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Eudesmol Isomers Induce Caspase-Mediated Apoptosis in Human Hepatocellular Carcinoma HepG2 Cells

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Abstract: Eudesmols are naturally occurring sesquiterpenoid alcohols that present cytotoxic effect to cancer cells. Herein, all eudesmol isomers displayed cytotoxicity to different tumour cell lines. α-Eudesmol showed IC_{50} values ranging from 5.38 ± 1.10 to 10.60 ± 1.33 μg/mL for B16-F10 and K562 cell lines, β-eudesmol showed IC_{50} values ranging from 16.51 ± 1.21 to 24.57 ± 2.75 μg/mL for B16-F10 and HepG2 cell lines, and γ-eudesmol showed IC_{50} values ranging from 8.86 ± 1.27 to 15.15 ± 1.06 μg/mL for B16-F10 and K562 cell lines, respectively. In addition, in this work, we studied the mechanisms of cytotoxic action of eudesmol isomers (α-, β- and γ-eudesmol) in human hepatocellular carcinoma HepG2 cells. After 24-hr incubation, HepG2 cells treated with eudesmol isomers presented typical hallmarks of apoptosis, as observed by morphological analysis in cells stained with haematoxylin–eosin and acridine orange/ethidium bromide. None of eudesmol isomers caused membrane disruption at any concentration tested. Moreover, eudesmol isomers induced loss of mitochondrial membrane potential and an increase in caspase-3 activation in HepG2 cells, suggesting the induction of caspase-mediated apoptotic cell death. In conclusion, the eudesmol isomers herein investigated are able to reduce cell proliferation and to induce tumour cell death by caspase-mediated apoptosis pathways.

Several studies have demonstrated that natural products and/or natural product structures continued to play a highly significant role in drug discovery and development process. In the case of approved therapeutic agents (01/1981–12/2010), only 20.2% of the total number of small-molecule anticancer drugs is classifiable into the synthetic category [1].

Some studies have reported cytotoxic activity for plants belonging to the genus *Guatteria* of the Annonaceae plant family [2–5]. Our research group demonstrated that the leaf essential oil of *Guatteria friesiana* possesses *in vitro* and *in vivo* antitumour actions, without substantial systemic toxicity [5]. The main components identified in *G. friesiana* essential oil were eudesmol isomers (α-, β- and γ-eudesmol; fig. 1), which exhibit multiple biological activities, such as antihypertensive [6], anticonvulsant [7], anti-angiogenic [8], antitumour [9], antimicrobial [10], acaricidal [11], antimigraine [12], insecticidal [13], antitrypanosomal [14] and anti-inflammatory properties [15]. These three eudesmol isomers have been also reported previously as cytotoxic agents, displaying cytotoxicity to several human tumour cell lines [5,8,9,16,17]. Moreover, other plant species that contain eudesmol isomers also present cytotoxic activity [18,19]. In addition, Li *et al.* [20] reported that β-eudesmol induces c-Jun N-terminal kinases (JNK)-dependent apoptosis through the mitochondrial pathway in HL60 cells; however, the mechanism of cytotoxicity of α- or γ-eudesmol has not been investigated. Therefore, in the present work, we investigated the cytotoxic mechanism of eudesmol isomers (α-, β- and γ-eudesmol) in human hepatocellular carcinoma HepG2 cells.

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Materials and Methods

Eudesmols isolation. Guatteria friesiana leaves were collected at the Federal University of Amazonas (UFAM), Manaus, AM, Brazil, in January 2008. A voucher specimen (no. 7341) was deposited in the Herbarium of the Department of Biology, UFAM, Manaus, Amazonas, Brazil. Samples of leaves (250 g) were dried at room temperature for 5 days and then submitted to hydrodistillation for 4 hr in a Clevenger-type apparatus (Amitel, São Paulo, Brazil). α-, β- and γ-Eudesmol were isolated from the essential oil extracted from the leaves of *G. friesiana,* according to the method described by Costa *et al.* [10].

