Evaluation of the antiproliferative activity of red propolis hydroalcoholic extract and its fractions obtained by partition

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1Universidade de Franca. Avenida Dr. Armando Sales de Oliveira, 201, 14404-600, Franca, São Paulo, Brazil. Tel.: +55-16-3711-8871; Fax: +55-16-3711-8878, *email: denisecrispim2001@yahoo.com
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Abstract. Squareisi IS, De Freitas KS, Lemes DC, Ccana-Ccapatinta GV, Mejia JAA, Bastos JK, Veneziani RCS, Ambrosio SR, Tavares DC. 2018. Evaluation of the antiproliferative activity of red propolis hydroalcoholic extract and its fractions obtained by partition. Biofarma J Nat Prod Biochem 18: 66-69. The present study aimed to evaluate the cytotoxicity of red propolis hydroalcoholic extract (RPHE) and its fractions obtained by partition, hexanes (HF), dichloromethane (DF), ethyl acetate (AF) and n-butanol (BF), on tumor and non-tumor cell lines. For this purpose, the XTT colorimetric assay was performed on human lung fibroblasts (GM07492A, non-tumor cell), breast adenocarcinoma (MCF-7), glioblastoma (U343) and cervix adenocarcinoma (HeLa) cells. The results showed that RPHE, HF and DF presented not only cytotoxic potential to all tumor cell lines but also to normal cell line, indicating selectivity absence. HF presented the lowest IC50 (half minimal inhibitory concentration; 33.8-133.3 µg/mL), with significant difference from those observed for RPHE (137.0-262.7 µg/mL). BF and AF revealed an IC50 which was higher than 1250 µg/mL in all cell lines. The results showed that red propolis has substances with antiproliferative activity, indicating that its hexanes fraction may have substances with antitumor potential.

Keywords: cytotoxicity, red propolis, XTT colorimetric assay

INTRODUCTION

Propolis is a product of honeybee hives, containing mainly beeswax and a resin obtained from various plant sources such as apical buds, young leaves and exudates (Salatino & Salatino 2018). Red propolis, with Dalbergia ecastaphyllum (Daugsch et al., 2008; Silva et al., 2008) and Symphonia globulifera (Ccana-Ccapatinta et al., 2020) as its main botanical sources, has recently stood out as a natural medicinal product due to its various biological properties such as anti-inflammatory (Batista et al. 2018), antitumoral (Salatino & Salatino 2018) antioxidant, cytotoxic (de Oliveira Reis et al., 2019), antimicrobial (Miranda et al. 2019) activities and healing capacity (Picolotto et al. 2019). The biological activity of red propolis is mainly related to isoflavones, which act in synergy with the other compounds. Other compounds, such as vestitol, neovestitol, biochanin A and liquiritigenin, are identified in the fractions and extracts of Brazilian red propolis are also considered as important markers and have been associated with different biological effects (Rufatto et al. 2018; Nani et al. 2018).

Bio guided fractionation is efficient methods to improve the development of new drugs, highlighting the activity of each group of compounds, individually or in combination with others (Dos Santos et al., 2019). Thus, the present study aimed at evaluating the cytotoxicity of the red hydroalcoholic extract (RPHE) and its fractions, namely, hexanic (HF), dichloromethane (DF), ethyl acetate (AF) and n-butanol (BF) on tumor and non-tumor cell lines.

MATERIALS AND METHODS

Obtention and profiling of red propolis hydroalcoholic extract (RPHE) and its fractions

Red propolis (2 kg) was purchased from Cooperativa de apicoltore de Canavieiras (COAPER) in the city of Canavieiras (Bahia state, Brazil) in April of 2018. RPHE was prepared by exhaustive maceration of 500 g of red propolis in 1.5 L of ethanol: H2O (7:3 v/v) for seven days. The extract was then filtered, concentrated and lyophilized to result in 75g of RPHE. After resuspending RPHE in 500 mL of methanol: H2O (8:2 v/v), the fractioning of this extract was executed through a four-times partition with 500 mL of hexanes, dichloromethane, ethyl acetate, and n-butanol respectively to result in HF (22.8g), DF (43.6g), AF (0.90g), and BF (4.34g) after evaporation.

