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In vitro Antiproliferative Potential of *Celtis iguanaea* against Ovarian (OVCAR-3) and Colon (HT-29) Tumor Cell

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BZ and WARJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ALTGR and TMW managed the analyses of the study. Authors DM, DBG, GL, CADV and PZS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Natural products have been reported as a main source of anticancer molecules. The species *Celtis iguanaea* (Jacq.) Sarg., (Cannabaceae) is widely distributed in Brazil where it is known as "esporão-de-galo or taleira". The leaves are popularly used as anti-inflammatory, in the treatment of body pain and urinary infections. However, the antiproliferative potential against human cancer cells remain to be elucidated. In this study, extracts and different fractions from the leaves of *C. iguanaea* were tested *in vitro*, against a panel tumor cell lines. The hydroalcoholic extract was inactive, while dichloromethane extract showed promisor antiproliferative effects. In turn, the

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dichloromethane fraction showed potent cytostatic activity against ovarian cell line (OVCAR-3, GI_{50} = 4.65 μg/ml). However, the stronger antiproliferative effects with high selectivity were observed for the hexane fraction that exhibited activity against ovarian (GI_{50} = 3.99 μg/ml) and colon (HT-29, GI_{50} = 3.16 μg/ml). The antiproliferative effects observed are probably related to the presence of 3,7,11,15-tetramethyl-2-hexadecen-1-ol and gamma-sitosterol present in the hexane fraction and detected by GC/MS. This is the first report of antiproliferative activity of *C. iguanaea* and the results suggested that the molecules of fraction hexane are promising chemotherapeutic compounds, especially against tumor cell of ovarian and colon.

Keywords: Antitumor agents; esporão-de-galo; medicinal plants; chemotherapeutic compounds.

1. INTRODUCTION

Cancer is a complex group of diseases characterized by the rapid and disordered growth of abnormal cells (malignant) that invade the tissues and organs and can spread to other regions of the body. These cells tend to be very aggressive and uncontrollable, determining the formation of tumors or malignant neoplasms [1]. It is one of the main causes of death worldwide, been estimated more than 22 million deaths by the disease and complications of its treatment in the next two decades [2].

Anticancer agents have as aim the destruction of tumor cells, representing the main treatment at different stages of the disease [3]. However, most of these drugs have low selectivity, causing severe side effects. In addition, many cancers still exhibit modest clinical responses to the usual protocols or develop chemotherapy resistance [4]. Thus, in order to achieve more effective and safer molecules, pharmacological studies with substances isolated from plants have been intensified, as well as, synthetic derivatives from these natural compounds [5-7].

Celtis iguanaea (Jacq.) Sarg., (Cannabaceae) is a species with large geographic distribution, been present in temperate and tropical climate regions as in the United States and South America [8]. *C. iguanaea* is as a small shrub popularly known in Brazil as esporão-de-galo, sarã, taleira and gurrupiá [9-11]. In addition, the leaves of plant are popularly used to the treatment of body pain, rheumatism, asthma, cramping, dyspepsia [11], urinary infections [9] and for the control of diabetes mellitus [12].

Gastro-protective effect of the hexane fraction from *C. iguanaea* was showed [13], and no cytotoxic or genotoxic effects were founded [14-15]. Recently, the hydroalcoholic extract was effective in treatment hyperglycemia, hypercholesterolemia and consequent prevention of atherosclerosis in rats [16]. Among the chemical components, coumarins, mucilage, and flavonoids have already been described in leaves and stems of *C. iguanaea* [11]. In addition, the friedelane-pentacyclic triterpenes friedelin, and epifriedelinol were also reported [15]. The triterpenes and phytosterols are generally found in abundance in the plants and are structurally resemble to the molecule of cholesterol [17]. These compounds are recognized for their hypolipidemic, antioxidant [18] and anti-inflammatory effects [19], as well as, have been highlighted by their anticancer potential [20].

