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Original Article

Antiproliferative activity of essential oils from three plants of the Brazilian Cerrado: *Campomanesia adamantium* (Myrtaceae), *Protium ovatum* (Burseraceae) and *Cardiopetalum calophyllum* (Annonaceae)

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Abstract

Essential oils, which may be extracted from several parts of plants, have different biological activities. The Brazilian Cerrado has a large variety of plants that yield essential oils, even though many have not been studied yet. Taking into account the biodiversity of this biome, this study aimed at evaluating the antiproliferative activity of essential oils extracted from three species of plants of the Cerrado in Goiás state: *Campomanesia adamantium* (Cambess.) O. Berg, *Protium ovatum* (Engl. in Mart.) and *Cardiopetalum calophyllum* (Schltdl.). Essential oils were extracted from both *C. adamantium* and *C. calophyllum* leaves and from *P. ovatum* leaves and green fruits by hydrodistillation carried out by a Clevenger-type apparatus. The chemical composition of the essential oils was determined by Gas Chromatography coupled to Mass Spectrometry (GC-MS). The following major chemical constituents were identified in the essential oils under investigation: β-myrcene (62.00%), spathulenol (28.78%), germacrene-B (18.27%), β-caryophyllene oxide (16.40%), β-caryophyllene (14.00%), α-pinene (11.30%), viridiflorol (9.99%), limonene (7.30%) and (*Z*,*E*)-pharnesol (6.51%). The antiproliferative activity was evaluated in different human tumor cell lines: breast adenocarcinoma (MCF-7), cervical adenocarcinoma (HeLa) and glioblastoma (M059J). A normal human cell line was included (GM07492A, lung fibroblasts). Results showed that essential oils from *C. adamantium* leaves got the lowest values of IC_{50} in all strains of tumor cells under evaluation. They were significantly lower than the ones of the normal cell line, an evidence of selectivity. It is worth mentioning that this is the first report of the antiproliferative activity of essential oils from *C. adamantium*, *P. ovatum* and *C. calophyllum* against human tumor cells.

Keywords: essential oils, Cerrado, antiproliferative activity, tumor cells.

Atividade antiproliferativa dos óleos essenciais de três plantas do Cerrado brasileiro: *Campomanesia adamantium* (Myrtaceae), *Protium ovatum* (Burseraceae) e *Cardiopetalum calophyllum* (Annonaceae)

Resumo

Os óleos essenciais podem ser extraídos de várias partes das plantas e apresentam diversas atividades biológicas. O Cerrado brasileiro possui uma grande variedade de plantas produtoras de óleos essenciais muitas delas ainda não estudadas. Levando-se em consideração a biodiversidade desse bioma, o objetivo deste trabalho foi avaliar a atividade antiproliferativa dos óleos essenciais extraídos de três espécies de plantas ocorrentes no Cerrado do estado de Goiás: *Campomanesia adamantium* (Cambess.) O. Berg, *Protium ovatum* (Engl. in Mart.) e *Cardiopetalum calophyllum* (Schltdl.). Os óleos essenciais foram obtidos das folhas de *C. adamantium* e *C. calophyllum* e das folhas e frutos verdes de *P. ovatum* por hidrodestilação, usando o aparelho do tipo Clevenger. A composição química dos óleos essenciais foi determinada pelo método de Cromatografia Gasosa acoplada à Espectrometria de Massas (CG-EM). Os constituintes químicos majoritários identificados nos óleos essenciais estudados foram: β-mirceno (62,00%), espatulenol (28,78%), germacreno-B (18,27%), óxido de β-cariofileno (16,40%), β-cariofileno (14,00%), α-pineno (11,30%), viridiflorol (9,99%), limoneno (7,30%) e (*Z*,*E*)-farnesol (6,51%). A atividade antiproliferativa foi avaliada em diferentes linhagens de células tumorais humanas: adenocarcinoma de mama (MCF-7), adenocarcinoma cervical (HeLa) e gliobastoma (M059J), além de, uma linhagem celular humana normal (GM07492A, fibroblastos pulmonares). O óleo essencial das folhas de *C. adamantium* exibiu menores valores de CI_{50} em todas as linhagens celulares tumorais avaliadas, sendo menores que aquele obtido na linhagem celular normal, indicando seletividade. Este é o primeiro relato da atividade antiproliferativa dos óleos essenciais de *C. adamantium*, *P. ovatum* e *C. calophyllum* contra células tumorais humanas.

Palavras-chave: óleos essenciais, Cerrado, atividade antiproliferativa, células tumorais.

1. Introduction

The Brazilian Cerrado (savanna) biome, which is a great source of medicinal plants, comprises several pharmacologically active species that have been used in folk medicine and contribute to significant knowledge of bioactive compounds and for the development of herbal remedy (Ribeiro et al., 2016). Essential oils yielded by these medicinal plants have been defined as complex mixtures of volatile, lipophilic, usually odoriferous, liquid and oily – at room temperature – substances which have broad biological application (Santos et al., 2013).