The chemical profiles of RPHE and its fractions were obtained by HPLC-DAD analysis using a Synergi Polar-RP (150 x 4.60 mm, 4 µm) column as stationary phase and a gradient mobile phase of H2O (A) and acetonitrile (B) starting from 23% up to 100% of (A) in 32 min at 1.2 mL/min. The detection wavelength was 220 nm and the compounds identification was performed by co-injection of
standard concentrations available in our laboratory (Figure 1): liquiritigenin (1), formononetin (2), liquiritigenin (3), vestitol (4), neovestitol (5), medicarpin (6), 7-O-methylvestitol (7), mixture of guttiferone E and xanthochymol (8), oblongifolin B (9).

Evaluation of antiproliferative activity

In this study, three different tumor cell lines were used: human breast adenocarcinoma (MCF-7), human cervical adenocarcinoma (HeLa) and human glioblastoma (U343). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate possible selective activity of the natural medicinal product under investigation. Different cell lines were maintained as monolayers in plastic culture medium (HAM-F10 + DMEM, 1:1, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich) and 2.38 mg/mL Hepes (Sigma-Aldrich). Cells were incubated at 36.5°C in humidified 5% CO₂ atmosphere.

Evaluation of the antiproliferative activity of the samples was performed using the in vitro toxicology colorimetric assay-XTT Kit (Roche Diagnostics) according to the manufacturer’s guidelines. For the experiments, 1 x 10⁴ cells were seeded in 96-well microplates, each well received a maximum of 100 µL culture medium (HAM F10 + DMEM, 1:1) supplemented with 10% fetal bovine serum containing different sample concentrations, which ranged from 9.77 to 1250 µg/mL. Negative (untreated), solvent (1% DMSO) and positive (25% DMSO) control wells were also included. After incubation with the substances at 37 °C for 24 h, the culture medium was removed, the cells were washed with 100 µL PBS to remove treatments and exposed to 100 µL HAM-F10 culture medium without phenol red. Then, 25 µL of XTT were added to each well and the microplates were incubated at 37 °C for 17 h. The absorbance of the samples was determined using a multi plate reader (Asys-UVM 340/Microwin 2000 ELISA) at a wavelength of 450 nm and a reference length of 620 nm. The number of soluble products formed (formazan) was proportional to the number of viable cells. The negative control group was designated as 100% and the results were expressed as a percentage of the negative control. The experiments were performed in triplicate.

Statistical analysis

Cytotoxicity was assessed using the IC₅₀ value (50% cell growth inhibition) as a response parameter, which was calculated with the GraphPad Prism 5.0 program (GraphPad Sofware, San Diego, CA, USA) by plotting cell survival against the respective concentrations of the natural products tested. One-way ANOVA was used for comparison of means (p < 0.05).

RESULTS AND DISCUSSION

Results

Table 1 presents the results expressed by inhibitory concentration of 50% cell growth (IC₅₀). The results showed that, for each cell line-GM07492A (human lung fibroblasts, non-tumor cells), MCF-7 (breast adenocarcinoma), U343 (glioblastoma) and HeLa (cervix adenocarcinoma)-RPHE revealed an IC₅₀ of 137.0, 262.7, 138.2 and 137.4 µg/mL, HF an IC₅₀ of 33.8, 133.3, 33.8 and 34.5 µg/mL, DF an IC₅₀ of 78.9, 237.6, 71.6 and 99.6 µg/mL, and AF and BF an IC₅₀ of higher than 1250 µg/mL in all cell lines. RPHE, HF and DF therefore showed cytotoxic activity to all tumor cell lines, but also to the normal cell line, indicating selectivity absence. HF presented the highest cytotoxic potential with the lowest IC₅₀, being significantly different from those observed for RPHE. The cytotoxicity of HF might be attributed to its chemical composition, consisting predominantly of polyphenylated benzophenones 8 and 9 (guttiferone E, xanthochymol and oblongifolin B), described in the literature as pro apoptotic compounds.
Table 1. Inhibitory concentrations (IC\(_{50}\), \(\mu\)g/mL) obtained from RPHE-treated non-tumoral (GM07492A) and tumoral (MCF-7, U343 and HeLa) cell cultures and its fractions.