Although the leaves of the plant are popularly used to combat various diseases and some of the chemical compounds identified, the anti-proliferative activity has not yet been investigated. In this context, this study aimed to advance in the chemical study, as well as investigate the antiproliferative effects of extracts and fractions of *C. iguanaea* leaves against a panel of human tumor cells.

2. MATERIALS AND METHODS

2.1 Standards and Chemicals

All solvents used in the chromatographic and spectroscopic analyzes were of analytical grade and the water was distilled and deionized. The solvents used were ethyl acetate, methylene chloride, ethanol, hexane, methanol and *n*-butanol (Vetec®, Rio de Janeiro, Brazil).

2.2 Plant Material

The leaves of *C. iguanaea* were collected in Chapecó (SC), Brazil (27°01'55.14"S and 52°47' 29.42"O) in September 2015. The plant material was identified by Adriano Dias de Oliveira, herbarium curator of Community University of the

Region of Chapecó (Unochapecó), where a voucher specimen was deposited (#3463).

2.3 Preparation of Extracts and Fractions of *Celtis iguanaea*

The leaves of *C. iguanaea* were dried at room temperature (25 ± 5°C), pounded into knife mill (Ciemlab®, CE430), selected in tamis (425 µm; 35 Tyler/Mesch), identified and stored protected from light. Using maceration (5 days) at room temperature, dry-milled leaves of *C. iguanaea* (100 g) were extracted successively with dichloromethane and in sequence, ethanol 70% (1:20, w/v). After filtration through Büchner funnel, the dichloromethane (DEC) and hydroalcoholic (HEC) extracts were concentrated by evaporation under reduced pressure, lyophilized, weighed and stored in a freezer at -20°C.

A second sample of dry-milled *C. iguanaea* leaves (500 g) was extracted by maceration with MeOH (2 litres). After evaporation under reduced pressure and lyophilisation (38.2% yield), the methanolic extract (41.18 g) was diluted with H_2O (500 ml) and submitted to liquid: liquid partition with hexane, dichloromethane, EtOAc and *n*-butanol successively, affording after solvent removal under reduced pressure and lyophilised, the respective fractions (Hex 7.16 g; DCM 1.31 g; EtOAc 1.46 g; *n*-BuOH 1.37 g). All samples were stored in a freezer at -20°C previously to chemical and biological evaluations.

2.4 Chemical Analysis

The chemicals analysis was performed by gas chromatography coupled to mass spectrometry (GC/MS Shimadzu, QP2010 S). Aliquots (0.0010 g) of the hexane and dichloromethane fractions were diluted in dichloromethane (2 ml) and were injected (1 µl) using a split ratio of 1:40. GC–MS analysis was performed using Shimadzu QP2010S advanced standard gas chromatograph mass spectrometer (Tokyo, Japan). A DB-1 (30 m; 0.25 mm, 0.25 mm) and a dimethyl polysiloxane analytical column were used for the separation. Helium was used as the carrier gas at a flow rate of 0.80 ml/min. The injector (splitless mode, 1:40 split ratio) was maintained at 300°C. The initial column temperature was set at 80°C and kept for 1 min. It was then mounted on a ramp for 5°C min up to 190°C min, then at 20°C min 1 up to 300°C min and, finally, at 15°C min 1 up to 310°C, where it was kept for 10 min. MS detector in a scan (m/z = 29-500 Da) operating at 70 eV. and positive electric impact ionization methods were employed. The constituents were identified by comparing the obtained mass spectra with those present in the data system library (NIST version 8.0) with the values described in the literature [21-22].