The essential oil (300 mg) was fractionated by silica gel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany) column chromatography (1.5 × 43.0 cm) using petroleum ether with increasing amounts of CH_2Cl_2 (0%, 5%, 10%, 20%, 50% and 80%) followed by CH_2Cl_2 with increasing amounts of EtOAc (0%, 5%, 10%, 20% and 50%) as eluent. Forty-five fractions (30 mL) were obtained. The eluted fractions were evaluated and pooled according to TLC and NMR analysis, resulting in 10 groups of fractions (GF1-GF10). Additional chromatographic separation of GF6 (200 mg) was carried out by preparative TLC with 10% $AgNO_3$ [21], doped silica gel and 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. The EI-MS and NMR data of the isolated eudesmols were in accordance with literature values [22–24].

Cell culture. Cytotoxicity was determined in tumour cells namely HepG2 (human hepatocellular carcinoma), K562 (human chronic myelocytic leukaemia) and B16-F10 (mouse melanoma), all donated by Hospital A.C. Camargo, São Paulo, SP, Brazil. Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco-BRL, Gaithersburg, MD, USA) medium supplemented with 10% foetal bovine serum (Cultilab, Campinas, SP, Brazil), 2 mM L-glutamine (Vetec Química Fina, Duque de Caxias, RJ, Brazil) and 50 μg/mL gentamycin (Novafarma, Anápolis, GO, Brazil). Adherent cells were harvested by treatment with 0.25% trypsin EDTA solution (Gibco-BRL). All cell lines were cultured in cell culture flasks at 37°C in 5% CO_2 and subcultured every 3–4 days to maintain exponential growth. Cytotoxicity experiments were conducted with cells in exponential growth phase.

Human lymphocyte cells were obtained by primary culture. Heparinized blood (from healthy, non-smoker donors who had not taken any drug at least 15 days prior to sampling) was collected, and peripheral blood mononuclear cells (PBMC) were isolated by a standard protocol using Ficoll density gradient (Ficoll-Paque Plus; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PBMC were washed and resuspended at a concentration of 0.3×10^6 cells/mL in RPMI 1640 medium supplemented with 20% foetal bovine serum, 2 mM glutamine, 50 μg/mL gentamycin at 37°C with 5% CO_2. In addition, concanavalin A (ConA; Sigma Chemical Co., St Louis, MO, USA) was used as a mitogen to trigger cell division in T lymphocytes. ConA (10 μg/mL) was added at the beginning of culture, and after 24 hr, cells were treated with the test drugs.

For all experiments, cell viability was performed by trypan blue exclusion (TBE) assay. Over 90% of the cells were viable at the beginning of the culture.

Cell proliferation assay. Cell growth was quantified by methyl-[^{3}H]-thymidine incorporation assay, as described by Griffiths and Sundaram [25] with minor modifications. Methyl-[^{3}H]-thymidine is a radiolabelled DNA precursor incorporated into newly synthesized DNA, in which the amount of incorporated methyl-[^{3}H]-thymidine is related to the rate of proliferation. In all experiments, 100 μL solution of cells (0.7×10^5 cells/mL for adherent cells – HepG2 and B16-F10 – or 0.3×10^6 cells/mL for suspended cells – K562 and PBMC) was seeded in 96-well plates. After 24 hr, the drugs (0.39–25 μg/mL), dissolved in dimethyl sulfoxide (DMSO; LGC Biotechnology, São Paulo, SP, Brazil), were added to each well and incubated for 72 hr. Doxorubicin (doxorubicin hydrochloride; Eurofarma, São Paulo, SP, Brazil) was used as a positive control. Six hours before the end of incubation time, 1 μCi of methyl-[3H]-thymidine (PerkinElmer, Boston, MA, USA) was added to each well. After this period, cultures were harvested using a cell harvester (Brandel Inc., Gaithersburg, MD, USA) to determine the ^{3}H-thymidine incorporation using a liquid scintillation cocktail HidexMaxilight (PerkinElmer Life Sciences, Groningen, GE, Netherlands) and a plate CHAMELEON V multilabel Counter (Mustionkatu 2, Turku, Finland) with MIKROWINHIDEX 2000 v. 4.38 software (Microtek Laborsysteme GmbH, Overath, Germany). The drug effect was quantified as the percentage of control radioactivity using the formula $[(C-T)/C] \times 100$, where 'T' and 'C' represent the radioactivity of the treated and control samples, respectively.

