Biomedicine & Pharmacotherapy 81 (2016) 48–55

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Combinatorial effects of geopropolis produced by *Melipona fasciculata* Smith with anticancer drugs against human laryngeal epidermoid carcinoma (HEp-2) cells

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 30 March 2016
Accepted 31 March 2016

Keywords:
Geopropolis
Chemotherapeutic agents
Apoptosis
P-glycoprotein

ABSTRACT

The identification of natural products exerting a combined effect with therapeutic agents could be an alternative for cancer treatment, reducing the concentration of the drugs and side effects. Geopropolis (Geo) is produced by some stingless bees from a mixture of vegetable resins, gland secretions of the bees and soil. It has been used popularly as an antiseptic agent and to treat respiratory diseases and dermatosis. To determine whether Geo enhances the anticancer effect of carboplatin, methotrexate and doxorubicin (DOX), human laryngeal epidermoid carcinoma (HEp-2) cells were treated with Geo alone or in combination with each drug. Cell growth, cytotoxicity and apoptosis were evaluated using 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release, and flow cytometry. Scratch assay was used to analyze cell migration and transmission electron microscopy to observe morphologic alterations. The influence of Geo on drug resistance was also investigated assessing P-glycoprotein (P-gp) action. Geo inhibited cell proliferation and migration. The combination Geo + DOX led to the highest cytotoxic activity and induced apoptosis, leading to loss of membrane integrity. Geo had no effect on P-gp-mediated efflux of DOX. Data indicate that Geo combined with DOX could be a potential clinical chemotherapeutic approach for laryngeal cancer treatment.

1. Introduction

The identification of natural products exerting a combined effect with therapeutic agents could be an alternative for cancer treatment, reducing the concentration of the drugs and side effects [1–3]. Geopropolis (Geo) is produced by some indigenous stingless bees in tropical and subtropical zones from a mixture of vegetable resins, gland secretions of the bees and soil. Geo has been used popularly because of its medicinal use as an antiseptic agent, to treat respiratory diseases and dermatosis, and to strength our natural defenses. Geo displayed an antimicrobial activity against oral pathogens and inhibited *Streptococcus mutans* biofilms in vitro, and exerted anti-inflammatory effects in mice [4,5]. The chemical composition of Geo produced by *Melipona fasciculata* Smith was previously investigated by gas chromatography coupled with mass spectrometry, revealing that its major compounds were carbohydrates and their derivatives, triterpenes, anacardic acid, alkylresorcinols and sugar alcohols [6].

Different chemotherapeutic agents such as carboplatin (CARB), methotrexate (MXT) and doxorubicin (DOX) have been widely used against neoplastic cells [7–9]. However, these therapeutic strategies may be unsatisfactory due to side effects and drug resistance [10–12]. Multidrug resistance (MDR) may be defined as resistance to various types and/or molecular structure of chemotherapeutic agents comprising different mechanisms of action, and it is a serious problem in cancer chemotherapy [13]. Among the mechanisms of MDR, the overexpression of efflux transporters in tumor cells, especially P-glycoprotein (P-gp), has attracted attention. P-gp acts as an efflux pump to expel chemotherapeutic agents from the cells, decreasing the intracellular concentration of the agents and cell resistance to them.

Laryngeal cancer is the most common malignant neoplasm among head and neck tumors. Moreover, it represents approximately 1% of all new cancer diagnoses lately and

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http://dx.doi.org/10.1016/j.biopha.2016.03.049
0753-3322/

chemotherapy has been used for its treatment [14,15]. Immortalized cell lines have been a valuable tool for investigating detailed molecular, biochemical, genetic, and immunological properties of head and neck cancer, such as HEp-2, HEp-3, KB, FaDu, HN-1, UM-SCC-22B, UM-SCC-30, CAL27, MDA-1483, MDA-886LN, MDA-686LN, T1/CUHK, T2/CUHK, among others, as reviewed by Lin et al. [16]. HEp-2 cells contain marker chromosomes of HeLa cells. This line was originally thought to be derived from an epidermoid carcinoma of the larynx, but HeLa marker chromosomes and DNA fingerprinting were subsequently found.

Previous works from our group revealed the sensitivity of canine osteosarcoma and HEp-2 cells to Geo [6,17]. In continuity of such investigation, the effects of Geo in combination with chemotherapeutic agents were analyzed, using lower concentrations of drugs and aiming at fewer side effects to patients. The goal of this study was to determine whether Geo alone or combined with CARB, MXT and DOX could inhibit the growth of HEp-2 cells, induce apoptosis and morphological alterations, and affect the action of P-glycoprotein (P-gp), opening perspectives for new therapies including this bee product.

2. Materials and methods

This work was approved by the Ethics Committee, UNESP, Campus of Botucatu (CEP 1.356.549-2015).

2.1. Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin Rubidox® (Bergamo, Brazil), lyophilized methotrexate (Cruz Vermelha, Botucatu, SP Brazil), carboplatin Darrow-Vancel® Laboratories A/S (London, UK), dimethyl sufoxide (DMSO—VETEC Sigma Aldrich, USA), trypsin (TrypLE™ Express Gibco,USA), annexin V-FITC/PI apoptosis kit (Becton Dickinson, USA), lactate dehydrogenase (LDH) test kit (Sigma Aldrich, USA), resin (Araldite®, Brazil) and verapamil hydrochloride (VRP—Sigma Aldrich, USA) were used.

