eudesmol, and 7.1 to 20.6 µg/mL in the SF-295 and MDA-MB-435 cell lines for γ-eudesmol, respectively. In the preclinical anticancer drug-screening program used in this study, a crude extract/oil that shows IC 50 values below 30 µg/mL and a lead compound that shows IC 50 values below 1 µg/mL are considered promising [23–26]. Therefore, we considered that EOGF presents potent cytotoxic activity, α- and γ-eudesmol were considered to be moderate cytotoxic compounds, and β-eudesmol presents only weak cytotoxic activity. The potent cytotoxic activity of the EOGF might be attributed to the mixture of its main constituents (α-, β- and γ-eudesmol), 5-FU, used as the positive control, showed IC 50 values ranging from 0.4 to 12.6 µg/mL for HCT-8 and HL-60 cell lines. The effects of EOGF on mice transplanted with sarcoma 180 tumor cells are presented in Fig. 2. EOGF showed antitumor activity with both administration routes (intraperitoneal, i.p.; oral, p.o.), but it was more potent by i.p. administration. On day 8, the average tumor weight of the control mice was 1.54 ± 0.16 g and 1.55 ± 0.13 g for i.p. and p.o. administration, respectively. In the presence of EOGF (50 or 100 mg/kg/day) by i.p. administration, the average tumor weights were 0.70 ± 0.08 and 0.87 ± 0.13 g, respectively. In the presence of EOGF (100 or 200 mg/kg/day) by p.o. administration, the average tumor weights were 1.45 ± 0.16 and 0.88 ± 0.24 g, respectively. Tumor growth inhibition rates were 43.4–54.2% and 6.6–42.8% for the EOGF treatment by i.p. and p.o. administration, respectively. In intraperitoneal administration, there were statistically significant differences between both doses in relation to the control group, but in oral administration only the highest dose of EOGF was statistically significant when compared to the control group (p < 0.05). 5-Fluorouracil (5-FU), used as the positive control at a dose of 25 mg/kg/day by i.p. administration, reduced tumor weight by 66.78%.

Systemic toxicological evaluation

Body mass loss, organ weight alteration, and leukogram were determined. The mice were weighed at the beginning and end of the experiment, as cited above. Peripheral blood samples of the mice were collected from the retro-orbital plexus under light ether anesthesia, and the animals were sacrificed by cervical dislocation. After sacrifice, the livers, kidneys, and spleens were removed and weighed. The wet weight of each organ was expressed as grams per 100 grams of body mass and compared to the control group. For the hematological analysis, an aliquot of blood from each animal was mixed with ethylenediaminetetra-acetic acid (EDTA), and the hematological parameters (total and differential leukocyte counts) were determined by standard manual procedures using light microscopy.
Britto ACS et al. In Vitro and... Planta Med

Table 1  Cytotoxic activity of the essential oil from the leaves of Guatteria friesiana (EOGF) and its main components (α-, β-, and γ-eudesmol) on cancer cell lines.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cell line* (IC_{50}* [µg/mL])</th>
<th>HCT-8</th>
<th>SF-295</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOGF</td>
<td>9.4</td>
<td>7.8–11.4</td>
<td>1.7</td>
</tr>
<tr>
<td>α-Eudesmol</td>
<td>5.1</td>
<td>4.5–8.9</td>
<td>10.2</td>
</tr>
<tr>
<td>β-Eudesmol</td>
<td>25.1</td>
<td>&gt; 25</td>
<td>24.9</td>
</tr>
<tr>
<td>γ-Eudesmol</td>
<td>10.2</td>
<td>10.6–24.3</td>
<td>8.3</td>
</tr>
<tr>
<td>5-FU *</td>
<td>12.6</td>
<td>9.7–16.3</td>
<td>0.1–1.9</td>
</tr>
</tbody>
</table>