Analysis of mechanisms involved in the cytotoxic activity. The following experiments were performed to elucidate the mechanisms involved in cytotoxic action of the drugs. In all experiments, 2 mL solution of HepG2 cells (0.7×10^5 cells/mL) was seeded in 24-well plates and incubated overnight to allow the cells to adhere to the plate surface. Then, the cells were treated for 24 hr with the drugs. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested drug. Doxorubicin (1 μg/mL) was used as a positive control.

TBE assay. After 24-hr drug exposure, cells were harvested by trypsinization and stained with a solution of 0.02% trypan blue. Viable cells were counted using a Neubauer chamber and the formula cell number/mL = $A \times B \times 10^4$, where 'A' and 'B' represent the average number per corner square and dilution factor from trypan blue, respectively.

Morphological analysis performed with acridine orange/ethidium bromide staining. After 24-hr drug exposure, cells were harvested by trypsinization, pelleted and resuspended in 25 μL saline. Thereafter, 1 μL aqueous solution of acridine orange (AO; Sigma Chemical Co.) and ethidium bromide (EB; Sigma Chemical Co.; AO/EB, 100 μg/mL) was added, and the cells were observed under a fluorescence microscope using the 60× objective (=600× total magnification; Olympus BX41, Tokyo, Japan). Three hundred cells were counted per sample and classified as viable, apoptotic or necrotic cells, as previously described [26].

Morphological analysis with haematoxylin–eosin staining. After 24-hr drug exposure, cells were harvested by trypsinization, transferred to cytospin slides, fixed with methanol for 30 sec. and stained with haematoxylin–eosin. Morphological changes were examined by light microscopy using the 60× objective (=600× total magnification; Olympus BX41) using IMAGE-PRO EXPRESS software (Media Cybernetics Inc., Silver Spring, MD, USA).

Cell membrane integrity. After 24-hr drug exposure, cells were harvested by trypsinization and stained with a solution of 50 μg/mL propidium iodide in phosphate-buffered saline, as previously described [27]. Cell fluorescence was determined by flow cytometry (using the FL-2 channel) in a FACSCalibur cytometer (Bencton Dickinson, San Diego, CA, USA) with CELLQUEST software (BD Biosciences, San Jose, CA, EUA). Ten thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

Measurement of mitochondrial transmembrane potential. After 24-hr drug exposure, cells were harvested by trypsinization and incubated with rhodamine 123 (5 μg/mL; Sigma Chemical Co.) at 37°C for 15 min. in the dark, as previously described [28]. Then, the cells were pelleted and resuspended in saline. The cells were then incubated again in saline at 37°C for 30 min. in the dark, and cell fluorescence was determined by flow cytometry (using the FL-1 channel) in a FACSCalibur cytometer (Bencton Dickinson) with CELLQUEST software (BD Biosciences). Ten thousand events were evaluated per experiment, and cellular debris was omitted from the analysis.

Caspase-3 activation assay. Caspase-3/CPP32 colorimetric assay kit (BioVision Incorporated, Milpitas, CA, USA) was used to investigate caspase-3 activation in treated cells based on the cleavage of Asp-Glu-Val-Asp (DEVD)-pNA. Briefly, cells were lysed by incubation with cell lysis buffer on ice for 10 min. and then centrifuged at $10{,}000 \times g$ for 1 min. To each reaction mixture, 50 μL cell lysate (100–200 μg total protein) was added. Enzyme reactions were carried out in a 96-well flat-bottom microplate. The absorbance was measured at 405 nm using SPECTRAMAX 190 absorbance microplate reader (Molecular Devices, Sunnyvale, CA, USA). The drug effect was quantified as the comparison of the absorbance of pNA from a treated sample with an untreated sample.

Statistical analysis. Data are presented as mean ± standard error of the mean. Differences among experimental groups were compared by one-way analysis of variance (ANOVA) followed by Newman–Keuls test ($p < 0.05$). All analyses were carried out using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA).

Table 1.
Cytotoxic activity of eudesmol isomers (α-, β- and γ-eudesmol) on tumour and normal cell lines.