2.2. Cell cultures

HEp-2 cells and African green monkey kidney (Vero) cells were obtained from the Virology Laboratory of Biotechnology Institute (IBTec, UNESP) and were mycoplasma free. Such cells were used to investigate the selectivity of Geo alone or associated to drugs against tumor or non-tumoral cells.

HEp-2 cells were grown in Minimal Essential Medium (MEM—Cultilab, Brazil) containing penicillin/streptomycin (1%) and 10% fetal bovine serum (FBS). Vero cells were maintained in Dulbecco's modified Eagle's Medium (DMEM—Cultilab, Brazil) supplemented with 10% FBS and penicillin/streptomycin (1%). Cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2.

Sub-confluent cells were detached using trypsin-EDTA, plated at 2×10^5 cells/ml in a 96-well plate and incubated for 24 h at 37 °C for adherence.

2.3. Geopropolis sample

Geo was produced in Palmeirândia, Maranhão State, northeast Brazil, by *Melipona fasciculata* Smith and samples were frozen at −20 °C before extraction. Geopropolis (40 g) was ground and macerated in 70% ethanol at room temperature under moderate shaking. After 24 h, the extract was filtered and the dry weight of geopropolis hydroalcoholic extract was calculated (9.6 mg/ml). Cells were treated with different Geo concentrations (25, 50 and 100 μg/ml) [6].

2.4. Chemotherapeutic agents combined with Geo

HEp-2 cells were incubated with DOX (0.5 and 1 μM), MXT (50 and 100 μM) and CARB (100 and 200 μM) with or without Geo (25 μg/ml) for 24, 48 and 72 h. Drugs concentrations were established according to literature and on previous assays standardized in our laboratory, in order to obtain the optimal concentrations to study the combination with Geo [18–20]. Before the assays, Geo and the drugs were filtered using a PES membrane (pore size 0.22 μm—TPP, Switzerland). Control cells were incubated with medium alone. All experiments were performed in triplicate with 3 repetitions of the assays.

2.5. Viability assay

MTT assay was performed to assess cell viability [21]. HEp-2 and Vero cells were exposed to various concentrations of the variables for 24, 48 and 72 h. The effects of ethanol 70% (Geo vehicle) were assayed over time as well. After, 100 μl of MTT solution (1 mg/ml) were added to each well and cells were incubated for 3 h. The formazan product were dissolved in DMSO (100 μl) and absorbances were measured using an automated plate reader (BioTek Instruments, USA) at 540 nm. Absorbance from untreated cells was considered as 100% cell viability, and percentage (%) of cell viability was calculated according to the formula: % = [mean experimental absorbance/mean control absorbance] × 100%.

In terms of comparison, the 50% growth inhibitory concentration (IC_{50}) of Geo extract was determined by interpolation of the graph of Geo concentration vs cell viability.

2.6. Cytotoxicity assay

To analyze the cytotoxic effects of Geo, cell membrane damage was assessed by measuring the release of lactate dehydrogenase (LDH) into the incubation medium using the LDH test kit (Sigma Aldrich, USA) and MRX revelation Dynex technologies analyzer (Germany).

2.7. In vitro scratch assay

The wound-healing assay was used to assess the in vitro migration ability of HEp-2 cells, culturing the cells in 24-well plates until formation of a single-layer confluence [22]. After starving overnight in serum-free medium, 200-μl pipette tips were used to make scorings in the cell layer; followed by incubation for 24 and 48 h with Geo (50 and 100 μg/ml). The concentration 25 μg/ml was not used because it exerted only a mild effect on cell viability.

Cell migration was observed in an optical microscope and measures were achieved using the software Image J (image processing and analysis in JAVA; http://rsbweb.nih.gov/ij).

2.8. Apoptosis analysis by flow cytometry

The induction of apoptosis by the variables was assessed by annexin V-FITC/PI apoptosis kit (Becton Dickinson, USA). Cells were seeded (1.5×10^5 cells/ml) in 24-well plates overnight and treated with the combination that led to the highest cytotoxic activity: DOX (1 μM) and Geo (25 μg/ml) for 72 h. Untreated cells were used as a control. Cells were centrifuged (200g/10 min) and washed twice with PBS. Staining was performed according to the manufacturer's instructions and samples were acquired in a FACSCanto™ II (BD Biosciences, USA) Flow Cytometer with emission filters of 515–545 nm for FITC (green) and 600 nm for PI (red) using FACSDiva (BD Biosciences) software and analyzed using FlowJo software vX 10.6 (Tree Stars Inc.). The percentages of

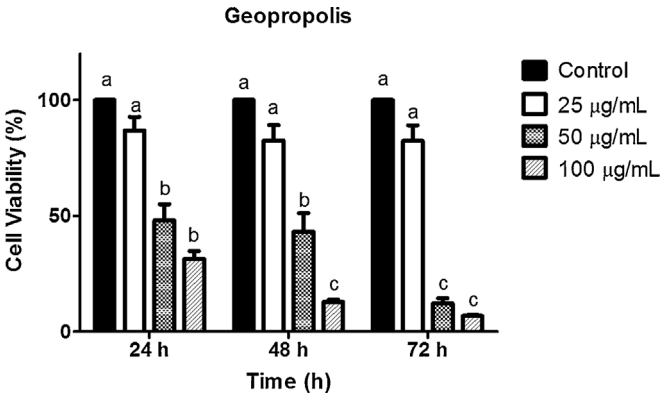


Fig. 1. Viability (%) of HEp-2 cells after incubation with geopropolis (25, 50 and 100 μg/ml) for 24, 48 and 72 h determined by MTT assay. Data represent means ± standard-deviation of three experiments. Different letters indicate significant differences between the treatments ($p < 0.05$).

early apoptotic (AV+, PI−), late apoptosis or necrotic (AV+, PI+) and live cells (AV−, PI−) were determined.