2.5 Antiproliferative Assay

The antiproliferative effects of dichloromethane (DEC) and hydroalcoholic (HEC) extracts, and fractions was investigated using the protocol previously described [23]. A panel of nine human cancer cell lines [U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype multiple drugs resistance), 786-0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-03 (ovarian), HT-29 (colon adenocarcinoma) and K-562 (chronic myeloid leukemia)], kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA, and one immortalized human cell line (HaCat, keratinocyte) provided by Dr. Ricardo Della Coletta (University of Campinas) was used. Stock and experimental cultures were grown in complete medium [RPMI-1640 supplemented with 5% fetal bovine serum and 1% penicillin: streptomycin mixture 1000 U/ml: 1000 µg/ml]. Stock samples solution was prepared in DMSO (0.1 mg/ml) followed by successive dilutions in complete medium affording the final concentration of 0.25, 2.5, 25 and 250 µg/ml. Doxorubicin, at final concentrations of 0.025, 0.25, 2.5 and 25 µg/ml, was used as positive control. Cells in 96-well plates (100 µl cells/well, cell densities: 3 to 7 x 10-4 cells/ml) were exposed to the four concentrations of samples and control (100 µl/well) in triplicate, for 48 h at 37°C and 5% of CO_2. Before (T0 plate) and after (T1 plates) sample addition, cells were fixed with 50% trichloroacetic acid (50 µl well) and submitted to sulforhodamine B assay for cell proliferation quantitation at 540 nm. The analysis was from a single experiment with experimental triplicate. The GI_{50} (concentration that produces 50% cell growth or cytostatic effect) values were determined through non-linear regression, type sigmoidal, using Origin 8.0 software (OriginLab Corporation). The selectivity index (SI) was calculated as presented in Equation 1.

$$SI = GI_{50}\ HaCaT / GI_{50}\ \text{tumor cell line} \quad (1)$$

3. RESULTS AND DISCUSSION

Several molecules extracted from natural products are used for the treatment of cancer. These compounds, including alkaloid, diterpenoid, flavonoids, sesquiterpenes lactones, and polyphenolic were widely tested and demonstrated properties against multiple types of cancer with effects in numerous molecular targets in both cell culture and animal models. [7]. In this work, was evaluated the antiproliferative potential of the extracts and fractions of the *C. iguanaea* leaves besides of analyses in the chemical composition. The better results were observed in the apolar fractions, especially against ovarian (OVCAR-3) and colon (HT-29) human tumor cells lines. To evaluate the results obtained, we assumed the National Cancer Institute (NCI) criteria described by Fouche et al. [24] that consider as a promising antiproliferative sample that one with $GI_{50} \leq$ to 30 µg/ml.

In the Fig. 1 is represented the antiproliferative effects of hydroalcoholic (HEC) and dichloromethane (DEC) extracts from leaves of *C. iguanaea*. HEC (Fig. 1a) was inactive (GI_{50} > 250 µg/ml). On the other hand, the DEC (Fig. 1b) showed moderated effect antiproliferative against ovarian (OVCAR-3), lung (NCI-H460), and glioma (U-251) (GI_{50} = 28.46, 32.31 and 37.99 µg/ml, respectively) (Fig. 1 and Table 1).

In a bioguided way the four fractions obtained of partitioning of the methanolic extract from *C. iguanaea* (hexane, dichloromethane, ethyl acetate, and *n*-butanol), also were evaluated against tumor cells. The *n*-butanol and the ethyl acetate fractions were inactive while the dichloromethane fraction showed important cytostatic effect against ovarian cell (OVCAR-3, GI_{50} = 4.6 µg/ml). However, the better effects were observed to the hexane fraction that showed a moderate activity against glioblastoma (U-251, GI_{50} = 6.40 µg/ml), ovarian (OVCAR-3, GI_{50} = 3.99 µg/ml), and colon (HT-29, GI_{50} = 3.16 µg/ml) human tumor cell (Table 2).

In this study was possible observed that the antiproliferative effects of *C. iguanaea* leaves were more representative in the extract and fractions of reduced polarity. This may partly explain the higher affinity and better permeation across cellular membranes by lipophilic compounds [25]. In addition, the compounds presented in the extract and fractions of low polarity showed also, effects on different cell lines. The dichloromethane fraction moderately inhibited the OVCAR-3 growth while the hexane fraction showed a similar activity against glioblastoma, ovarian and colon tumor cell lines [24].