Cell lines	Histotype	α-Eudesmol	β-Eudesmol	γ-Eudesmol	Doxorubicin
HepG2	Hepatocellular carcinoma	9.71 ± 1.56 (43.67)	24.57 ± 2.75 (110.49)	9.50 ± 1.25 (42.72)	0.62 ± 0.15 (1.14)
K562	Chronic myelocytic leukaemia	10.60 ± 1.33 (47.67)	22.50 ± 1.04 (101.18)	15.15 ± 1.06 (68.13)	2.92 ± 1.13 (5.37)
B16-F10	Melanoma	5.38 ± 1.10 (24.19)	16.51 ± 1.21 (74.25)	8.86 ± 1.27 (39.84)	0.03 ± 0.15 (0.06)
PBMC	Normal lymphocyte	12.01 ± 1.53 (54.01)	19.80 ± 1.09 (89.04)	10.71 ± 1.75 (48.16)	4.17 ± 1.10 (7.67)

PBMC, peripheral blood mononuclear cells. Data are presented as mean of IC_{50} values ± S.E.M., in μg/mL (μM), obtained by non-linear regression from four or six experiments by methyl-[^{3}H]-thymidine incorporation assay after 72-hr incubation. Doxorubicin was used as a positive control.

Results

Eudesmol isomers displayed cytotoxicity to different tumour cell lines. Tumour cell lines were treated with increasing concentrations of eudesmol isomers (α-, β- and γ-eudesmol) for 72 hr and analysed by methyl-[^{3}H]-thymidine incorporation assay. Table 1 shows the IC_{50} values obtained. α-Eudesmol showed IC_{50} values ranging from 5.38 ± 1.10 to 10.60 ± 1.33 μg/mL for B16-F10 and K562 cell lines, β-eudesmol showed IC_{50} values ranging from 16.51 ± 1.21 to 24.57 ± 2.75 μg/mL for B16-F10 and HepG2 cell lines, and γ-eudesmol showed IC_{50} values ranging from 8.86 ± 1.27 to 15.15 ± 1.06 μg/mL for B16-F10 and K562 cell lines, respectively. Doxorubicin, used as a positive control, showed IC_{50} values from 0.03 ± 0.15 to 2.92 ± 1.13 μg/mL for B16-F10 and K562 cell lines, respectively. In addition, the cytotoxicity of eudesmol isomers was also evaluated against normal cells (PBMC). The results, presented in table 1, showed that α-, β- and γ-eudesmol were also cytotoxic to normal cells.

All subsequent experiments were conducted on HepG2 cell line to determine the mechanism of action of eudesmol isomers in this cell line. For this, cells were incubated for 24 hr with the drugs at concentrations of 5 and 10 μg/mL for α- and γ-eudesmol and 10 and 20 μg/mL for β-eudesmol. These concentrations were chosen based on their IC_{50} value in this cell line (9.71 μg/mL for α-eudesmol, 9.50 μg/mL for γ-eudesmol and 24.57 μg/mL for β-eudesmol).

Eudesmol isomers induce apoptosis in HepG2 cells

TBE assay. The cytotoxic effect of eudesmol isomers on the number of viable cells was measured by the TBE assay (fig. 2A). Although α-, β- and γ-eudesmol reduced proliferation of HepG2 cells in a concentration-dependent manner ($p < 0.05$), no increase in the number of non-viable cells was observed ($p > 0.05$), suggesting a lack of effect of these compounds on membrane integrity. Doxorubicin, used as a positive control, also reduced the proliferation of HepG2 cells without affecting cell membrane integrity.

Morphological analysis performed with AO/EB staining. The percentages of viable, apoptotic and necrotic cells were calculated in morphological analysis using AO/EB staining cells (fig. 2B). Uniformly green live cells with normal morphology were seen in the HepG2 control group, representing more than 90% of cells counted. After α-, β- and γ-eudesmol treatment of HepG2 cells for 24 hr, an increasing number of apoptotic cells was observed ($p < 0.05$). No significant increase in the percentage of necrotic cells was seen ($p > 0.05$). In addition, doxorubicin-treated cells also showed apoptotic characteristics.

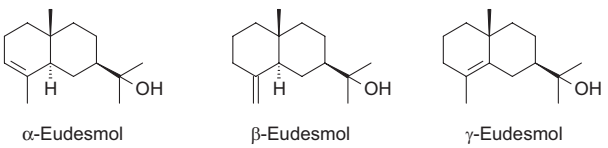

Fig. 1. Chemical structure of eudesmol isomers.