| Treatment | GM07492A | MCF-7 | U343 | HeLa |
|-----------|----------|-------|------|------|
| RPHE      | 137.0 ± 4.2 | 262.7 ± 5.9 | 138.2 ± 2.3 | 137.4 ± 0.3 |
| HF        | 33.8 ± 0.6\(^a\) | 133.3 ± 1.3\(^a\) | 33.8 ± 0.3\(^a\) | 34.5 ± 0.1\(^a\) |
| DF        | 78.9 ± 1.7\(^a\) | 237.6 ± 26.6 | 71.6 ± 0.6\(^a\) | 99.6 ± 27.3 |
| AF        | > 1250 | > 1250 | > 1250 | > 1250 |
| BF        | > 1250 | > 1250 | > 1250 | > 1250 |

Note: RPHE: red propolis hydroalcoholic extract; HF: hexanes fraction; DF: dichloromethane fraction; AF: ethyl acetate fraction; BF: n-butanol fraction. GM07492A: non-tumor human lung fibroblast; MCF-7: human breast adenocarcinoma; U343: human glioblastoma; HeLa: human cervical adenocarcinoma. Values are mean ± SD; n: 3. *Significantly different from the RPHE treatment (\(p < 0.05\))

Discussion

In this sense, Lin et al. (2019) reported cytotoxicity of a mixture of guttiferone E and xanthochymol in five types of human cancer cell lines: leukemia (HEL, IC\(_{50}\) = 11.27 \(\mu\)g/mL; K562, IC\(_{50}\) = 10.92 \(\mu\)g/mL), cervical (HeLa, IC\(_{50}\) = 5.46 \(\mu\)g/mL), breast (MCF-7, IC\(_{50}\) = 4.68 \(\mu\)g/mL) and lung (A549, IC\(_{50}\) = 6.10 \(\mu\)g/mL). These results demonstrate that these two substances present in the hexanes fraction of red propolis corroborate their cytotoxic potential.

Novak et al. (2017) observed that a fraction of the ethanolic extract of Brazilian red propolis, containing xanthochymol and formononetin, showed antiproliferative effect in acute promyelocytic leukemia cell lines (HL-60, IC\(_{50}\) = 20.5 \(\mu\)g/mL), human chronic myeloid leukemia (K562), IC\(_{50}\) = 30.3 \(\mu\)g/mL, multiple myeloma (RPMI8226, IC\(_{50}\) = 32.6 \(\mu\)g/mL) and murine melanoma (B16F10, IC\(_{50}\) = 25.7 \(\mu\)g/mL). This fraction showed more promising antiproliferative effect than that observed by the ethanolic extract.

Through the MTT test, the ethyl acetate fractions of the red propolis extract revealed cytotoxic activity in HT-29 (human colorectal adenocarcinoma) and HCT-116 (human colorectal carcinoma) and non-tumor cell line Vero (monkey kidney epithelium). IC\(_{50}\) values ranged from 40.32 to 105.23 \(\mu\)g/mL, with the lowest IC\(_{50}\) values corresponding to the tumoral lines tested, indicating selectivity. Chemical analysis of the fractions indicated the presence of formononetin, vestitol, biochanin A, liquiritigenin and the guttiferone E xanthochymol mixture (Santos et al. 2019).

The antiproliferative activity of the hydroethanolic extract of red propolis and two of its fractions (J and L) was evaluated in human laryngeal epidermoid carcinoma cells (HeLa) by Da Silva Frozza et al. (2017). The chemical profile of fraction J revealed the presence of formononetin, liquiritigenin, medicarpin, vestitol, isovestitol and (3S)-ferreirin, and of fraction L, only liquiritigenin. The results showed an IC\(_{50}\) of 145.40 \(\mu\)g/mL for the extract, 60.90 \(\mu\)g/mL for the J fraction and 74.60 \(\mu\)g/mL for the L fraction. Thus, the fractions showed a greater cytotoxic effect than the extract. Fractionation of the extract leads to a decrease in the number of chemicals present in the fraction and may increase the concentration of active compounds when compared to the exposure of cells to the crude extract (da Silva Frozza et al. 2017).

The results showed that red propolis presents substances with antiproliferative activity, indicating that the hexanes fraction may have substances with antitumor potential. Further studies should be conducted with guttiferone E, xanthochymol and oblongifolin B, substances predominantly identified in the hexanes fraction.

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