It is important to highlight that one of the main problems related to the classic treatment with chemotherapy is the low selectivity, which can promote numerous adverse reactions in the organism. Thus, Suffness and Pezzuto [26] proposed the calculation of the selectivity index (SI = IC_{50} HaCat/IC_{50} tumor cell line) for the prospection of antiproliferative compounds. Thereby, it is possible to verify the activity of a compound on tumor cells compared to its effect on normal cells. The extracts or

Table 1. Antiproliferative effect of hydroalcoholic (HEC) and dichloromethane (DEC) extracts from *Celtis iguanaea* leaves against different cell lines

Cell lines	GI_{50} (µg/ml)		
	HEC	DEC	DOXO
U-251	*	37.9	0.26
MCF-7	*	71.9	0.16
NCI/ADR-RES	*	119.3	0.31
786-0	*	47.0	0.04
NCI-H460	*	32.3	0.10
PC-3	*	66.6	0.78
OVCAR-3	62.3	28.4	1.07
HT-29	*	72.3	1.20
K-562	*	54.2	0.38
HaCat	*	42.0	0.25

*Note: Doxorubicin (DOXO). Human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), kidney (786-O), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); Human immortalized keratinocyte (HaCat). GI_{50} = 50% growth inhibition. * effective concentration higher than the highest tested concentration (250 µg/ml).*

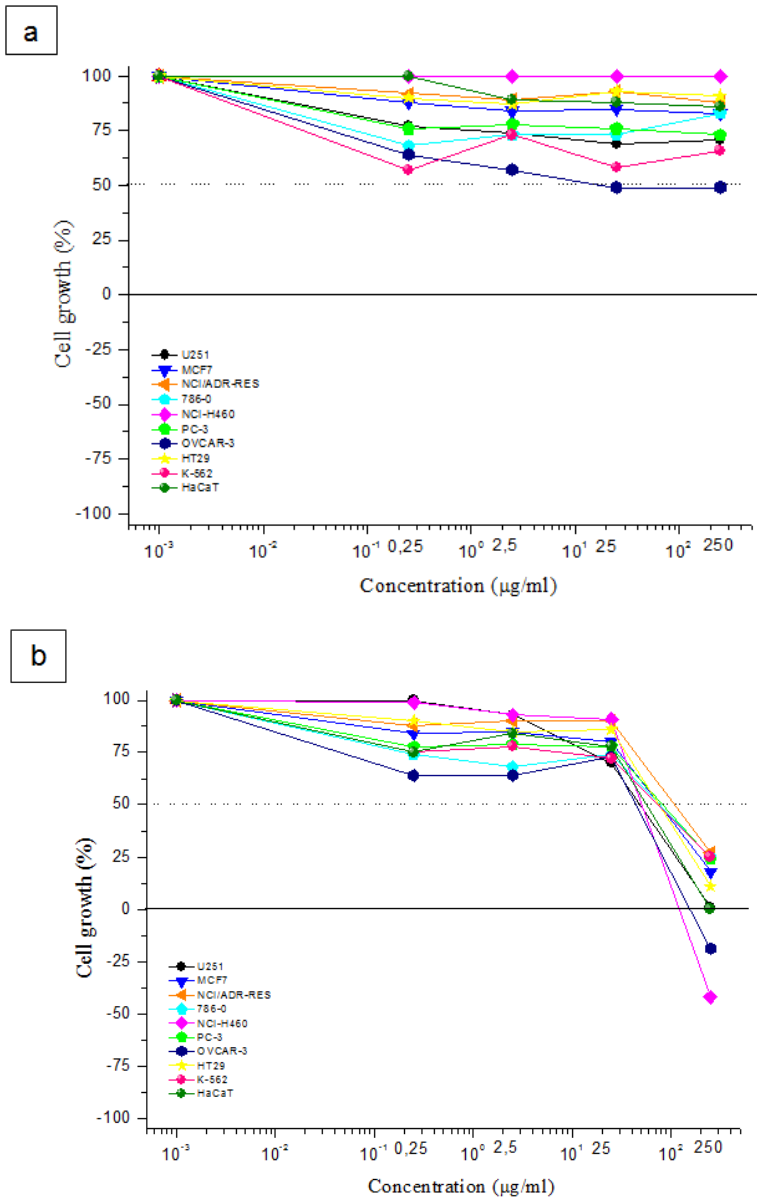

Fig. 1. *In vitro* antiproliferative effects of extracts from *Celtis iguanaea*. A: Hydroalcoholic extract (HEC); B: Dichloromethane extract (DEC)