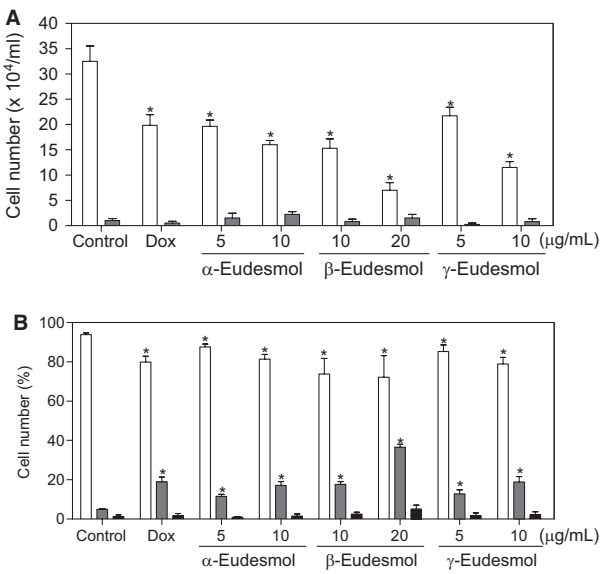

Fig. 2. Effect of eudesmol isomers (α-, β- and γ-eudesmol) on viability of human hepatocellular carcinoma HepG2 cells after 24-hr incubation. (A) Cell viability measured by trypan blue exclusion assay – viable cells (white bar) and non-viable cells (grey bar). (B) Cell viability measured by fluorescence microscopy using acridine orange/ethidium bromide – viable cells (white bar), apoptotic cell (grey bar) and necrotic cell (black bar). Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin (Dox, 1 μg/mL) was used as a positive control. Data are presented as mean values ± S.E.M. from four or six experiments. *$p < 0.05$ compared with negative control by ANOVA followed by Student–Newman–Keuls test. DMSO, dimethyl sulfoxide.

Morphological changes using haematoxylin–eosin staining. Morphological changes were investigated using

haematoxylin–eosin staining (fig. 3). In the presence of α-, β- and γ-eudesmol, cells presented morphology consistent with apoptosis, including abundant vacuoles, reduction in cell volume, chromatin condensation and fragmentation of the nuclei. Doxorubicin, used as a positive control, also showed morphology consistent with apoptosis.

Cell membrane integrity. The cell membrane integrity was measured using propidium iodide, as a parameter of the viability (fig. 4). After 24 hr of exposure, none of eudesmol isomers caused membrane disruption at any concentration tested ($p > 0.05$). Doxorubicin, used as a positive control, also did not affect membrane integrity.

Mitochondrial membrane potential. The cell treated with α-, β- and γ-eudesmol-induced mitochondrial depolarization, as measured by incorporation of rhodamine-123, suggesting mitochondrial-mediated apoptotic cell death. The mitochondrial depolarization occurred in a concentration-dependent manner ($p < 0.05$, fig. 5A).

Caspase-3 activation. A remarkable activation of caspase-3 was recorded in lysates from HepG2 cells treated with α-, β- and γ-eudesmol ($p < 0.05$, fig. 5B), suggesting caspase-mediated apoptotic cell death. Doxorubicin, used as a positive control, also induced apoptotic features.

Discussion

In the current study, the cytotoxic activity of eudesmol isomers (α-, β- and γ-eudesmol) was investigated. All eudesmol isomers displayed cytotoxicity to different tumour cell lines. When tested against HepG2, K562 and B16-F10 cells, α- and γ-eudesmol were more potent than β-eudesmol, suggesting that the β-exocyclic double bonds reduce the cytotoxicity of eudesmol compounds.

HepG2 cells treated with eudesmol isomers presented typical hallmarks of apoptosis. As observed in the cytotoxic assay, α- and γ-eudesmol exhibited stronger activity than β-eudesmol in pro-apoptotic activity. The cytotoxic mechanism of β-eudesmol had been previously examined on human leukaemia HL60 cells [20]; however, the cytotoxic mechanism of α- and γ-eudesmol was evaluated for the first time in this communication.