2.9. Morphological analysis

The morphological characteristics of cells treated with DOX (1 μM), Geo (25 μg/ml) or their combination for 72 h was determined by transmission electron microscopy (FEI Tecnai™, USA). Cells were trypsinized and centrifuged at 200*g* for 10 min. Afterwards, 2 ml of cell suspension containing 2×10^5 cells/well were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde buffered with 0.1 M NaH_2PO_4 + $NaHPO_4$ (pH 7.3) and post-fixed in 0.5% osmium tetroxide (OsO_4). After dehydration in ethanol, cells were embedded in resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Characteristic signs of apoptosis or necrosis (apoptotic bodies, condensation of chromatin and loss membrane integrity) were investigated.

2.10. Effect of a P-glycoprotein inhibitor (verapamil) on HEp-2 cells

The sensitivity of HEp-2 cells to DOX and the effects of a P-gp inhibitor (verapamil hydrochloride—VRP) were determined using the MTT assay (described above). The concentration of VRP (5 μM) was based on literature [19] and exerted no cytotoxic effects (data not shown).

VRP was dissolved in ethanol 70% prior to the incubation with the cells. Cells were treated with DOX (1 μM), Geo (25 μg/ml) or their combination in the presence of VRP.HCL for 72 h. Untreated HEp-2 cells were used as control. Assays were carried out in triplicate.

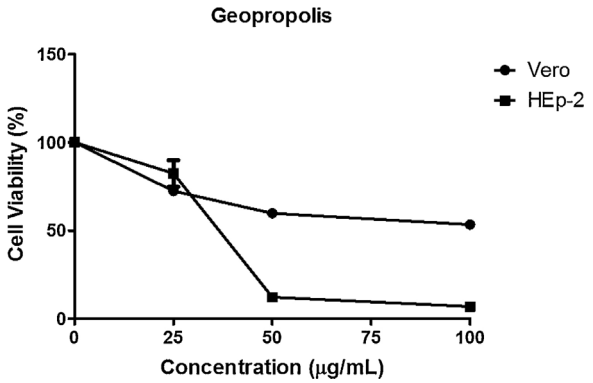


Fig. 3. Viability (%) of HEp-2 and Vero cells after 72 h incubation with geopropolis (25, 50 and 100 μg/ml) by MTT assay. Data represent means ± standard-deviation of three experiments.

2.11. Statistical analysis

Data were plotted in GraphPad Prism 4.01 using the means and standard-deviation. One-way ANOVA was used for multiple comparisons followed by Tukey test. Significant differences were considered at $p < 0.05$.

3. Results

3.1. Effects of geopropolis extract on the growth of HEp-2 and Vero cells

Fig. 1 shows the effects of Geo on the growth of HEp-2 cells after 24, 48 and 72 h incubation with concentrations ranging from 25 to 100 μg/ml. The highest inhibitory effect was seen after 72 h. Ethanol 70% (Geo solvent) showed no inhibitory effect on HEp-2 cells (Fig. 2), and the effects of Geo on the growth of Vero cells are seen in Fig. 3.

Geo inhibited the growth of HEp-2 cells in a dose- and time-dependent manner, with IC_{50} values of 66.86 ± 16.08 at 24 h,

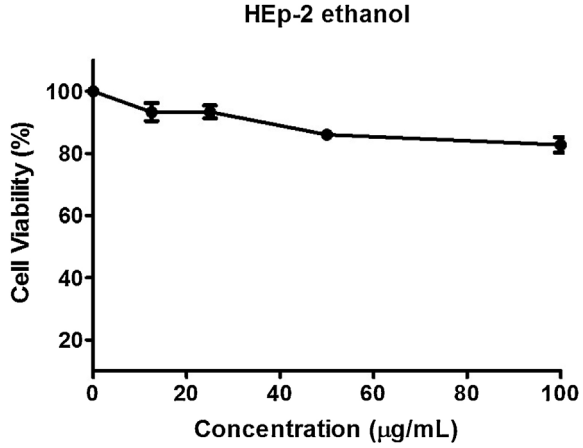


Fig. 2. Viability (%) of HEp-2 cells after 72 h incubation with 70% ethanol (geopropolis solvent) by MTT assay. Ethanol concentrations were equivalent to those found in 25, 50 and 100 μg/ml of Geo (0.15, 0.29 and 0.59%). Data represent means ± standard-deviation of three experiments ($p > 0.05$).