Most cancer treatments have been considered one of the most challenging problems in medicine nowadays. The search for new drugs that display activity against several types of cancer has become one of the most interesting subjects in the field of natural products. As a result, several experimental and epidemiological studies have shown that some plants may promote chemopreventive and/or antineoplastic activity (Oliveira et al., 2014). It is known that 50% of therapeutic agents used in cancer treatments have natural origin, mainly essential oils, which have drawn researchers' interest worldwide due to the fact that some have important antiproliferative activity (Al-Hajj et al., 2017).

Species of the genus *Campomanesia* have been widely used as infusions to treat ulcers, diarrhea and inflammation by popular medicine (Matos et al., 2015). More specifically, the species *C. adamantium* is a plant of the Myrtaceae family – also popularly known as guavira, gabiroba and guabiroba in Portuguese – whose bush is from 0.5 to 1.5 m high (Oliveira et al., 2016). A study of essential oils from *C. adamantium* reported high concentration of sesquiterpenes in the essential oil from fresh leaves (Matos et al., 2015).

The species *Protium ovatum* (Burseraceae) – also popularly known as almecega in Portuguese – can be found in both biomes Cerrado and the Amazon Forest. Its main characteristic is to yield resins whose anti-inflammatory, immunostimulant, repellent, antinociceptive and antineoplastic activity has already been proven (Estevam et al., 2017).

Cardiopetalum calophyllum (Annonaceae) – also popularly known as imbirinha – is a typical plant of the Cerrado and can be found in central Brazil, Triângulo Mineiro, Goiás and Mato Grosso (Xavier et al., 2016a). Several biological properties of extracts from *C. calophyllum*, such as larvicide activity against *Aedes aegypti* larvae, leishmanicidal activity against both *Leishmania brasilienses* and *Leishmania amazonenses*, besides ovicidal and nymphicidal activity against *Rhodnius neglectus*, have been described in the literature (Xavier et al., 2016a).

Some biological activities of essential oils from *C. adamantium*, *C. calophyllum* and *P. ovatum*, such as leishmanicidal, trypanocidal, antibacterial, antioxidant and antifungal activity have also been reported in the literature (Estevam et al., 2017; Oliveira et al., 2016; Xavier et al., 2016a). However, the antiproliferative activity of essential oils from these three species of the Brazilian Cerrado against tumor cells has not been investigated yet.

In this study, cytotoxicity of essential oils extracted from both *C. adamantium* and *C. calophyllum* leaves and *P. ovatum* leaves and green fruits was screened against different cell lines. In sum, the antiproliferative activity of the essential oils these three plants found in the Brazilian Cerrado is described for the first time in this work.

2.Material and Methods

2.1. Collection and identification of plant material

Campomanesia adamantium leaves were collected on farms which belong to the Universidade de Rio Verde (UniRV), located in the Rio Verde region, in October 2014. The plant material was deposited at the Herbarium Professor Germano Guarin Neto, in agreement with voucher HJ 6561. *Cardiopetalum calophyllum* leaves were collected in Rio Verde city in March 2014 and the exsiccate was deposited at the herbarium of the Universidade Estadual de Montes Claros (UNIMONTES), in agreement with voucher 3815. *Protium ovatum* leaves and green fruits were collected at the Universidade de Rio Verde (UniRV), located in the Cerrado region, in July 2014. The plant was deposited at the Herbarium Jataiense Professor Germano Guarim Neto at exsiccate number HJ 742.

2.2. Essential oil extraction

Essential oils were extracted from *P. ovatum* leaves (100 g) and green fruits (100 g) and from *C. adamantium* and *C. calophyllum* leaves (100 g), which were reduced by a knife mill. They had their essential oils extracted by hydrodistillation carried out by a Clevenger-type apparatus at 100 °C for 2 h. Thereafter, the hydrolate was submitted to liquid-liquid partition in a separatory funnel. Three washes of the hydrolate were performed with three 10 mL portions of dichloromethane. Total oil yield was expressed as percentage (g/100 g fresh plant material). Essential oil samples were stored at −4 °C until further chemical and biological tests.

2.3. GC-MS analysis of essential oils

Gas Chromatography coupled to Mass Spectrometry (GC-MS) was carried out by a Shimadzu QP2010 with an AOC-20i auto-injector and a DB-5MS column (30 m x 0.25 mm, 0.25 mm in thickness). The carrier gas was He at 57.4 kPa (pressure) and at 1.0 mL/min (flow rate). The split ratio was 1/30, the injector temperature was 250 °C and the injected volume was 0.1 µL. Temperatures, which were increased at the rate of 3 °C/min, ranged between 60 and 240 °C. MS was recorded on the electron ionization (EI) mode, with ionization energy of 70 eV (scan time: 2 scan/s). Identification of volatile chemical constituents was based on their retention indices, relative to a homologous series of *n*-alkanes (C_{10}–C_{29}) and on the comparison between mass spectra and libraries (Wiley 7 and Nist 62) and references of previously published data (Adams, 2007).