Programmed cell death (apoptosis), the deletion of certain cells in tissues without concomitant inflammation, is advantageous in tissue homoeostasis. Induction of apoptosis in tumour cells is of great benefit in cancer chemotherapy. Several antitumour agents have been described as apoptosis inductors. Cells undergoing apoptosis show typical well-defined morphological changes including phosphatidylserine externalization, cytochrome c leakage from the mitochondria, caspase activation, reduction in cell and nuclear volume, chromatin condensation and DNA damage [29,30]. As observed by light microscopy using haematoxylin–eosin staining, cells treated with eudesmol isomers presented reduction in cell volume, chromatin condensation and fragmentation of the nuclei, suggesting that treated cells undergo apoptosis. These findings were confirmed by the fluorescence microscopy using AO/EB staining.

During the initial stages of apoptosis, the cell shrinks, whereas the membrane remains intact. During necrosis, cell swelling occurs as a result of the early failure of the membrane integrity. Interestingly, cells treated with eudesmol isomers did not decrease the cell membrane integrity. These data corroborated with TBE where eudesmol isomers

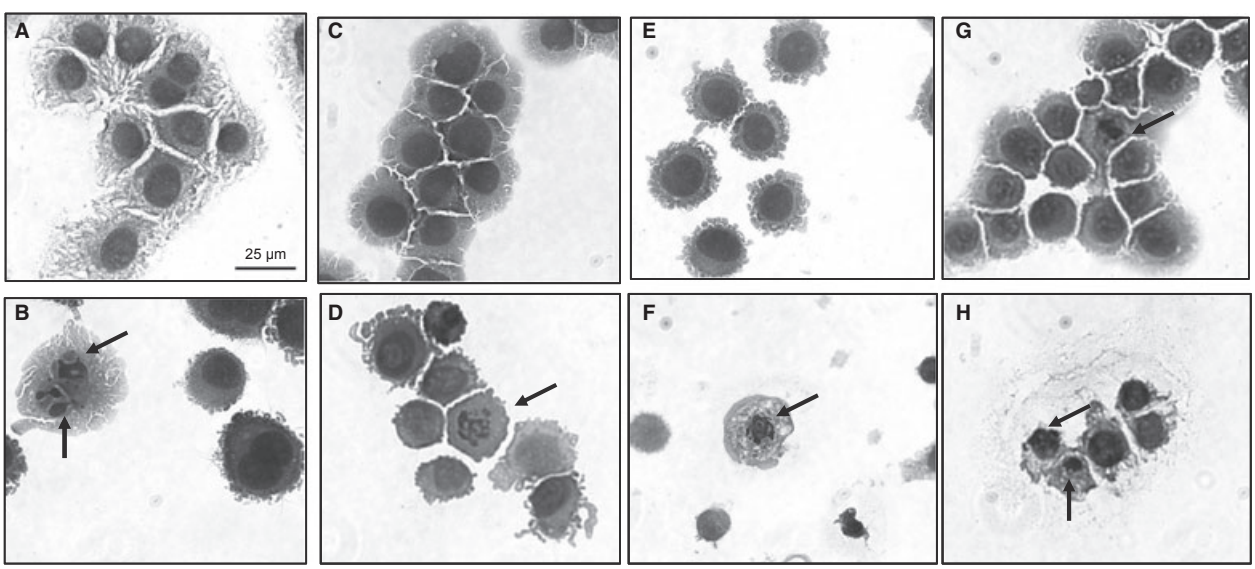

Fig. 3. Effect of eudesmol isomers (α-, β- and γ-eudesmol) on cell morphology of human hepatocellular carcinoma HepG2 cells. The cells were stained with haematoxylin–eosin and analysed by optical microscopy, using the 60× objective (=600× total magnification), after 24-hr incubation with α-eudesmol at concentrations 5 (C) and 10 µg/mL (D), β-eudesmol at concentrations 10 (E) and 20 µg/mL (F) and γ-eudesmol at concentrations 5 (G) and 10 µg/mL (H). Negative control (A) was treated with the vehicle (0.1% DMSO) used for diluting the tested substances. Doxorubicin (1 µg/mL) was used as a positive control (B). Black arrows show chromatin condensation. DMSO, dimethyl sulfoxide.