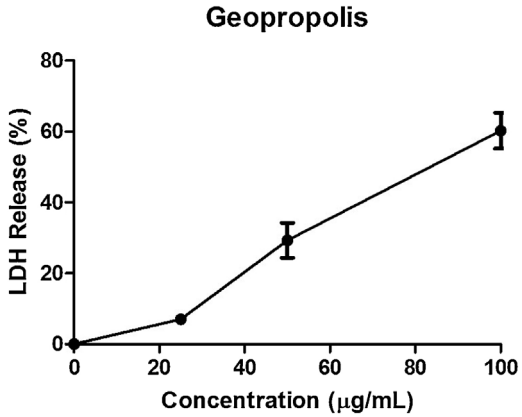


Fig. 4. LDH release (%) by HEp-2 cells after incubation with geopropolis (25, 50 and 100 μg/ml) for 72 h. Data represent means ± standard-deviation of three experiments.

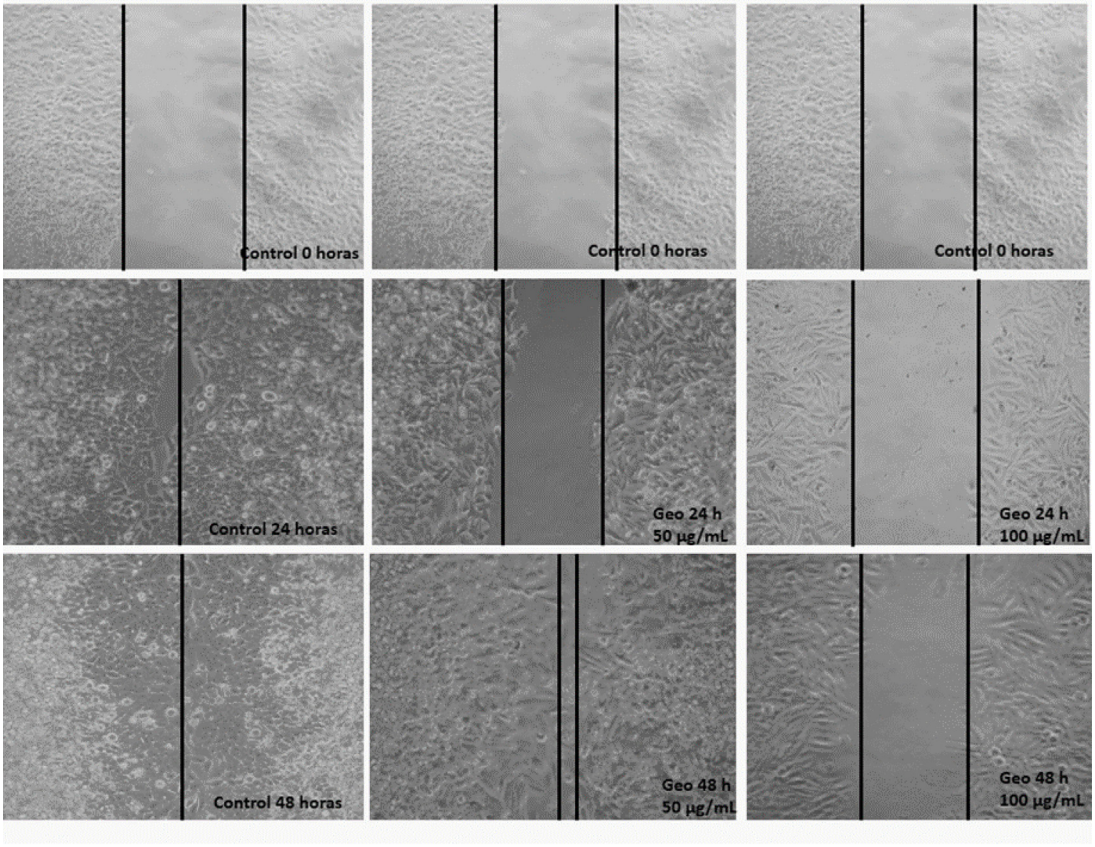
54.42 ± 19.63 at 48 h, and 44.10 ± 23.88 at 72 h. The IC_{50} obtained from three independent experiments for Vero cells after 72 h was 91.01 ± 10.33, showing that Geo exhibited a selective action for tumors cells over non-tumoral ones.

3.2. LDH release assay

Fig. 4 shows LDH release from HEp-2 cells after incubation with various concentrations of Geo for 72 h. Geo affected cell viability using 50 μg/ml (29.3%) and 100 μg/ml (60.2%). The concentration 25 μg/ml exhibited a mild effect on HEp-2 cells viability (7.0%) and was used in the next assays due to its lower cytotoxic effects, to determine if Geo would exert a better action in combination with the drugs.

3.3. In vitro migration assay

The wound healing assay showed that Geo (50 and 100 μg/ml) reduced significantly cell migration after 24 h, while Geo (100 μg/ml) was efficient only after 48 h (Fig. 5). Geo (25 μg/ml) did not affect cell migration (data not shown).