2.4. Cell lines and culture conditions

In this study, three different human tumor cell lines were used: breast adenocarcinoma (MCF-7), cervical adenocarcinoma (HeLa) and glioblastoma (M059J). A normal human cell line (lung fibroblasts, GM07492A) was included to evaluate whether the natural product under investigation had selective activity. 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 a humidified 5% CO_2 atmosphere.

2.5. Antiproliferative activity assay

Antiproliferative activity was measured by the in vitro Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics), in agreement with the manufacturer's instructions. In the experiments, cells (10^4 cells/well) were plated onto 96-well microplates. Each well got 100 µL HAM-F10/DMEM medium with essential oil at concentrations ranging from 3.91 to 500 µg/mL. Negative (no treatment), solvent (0.02% DMSO, dimethylsulfoxide, Sigma-Aldrich) and positive (doxorubicin, DXR, Pharmacia Brasil Ltda.) controls were included. After incubation at 36.5 °C for 24 h, the culture medium was removed. Cells were washed with 100 µL PBS (phosphate buffered saline) to remove the treatments and then exposed to 100 µL culture medium HAM-F10 without phenol red. Afterwards, 25 µL XTT was added and cells were incubated at 36.5 °C for 17 h. Sample absorbance was determined by a multi-plate reader (ELISA – Tecan – SW Magellan vs 5.03 STD 2P) at wavelength of 450 nm and reference length of 620 nm. Antiproliferative activity was assessed with the use of IC_{50}, the concentration that was able to inhibit 50% of the cell line growth as a response parameter, which was calculated by the GraphPad Prism program by plotting cell survival against the respective concentrations of the natural product under investigation. One-way ANOVA was used for comparing means ($P \leq 0.05$). Experiments were performed in triplicate. The selectivity index was calculated by dividing the IC_{50} value of the essential oils obtained for GM07492A cells by the IC_{50} value obtained for the tumor cell line.

3. Results and Discussion

Previous studies carried out by the members of this research group reported the content and the chemical composition of essential oils extracted from *C. adamantium* and *C. calophyllum* leaves and from *P. ovatum* leaves and green fruits. Mean contents of essential oils found after hydrodistillation of *C. adamantium* and *C. calophyllum* leaves and of *P. ovatum* leaves and green fruits were 0.32%, 0.06%, 0.30% and 0.50%, respectively (Oliveira et al., 2017; Xavier et al., 2016b; Estevam et al., 2017; Estevam et al., 2018).

Major constituents identified in essential oils from *C. adamantium* leaves were spathulenol (19.27%), germacrene-B (18.27%) and β-caryophyllene oxide (12.37%) (Oliveira et al., 2017), whereas, in the ones from *P. ovatum* leaves, they were spathulenol (17.60%), β-caryophyllene (14.00%) and β-myrcene (8.40%) (Estevam et al., 2017). Major constituents of essential oils from *P. ovatum* green fruits were and β-myrcene (62.0%), α-pinene (11.3%) and limonene (7.3%) (Estevam et al., 2018), while *C. calophyllum* leaves displayed spathulenol (28.78%), viridiflorol (9.99%) and (*Z*,*E*)-pharnesol (6.51%) (Xavier et al., 2016b).

Taking into account all essential oils which were investigated by this study, the treatment with essential oils from *C. adamantium* leaves led to the lowest IC_{50} values, from 77.2 ± 8.1 to 80.5 ± 8.8 µg/mL, in all tumor cell lines (Table 1). Such values were significantly lower than IC_{50} values found in normal cell line GM07492A, whose mean selectivity index was 4.7 (Table 1). In the case of essential oils from *C. calophyllum* leaves, IC_{50} values ranged from 216.8 ± 7.9 to 353.5 ± 1.2 µg/mL and selectivity was not observed. Regarding the species *P. ovatum*, significant reduction in cell viability was found in cell line M059J in the treatment with essential oils from leaves, whose IC_{50} value was 191.3 ± 26.0 µg/mL, significantly lower than the one found against normal line GM07492A (276.1 ± 14.5), with selectivity index equal to 1.4 (Table 1). The oil from its fruits had IC_{50} values which ranged from 306.0 ± 6.2 to 583.5 ± 54.6 µg/mL, with no selective effect (Table 1).