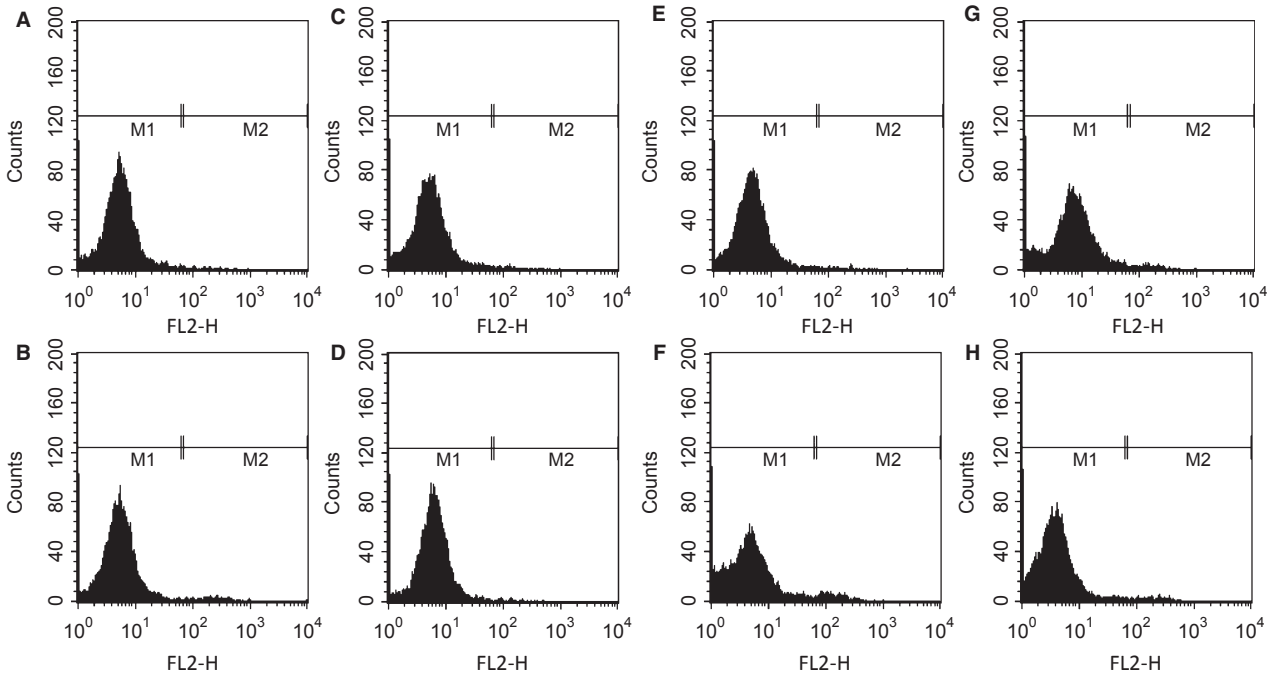

Fig. 4. Representative histograms of cell membrane integrity assay. Cell membrane integrity was measured by flow cytometry, using propidium iodide, on hepatocellular carcinoma HepG2 cells after 24-hr incubation with α-eudesmol at concentrations 5 (C) and 10 μg/mL (D), β-eudesmol at concentrations 10 (E) and 20 μg/mL (F) and γ-eudesmol at concentrations 5 (G) and 10 μg/mL (H). Negative control (A) was treated with the vehicle (0.1% DMSO) used for diluting the tested substances. Doxorubicin (1 μg/mL) was used as a positive control (B). Ten thousand events were analysed in each experiment. Units are arbitrary. DMSO, dimethyl sulfoxide.