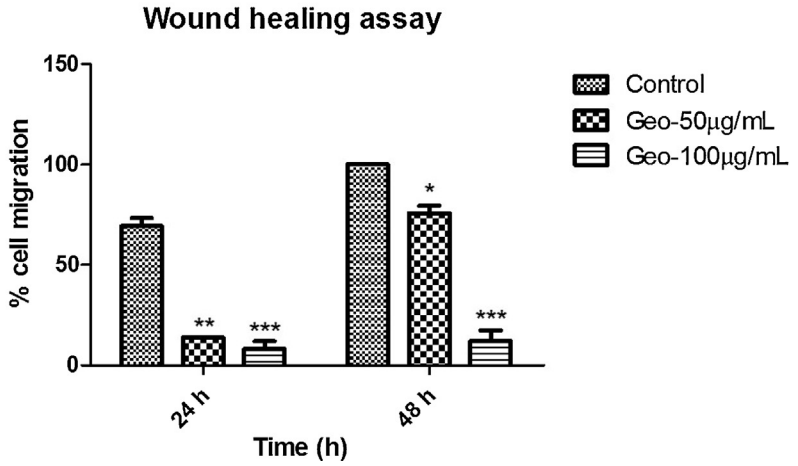


Fig. 5. Percentage (%) of HEp-2 cells migration in vitro after treatment with geopropolis (Geo—50 and 100 μg/ml) by wound healing assay. Data represent means ± standard-deviation of three experiments (*$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$ vs control).

3.4. Effects of Geo combined with DOX, MXT and CARB on HEp-2 cells viability

The sensitivity of HEp-2 cells to DOX (0.5 and 1 μM), MXT (50 and 100 μM) and CARB (100 and 200 μM) with or without Geo (25 μg/ml) was examined after 24, 48 and 72 h by MTT assay.

Geo combined with MXT affected HEp-2 viability only after 24 h compared to monotherapy (Fig. 6a). The co-treatment with Geo + CARB showed no inhibitory effect on HEp-2 cells (Fig. 6b). The combination DOX + Geo affected significantly the sensitivity of HEp-2 cells after 72 h compared to DOX and Geo alone, decreasing cell viability to 43.5% (Fig. 6c). Thus, the combination DOX (1 μM) and Geo (25 μg/ml) was chosen to evaluate apoptosis, morphological changes in HEp-2 cells and the action on P-gp.

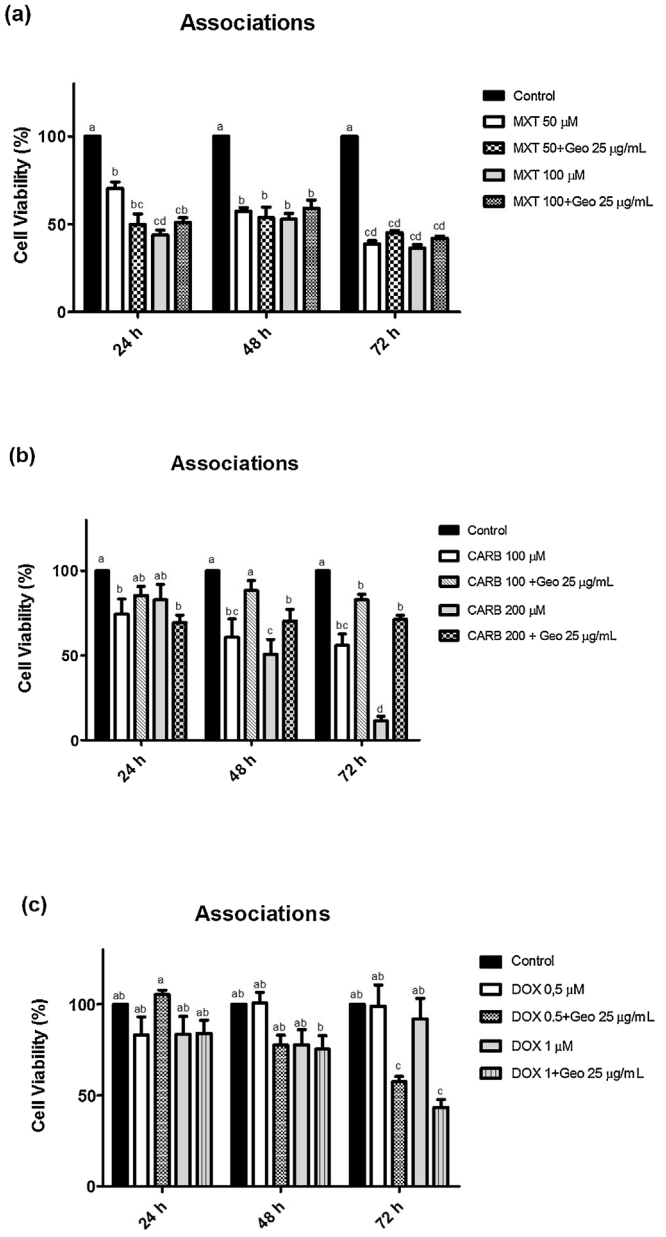


Fig. 6. Viability (%) of HEp-2 cells treated with geopropolis (Geo—25 μg/ml) and methothrexate (MXT—50 and 100 μM) (a); Geo (25 μg/ml) and carboplatin (CARB—100 and 200 μM) (b); Geo (25 μg/ml) and doxorubicin (DOX—0.5 and 1 μM) (c) after 24, 48 and 72 h incubation. Different letters indicate significant differences between the treatments ($p < 0.05$).