Table 2  Effect of the essential oil from the leaves of Guatteria friesiana (EOGF) on body and organ weights of mice transplanted with sarcoma 180 tumor cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg/day)</th>
<th>Increase in body weight (g)</th>
<th>Liver (g/100 g body weight)</th>
<th>Spleen (g/100 g body weight)</th>
<th>Kidney (g/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraperitoneal route</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% DMSO</td>
<td>–</td>
<td>2.25 ± 0.92</td>
<td>5.01 ± 0.19</td>
<td>0.49 ± 0.03</td>
<td>1.36 ± 0.06</td>
</tr>
<tr>
<td>5-FU</td>
<td>25</td>
<td>3.18 ± 0.56*</td>
<td>3.80 ± 0.15*</td>
<td>0.30 ± 0.03</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>EOGF</td>
<td>50</td>
<td>0.50 ± 0.09</td>
<td>5.22 ± 0.15</td>
<td>0.62 ± 0.05</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>2.00 ± 1.33</td>
<td>4.34 ± 0.14</td>
<td>0.52 ± 0.03</td>
<td>1.37 ± 0.03</td>
</tr>
<tr>
<td>Oral route</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% DMSO</td>
<td>–</td>
<td>2.27 ± 0.57</td>
<td>5.12 ± 0.30</td>
<td>0.42 ± 0.03</td>
<td>1.55 ± 0.10</td>
</tr>
<tr>
<td>EOGF</td>
<td>100</td>
<td>2.50 ± 0.71</td>
<td>4.99 ± 0.27</td>
<td>0.49 ± 0.04</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>1.16 ± 1.53</td>
<td>4.97 ± 0.39</td>
<td>0.71 ± 0.09*</td>
<td>1.68 ± 0.13</td>
</tr>
</tbody>
</table>

Mice were injected with sarcoma 180 (2.0 × 10⁶ cells/animal, s.c.). The animals were treated, starting one day after tumor implantation, for seven consecutive days. Data are presented as mean ± SEM of ten animals. *P < 0.05 compared with the 10% DMSO group using ANOVA followed by the Student-Newman-Keuls test.

Discussion

Many experimental studies have identified the anticancer potential for essential oils and some purified substances from those species [27–30]. The present work investigated the in vitro cytotoxic activity of EOGF and its main components (α-, β-, and γ-eudesmol) against tumor cell lines and the in vivo antitumor effect of EOGF on mice transplanted with sarcoma 180 tumor cells. The cytotoxic or antitumor activities of EOGF have not been previously subjected to investigation.

EOGF and its components α-, β-, and γ-eudesmol displayed cytotoxicity against several human tumor cell lines. γ-Eudesmol has been previously reported as a cytotoxic agent, with IC_{50} values ranging from 0.01 to 1.5 µg/mL against human hepatocarcinoma cell lines [31]. Ben Sghaier et al. [32] showed that β-eudesmol significantly inhibits the proliferation of K562 (human leukemia) cells with an IC_{50} value of 20 µg/mL. The cytotoxic effect of α-eu-

the liver and spleen organ weights (p < 0.05). In the peripheral blood from mice transplanted with sarcoma 180 tumor cells, 5-FU induced a decrease in total leukocytes (p < 0.05). However, unlike 5-FU, EOGF reduced tumor development without inducing a disturbance in the leukocytes count.

Fig. 2  Effect of the essential oil from the leaves of Guatteria friesiana (EOGF) on mice transplanted with sarcoma 180 tumor cells. The graph shows tumor weight (g). The animals were treated by intraperitoneal (A) or by oral (B) administration, starting one day after tumor implantation, for seven consecutive days. 5-Fluourouracil (5-FU, 25 mg/kg/day by i.p. route) was used as the positive control. The negative control was treated with the vehicle used for diluting the tested substance (10% DMSO). Data are presented as mean ± SEM of ten animals. *P < 0.05 compared with the 10% DMSO group using ANOVA followed by the Student-Newman-Keuls test.