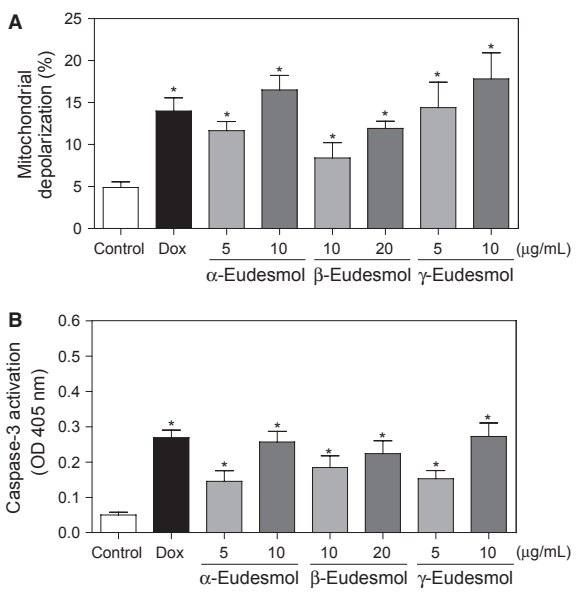

Fig. 5. Effect of eudesmol isomers (α-, β- and γ-eudesmol) on viability of human hepatocellular carcinoma HepG2 cells after 24-hr incubation. (A) Mitochondrial membrane depolarization determined by flow cytometry using rhodamine 123. (B) Caspase 3 activation measured by colorimetric assay. Negative control was treated with the vehicle (0.1% DMSO) used for diluting the tested substance. Doxorubicin (Dox, 1 μg/mL) was used as a positive control. Data are presented as mean values ± S.E.M. from four or six experiments. In flow cytometry analysis, 10,000 events were analysed in each experiment. $*p < 0.05$ compared with negative control by ANOVA followed by Student–Newman–Keuls test. DMSO, dimethyl sulfoxide.

reduced viable cell numbers without increasing the number of non-viable cells.

Moreover, eudesmol isomers induced loss of mitochondrial membrane potential and an increase in caspase-3 activation on HepG2 cells, suggesting that the eudesmol isomers reduce tumour cell proliferation triggering cell death by mitochondria- and caspase-mediated apoptosis. A regulatory cascade eventually leads to the activation of effector caspases, such as caspase-3. These caspases are responsible for the cleavage of cellular proteins, such as cytoskeletal components, leading to the typical morphological changes observed in cells undergoing apoptosis [31].

Li *et al.* [20] reported that β-eudesmol induces apoptosis in HL60 cells. β-Eudesmol-induced apoptosis was accompanied by cleavage of caspase-3, caspase-9 and poly (ADP-ribose) polymerase, but not caspase-8. β-Eudesmol treatment also down-regulated the expression of Bcl-2 but had no effect on the expression of Bax and Mcl-1. The decrease in mitochondrial membrane potential and the release of cytochrome c from mitochondria in HL60 cells were also observed. JNK were activated after β-eudesmol treatment, suggesting that β-eudesmol induces apoptosis in HL60 cells via the mitochondrial apoptotic pathway, which is controlled through JNK signalling. In our results, all eudesmol isomers were also able to induce mitochondria- and caspase-mediated apoptosis in HepG2 cells.

β-Eudesmol was also able to inhibit the proliferation and tube formation of endothelial cells (porcine brain microvascular endothelial cells – PBMEC, human dermal microvascular

endothelial cells – HDMEC and human umbilical vein endothelial cells – HUVEC) [8]. It also exhibited inhibitory effects on basic fibroblast growth factor (bFGF)-stimulated HUVEC migration and on tube formation by HUVEC. In addition, β-eudesmol blocked the activation of ERK1/2 in endothelial cells stimulated with vascular endothelial growth factor (VEGF) or bFGF, whereas β-eudesmol had no effect on the activation of p38 MAP kinase. In addition, Ma *et al.* [9] showed that β-eudesmol blocked the phosphorylation of cAMP response element binding protein (CREB) induced by VEGF in HUVEC. Therefore, β-eudesmol may inhibit angiogenesis by suppressing CREB and ERK signalling pathways. In fact, β-eudesmol significantly inhibited angiogenesis in subcutaneously implanted Matrigel plugs in mice and in adjuvant-induced granuloma in mice [8], as well as the growth of H (22) and S(180) mouse tumour *in vivo* [9]. However, the anti-angiogenic potential of α- or γ-eudesmol was not investigated.

Conclusion

In conclusion, all eudesmol isomers showed potential cytotoxic properties in cancer cells. Moreover, our findings suggest that these naturally occurring compounds reduce cell proliferation and induce tumour cell death by caspase-mediated apoptosis pathways.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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