As shown in Table 1, IC_{50} values of essential oils from *C. adamantium* leaves stood out, due to the fact that these values were lower than those found by Hussain et al. (2010) for essential oils from *Rosmarinus officinalis* leaves against cell lines MCF-7 (IC_{50} = 190.1 µg/mL) and LNCaP (IC_{50} = 180.9 µg/mL). These authors described that IC_{50} < 10 µg/mL means potentially very toxic, IC_{50} from 10 to 100 µg/mL means potentially toxic, IC_{50} from 100 to1000 µg/mL means potentially harmful and IC_{50} > 1000 µg/mL means potentially non-toxic.

The antiproliferative activity found by this study may be justified by the presence of major chemical constituents which were identified in essential oils from *C. adamantium*, *P. ovatum* and *C. calophyllum*. For instance, the compounds β-caryophyllene, β-caryophyllene oxide and germacrene-B have already been identified in essential oils from *Casearia*

Table 1. IC_{50} and selectivity index (SI) of essential oils from *C. adamantium* and *C. calophyllum* leaves and from *P. ovatum* leaves and green fruits against different cell lines.

Cell lines	*C. adamantium*		*C. calophyllum*		*P. ovatum*			
	Leaves		Leaves		Leaves		Fruits	
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI
GM07492A	369.8±15.3	-	216.8±7.9	-	276.1±14.5	-	306.0±6.2	-
MCF-7	77.2±8.1[a]	4.8	353.5±1.2	-	330.8±27.9	-	313.6±11.1	-
HeLa	80.5±8.8[a]	4.6	337.2±24.6	-	429.8±26.2	-	542.4±19.0	-
M059J	79.9±7.8[a]	4.6	311.4±31.2	-	191.3±26.0[a]	1.4	583.5±54.6	-

Doxorubicin (DXR) was used as positive control (IC_{50} values of 0.5 ± 0.2, 62.1 ± 2.0, 5.3 ± 1.3, 16.2 ± 2.5 µg/mL to GM07492A, MCF-7, HeLa and M059J, respectively). GM07492A (human lung fibroblasts), MCF-7 (human breast adenocarcinoma), HeLa (human cervical adenocarcinoma) and M059J (human glioblastoma). The selectivity index is the ratio between the IC_{50} value of the essential oils obtained for GM07492A cells and the value found for the tumor cell line. IC_{50} values are µg/mL, mean ± SD, n = 3. [a] Significantly different from the normal cell line (GM07492A) ($P \leq 0.05$).

lasiophyla leaves and have had their anticancerous activity described in the literature (Salvador et al., 2011). Bayala et al. (2014) described the anticancerous activity of the chemical constituents β-myrcene, α-pinene and limonene against several types of cancer. Essential oils from *Xylopia frutescens* leaves displayed anticancerous activity against tumor cell lines NCI-H358M and PC-3M. Viridiflorol was identified as one of their minor constituents (Ferraz et al., 2013). The terpene (*Z*,*E*)- pharnesol has already been described in the literature as a strong ally against cancer (Chagas et al., 2009). Nascimento et al. (2018) had previously observed that the IC_{50} value of spathulenol, a major constituent in essential oils from *C. adamantium* leaves, was 49.3 µg/mL against tumor cell line MCF-7. As a result, cytotoxicity of this essential oil may be attributed – at least, partially – to the antiproliferative activity of this sesquiterpene. In short, as mentioned before, the sesquiterpene spathulenol has stood out because its anticancerous activity has been identified in essential oils from several vegetal species, such as *Croton rhamnifolioides*, *Boswellia carterii* and *Commiphora pyracanthoides* (Chen et al., 2013; Vidal et al., 2016).

In sum, Li et al. (2009) highlights that the antiproliferative activity of essential oils can also be attributed to synergic effects of all terpenes that are part of the chemical composition of essential oils. There may also be other active compounds at low concentrations, responsible for the promising antiproliferative effect. Therefore, further studies to better investigate the action mechanism of essential oils from *C. adamantium*, *P. ovatum* and *C. calophyllum* against tumor cells are needed.

4. Conclusion

This study described, for the first time, the antiproliferative potential of essential oils from *C. adamantium* and *C. calophyllum* leaves and from *P. ovatum* leaves and green fruits. Results showed promising activity against tumor cell lines MCF-7, HeLa and M059J. Major chemical constituents of essential oils from *C. adamantium*, *C. calophyllum* and *P. ovatum* were the terpenes spathulenol, germacrene-B, β-caryophyllene oxide, β-caryophyllene, β-myrcene, α-pinene, viridiflorol, limonene and (*Z*,*E*)-pharnesol. The antiproliferative activity found by this study may be justified by the presence of these major constituents, since all of them have already had their anticancerous activity well described in the literature. Therefore, results of this study not only highlight *C. adamantium*, *P. ovatum* and *C. calophyllum* as potential sources in the search for new anticancerous agents but also reinforce the importance of further studies to evaluate their phytochemical, toxicological and pharmacological aspects.

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