DOX concentrations did not affect Vero cells viability ($p > 0.05$) after 72 h, indicating that this drug did not exert cytotoxic effects towards non-tumoral cells (Fig. 7).

3.5. Apoptotic effect of Geo and DOX on HEp-2 cells

To examine whether Geo (25 μg/ml), DOX (1 μM) and their combination could induce apoptosis after 72 h, cells were stained with Annexin V-FITC and analyzed by flow cytometry. As shown in Fig. 8, DOX or Geo alone induced cell apoptosis/necrosis (Q1 + Q2: 16.28 and 11.53%, respectively). The percentage of apoptotic cells increased in comparison to control group (9.31%). The percentage of apoptotic cells was significantly higher after incubation with Geo + DOX (30.11%) compared to DOX and Geo alone. A higher percentage of necrotic cells were seen after incubation of HEp-2 cells with the combination Geo + Dox (Q2 + Q3: 28.57%).

3.6. Morphological changes

HEp-2 cells treated with Geo + DOX exhibited fragmentations of cytoplasmic membrane (apoptotic bodies) and loss of membrane integrity, what is characteristic of late apoptotic/necrosis. As shown in Fig. 9, the combination increased the alterations in the cell morphology.

3.7. Effects of verapamil on sensitivity of HEp-2 cells to Geo + DOX

The sensitivity of HEp-2 cells to DOX was examined after co-treatment with a P-gp inhibitor: VRP (Fig. 10). DOX alone did not influence cell viability, whereas the co-treatment with VRP diminished the efflux of this drug by inhibiting P-gp, decreasing cell viability.

Geo + DOX decreased cell viability, and the co-incubation with VRP revealed that this combination exerted a similar action to DOX alone.

4. Discussion

Drug combination therapies are commonly used in cancer treatment in order to obtain better results and reduce drug resistance [23]. To address these problems, attention has been focused on identifying novel agents that can be combined with antitumor drugs to increase the therapeutic efficacy and decrease side effects. Recently, some studies have reported that some natural products combined with chemotherapeutic drugs enhanced the anticancer effects against various cell lines [24–27]. In the present study, the effects of Geo, a stingless bee product,

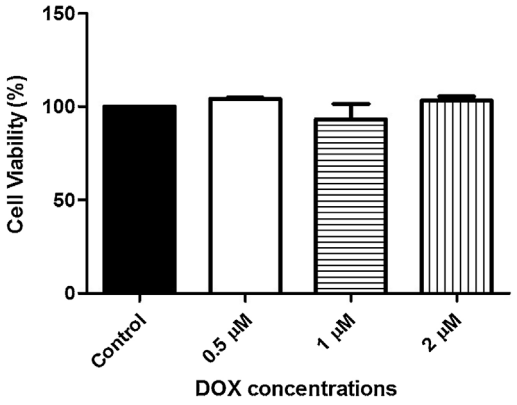


Fig. 7. Viability (%) of Vero cells after incubation with different concentrations of doxorubicin (DOX—0.5, 1 and 2 μM) for 72 h, by the MTT assay. Data represent means ± standard-deviation of three experiments ($p > 0.05$).