Table 2  Effect of the essential oil from the leaves of Guatteria friesiana (EOGF) on body and organ weights of mice transplanted with sarcoma 180 tumor cells.

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</tr>
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<td>10% DMSO</td>
<td>–</td>
<td>2.25 ± 0.92</td>
<td>5.01 ± 0.19</td>
<td>0.49 ± 0.03</td>
<td>1.36 ± 0.06</td>
</tr>
<tr>
<td>5-FU</td>
<td>25</td>
<td>3.18 ± 0.56*</td>
<td>3.80 ± 0.15*</td>
<td>0.30 ± 0.03</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td>EOGF</td>
<td>50</td>
<td>0.50 ± 0.09</td>
<td>5.22 ± 0.15</td>
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desmol was studied here for the first time. α-, β- and γ-Eudesmol seem to be responsible for the cytotoxicity of EOGF. The ability of β-eudesmol to suppress tumor growth through the inhibition of tumor neovascularization has been investigated in *in vitro* and *in vivo* models. β-Eudesmol inhibits angiogenesis in subcutaneously implanted Matrigel plugs in mice and in adjuvant-induced granuloma in mice, which can be due to the blockade of the ERK (extracellular signal-regulated kinase) and the CREB (cAMP response element binding protein) activation-signaling pathway [33, 34]. β-Eudesmol treatment (2.5–5 mg/kg) also inhibited the growth of H22 and S180 mouse tumors *in vivo* [34]. Therefore, β-eudesmol can contribute to the anticancer potential of EOGF as an antiangiogenic agent. In the Annonaceae family, other essential oils also present an anticancer potential. The essential oil obtained from the leaves of *Annona senegalensis* Pers. has shown mild to moderate cytotoxicity in a brine shrimp lethality bioassay and against human tumor cell lines [35]. In a similar study, the fruit essential oil of *Xylopia aethiopica*, another Annonaceae species, showed cytotoxic activity against the Hep-2 cell line [36]. However, until now, no study has been conducted evaluating the cytotoxicity of the components of the essential oil. In the *in vivo* study, we investigated the effect of EOGF on tumor growth using sarcoma 180 transplanted mice. Sarcoma 180 is an original mouse tumor, transplantable, and well-characterized experimental model used in the research of antitumor activity [21–23, 37]. Two administration routes were used: intraperitoneal (a parenteral route) and oral (gavage) (an enteral route). EOGF showed antitumor activity in both administration routes, but it was more potent by intraperitoneal administration. In fact, usually, the oral route is disadvantaged because of less absorption. The oral route offers maximum convenience, but absorption may be slower and less complete than when parenteral routes are used. Ingested drugs are subject to the first-pass effect, in which a significant amount of the agent is metabolized in the gut wall and the liver before it reaches systemic circulation. Thus, some drugs have low bioavailability when given orally [38]. α-, β- and γ-Eudesmol were not tested in an animal-bearing tumor due to the small amount of sample available. Since the chemotherapeutic agents, alone or in combination, commonly may cause hepatic dysfunction, renal toxicity, and hematopoietic depression as important side effects, the toxicity of EOGF or its constituents must be addressed. Thus, the toxicological aspects were also subject to investigation in the present study. The treatment with EOGF did not significantly affect the body mass, macroscopy of the organs (kidney and liver), or blood leukocyte counts. Moreover, spleen weights were increased in one group of EOGF-treated animals, which suggests an immunostimulatory action. In contrast, the clinically useful chemotherapeutic drug 5-FU, used here as the positive control, induced the development of leucopenia, which substantially increases the risk of infections [39]. Based on these results, we can conclude that EOGF possesses significant antitumor activity and has only low systemic toxicity. These effects could be assigned to its components α-, β- and γ-eudesmol. Therefore, further investigations to elucidate the mechanisms of the cytotoxic and antitumor effects exhibited are required.

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**Conflict of Interest**

None.

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