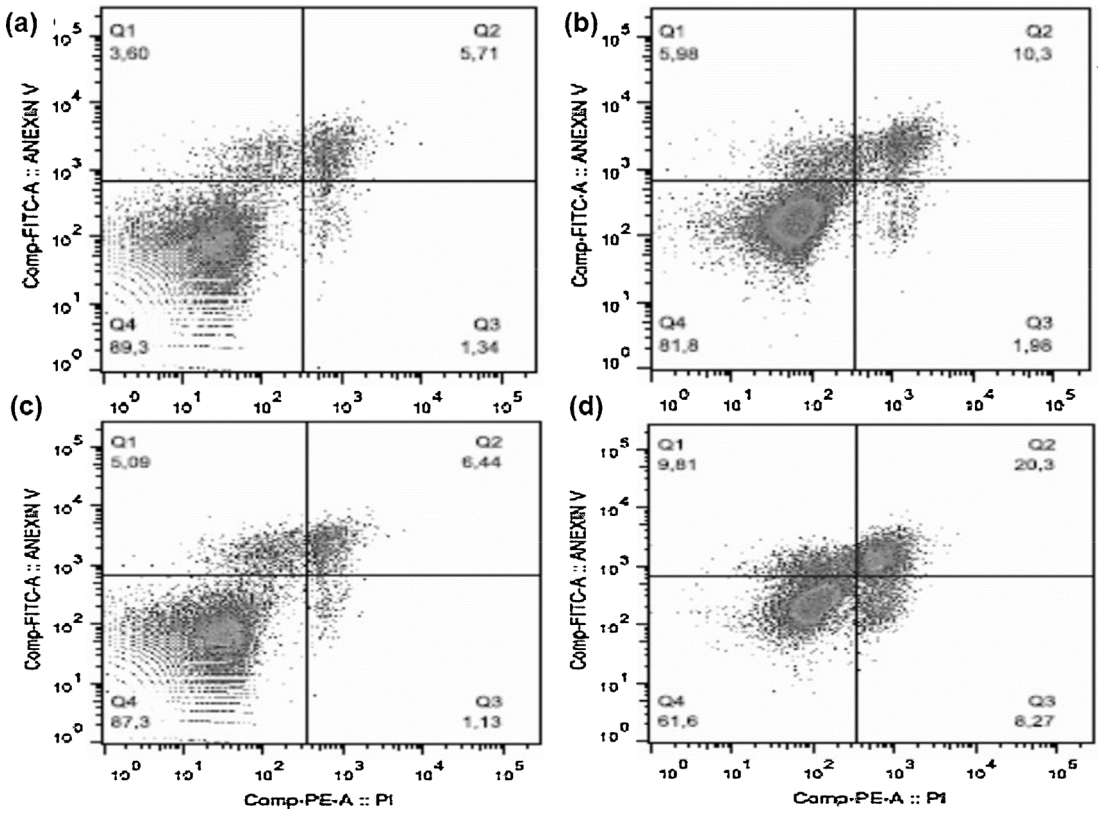


Fig. 8. Apoptosis induction in HEp-2 cells using flow cytometry by staining with annexin V/PI. The pseudocolor graphs show (a) control; (b) geopropolis (Geo—25 μg/ml) (c) doxorubicin (DOX—1 μM); and (d) DOX + Geo (1 μM/25 μg/ml) showing the highest cytotoxicity levels compared to control, as demonstrated by the percentage of early apoptotic cells (AV+), late apoptotic or necrotic cells (AV+/PI+ or PI+). Q1 represent early apoptotic cells; Q2 represents late apoptotic or necrotic cells; Q3 represents necrotic cells; Q4: live cells.

combined with DOX, MXT and CARB were investigated on HEp-2 cells. To determine whether Geo enhanced the anticancer effect of such drugs, HEp-2 cells were treated with Geo alone or in combination with each chemotherapeutic drugs and cell proliferation and migration, morphological changes, apoptosis and P-gp activity were evaluated.

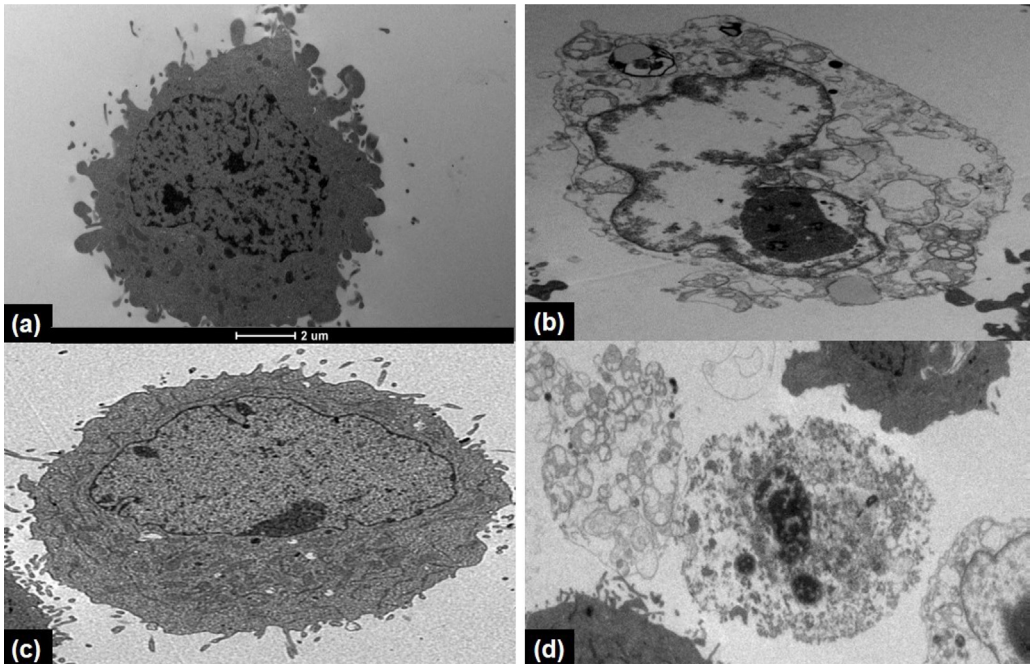


Fig. 9. Microscopical analysis of HEp-2 cells after 72 h. (a) control, (b) geopropolis (Geo—25 μg/ml), (c) doxorubicin (DOX—1 μM), and (d) combination DOX + Geo (1 μM/25 μg/ml), showing apoptotic/necrotic cells with loss of membrane integrity. Scale bar represents 2 μm.

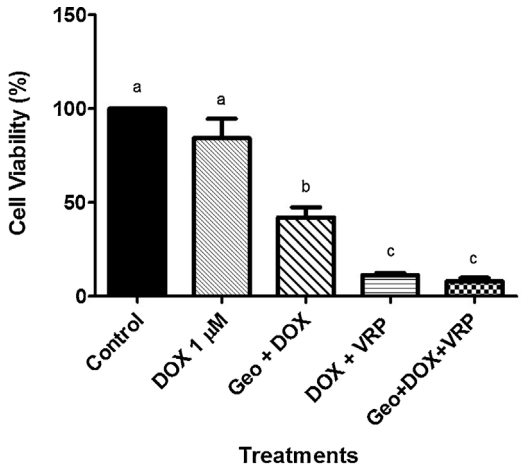


Fig. 10. Effects of a P-gp inhibitor (verapamil—VRP, 5 μM) on the viability (%) of HEp-2 cells incubated with doxorubicin (DOX—1 μM), geopropolis (Geo—25 μg/ml), and Geo + DOX for 72 h. Different letters indicate significant differences between the groups ($p < 0.05$).

The proliferation of HEp-2 cells was reduced by Geo treatment in a time- and dose-dependent manner, showing selectivity against tumor cells compared to non-tumoral Vero cells. LDH leakage assay was also performed as indicator of cytotoxicity. LDH leakage increased in a dose-dependent manner after incubation of HEp-2 cells with Geo. Moreover, our data showed that the migration capacity of tumor cells decreased significantly after Geo treatment *in vitro*. The biological effects of Geo may be determined by its chemical composition. One may speculate that the antiproliferative and cytotoxic activity exerted by Geo may be due to the presence of Geo constituents such as lupeol, amyrins and anacardic acid and derivatives found in our sample [6]. These compounds have already been described by their cytotoxic action against different tumor cell lines [28–31].

The combination Geo + DOX exhibited a higher efficacy by inhibiting HEp-2 cells growth than CARB and MXT, suggesting that Geo may act differentially in combination with drugs. In this work, Geo enhanced the inhibitory action of DOX against HEp-2 cells; in contrast, it diminished CARB activity. Sforcin and Bankova reported that possible interactions between propolis and other medicines should be investigated in humans [32]; therefore, further investigation should evaluate whether Geo enhances or diminishes the antitumoral action of drugs *in vivo*.

The antiproliferative potential of different chemotherapeutic agents in association with natural compounds may vary according to the sensitivity and cancer cell lines [33]. DOX is one of the widely used anticancer drugs in the treatment of various malignancies, but its clinical use is limited due to severe side effects to non-tumoral cells [34,35]. The mechanism of action of DOX comprises the inhibition of cell proliferation and induction of apoptosis [36]. However, DOX-mediated cytotoxicity is different towards cancer and normal tissues depending of concentration *in vivo* and *in vitro* [37,38]. The present study showed no cytotoxic effects of DOX (0.5, 1 and 2 μM) on VERO cells and demonstrated the effectiveness of Geo + DOX against HEp-2 cells. It has been reported that differences in DOX-mediated toxicity may be used as an alternative to improve the antitumor therapy with DOX [26,39].

To assess possible mechanisms involved in the anticancer activity of the combination Geo + DOX, the induction of apoptosis of HEp-2 cells was evaluated by flow cytometry. Apoptosis plays a fundamental role in protecting organisms against tumorigenesis [40]. Apoptosis dysregulation is commonly found in cancer cells and its induction has been described as a strategy in cancer therapy [41–43]. Our findings showed that the treatment of HEp-2 cells with Geo in combination with DOX induced apoptosis compared to Geo or DOX alone. These data are in agreement with the images obtained by transmission electron microscopy, showing the presence of apoptotic cells after treatment with Geo + DOX. Additionally, several studies have demonstrated that combination between DOX and different natural products can induce apoptosis [44–46]. Our data indicated for the first time that the combination of Geo extract and DOX exhibited a significant apoptotic potential.

To address whether the combination Geo + DOX could affect P-gp activity, HEp-2 cells were co-treated with VRP. DOX is a P-gp substrate and may modulate its expression, inducing cell resistance by increasing drug efflux [47]. P-gp is expressed by several cells and it is highly specific to eliminate hydrophobic compounds, such as chemotherapeutic agents, taxanes, topoisomerase inhibitors and antimetabolites [48,49]. VRP is a calcium channel blocker and a P-gp inhibitor at concentrations 5–10 μM. It has been associated with reversion of resistance caused by P-gp action *in vitro* [19,50]. The treatment concomitantly with VRP increased significantly the sensitivity of HEp-2 cells to DOX, decreasing its efflux. In the presence of VRP, the effects of the combination Geo + DOX were similar to DOX, suggesting that Geo did not affect P-gp-mediated efflux of DOX and indicating that Geo may affect cell viability by other mechanisms. Similarly, a decreased HEp-2 cell viability was seen after incubation with VRP, DOX and another natural product—curcumin, which had no effects on P-gp [19]. On the contrary, it has been suggested that propolis extract inhibited the function of MDR1 and increased the sensitivity to substrates of MDR1 in HeLa/TXL cells, suggesting that the propolis effects could be due to other constituents than caffeic acid phenethyl ester (CAPE) [51].

Altogether, our findings *in vitro* showed that Geo inhibited the proliferation and migration of human laryngeal carcinoma cells *in vitro.* Moreover, Geo enhanced the anticancer effect of DOX on HEp-2 cells by inducing apoptosis, morphological changes including apoptotic bodies, secondary apoptosis/necrosis and membrane dysruption, with no effects on P-gp action.

The effect of natural products associated to chemotherapeutic agents may be dependent on the type of agent, the characteristics of the tumor cells and/or the chemical composition of the natural product. The antitumor potential of the combination Geo + DOX should be further investigated in vivo not only to understand its mechanisms of action for clinical chemotherapeutic approaches but also using lower concentrations of drugs and aiming at fewer side effects to patients, since DOX may cause several adverse effects in different organs such as kidney, liver, and brain [52].

Conflict of interest

The authors declare that they have no conflict of interest.

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