Note: Concentration range: 0.25 - 250 µg/ml; exposition time: 48 h; human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), 786-O (kidney), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); human immortalized cell line: keratinocytes (HaCat)

molecules with IC equal to or above 2 exhibit significant selectivity. In this work, the hexane fraction showed high selectivity index (12.10 and 9.58) against HT-29 and OVCAR-3, respectively. For the same cell lines, doxorubicin (chemotherapy, positive control) presented values of 0.29 and 0.03, respectively. Furthermore, the dichloromethane and hexane fractions are strong candidates to the prospection of new antitumor molecules.

Based on these results, we analyze the chemical composition of the dichloromethane and hexane fractions by GC-MS. The mass fragmentation profile revealed comparatively, the presence of 3,7,11,15-tetramethyl-2-hexadecen-1-ol and

gamma-sitosterol in both fractions together with phytol and methyl esters of palmitic and stearic acids in hexane fraction (Table 3).

The hexane fraction (more active and selective) demonstrated 4 and 5 times more gamma-sitosterol and 3,7,11,15-tetramethyl-2-hexadecen-1-ol, respectively, than the ethyl acetate fraction (inactive). This chemical analysis may justify in part the antiproliferative effects, since it is known that the gamma sitosterol belongs to the phytosterol group, and this molecules is recognized for their beneficial effects on health such as reduction in plasma cholesterol levels, anti-inflammatory effects and anticancer potential [27].

The gamma sitosterol has already been described as cytotoxic against colon, liver, breast and lung human tumor cell lines [28,29]. The pharmacological mechanism was attributed to cell cycle arresting and apoptosis induction [29-31]. Besides, there are evidences that the phytosterols were able to reduce also, the cancer risk of prostate and ovary. Several mechanisms such as inhibition of carcinogen production, stimulation of apoptosis, and induction of the sphingomyelin cell cycle have been suggested as accounting for the anticarcinogenic effects of these compounds [32]. One phytosterol well documented as anticancer agent against prostate, breast and colon cancers is the beta sitosterol (BS) [33].

Interestingly, recent reports suggested that anticancer mechanism of BS is similar to the activity of colchicine in binding preferentially on β-tubulin isotypes such as βII and βIII in the αβ-tubulin dimer [34]. Furthermore, it was demonstrated that BS induced apoptosis by scavenging reactive oxygen species and suppressed the expression of β-catenin and PCNA antigens in human colon cancer cells [35]. Also, BS induced apoptosis by down regulation of the pro-apoptotic protein Bcl-2 and activation

Table 2. Antiproliferative effect of fractions obtained from *Celtis iguanaea* against different cell lines

Cell lines	GI_{50} (µg/ml)				
	Fractions				DOXO
	Hex	Dic	EtOAc	B	
U251	6.4	25.8	167.6	*	<0.025
MCF-7	58.0	27.8	196.6	*	<0.025
NCI/ADR-RES	32.5	27.9	*	*	0.078
786-0	30.2	25.7	*	*	0.025
NCI-H460	87.4	45.0	*	*	<0.025
PC-3	85.9	27.8	*	*	0.09
OVCAR-3	3.9	4.6	77.7	*	0.09
HT-29	3.1	38.9	*	*	0.10
K-562	27.8	28.4	64.4	*	0.11
HaCat	38.2	27.5	*	*	0.03

*Note: Hexane fraction (Hex), dichloromethane fraction (Dic), ethyl acetate fraction (EtOAc), n-butanol fraction (B), doxorubicin (DOXO). Human tumor cell lines: glioblastoma (U-251), breast (MCF-7), ovarian expressing the resistance phenotype (NCI/ADR-RES), kidney (786-O), non-small cells lung (NCI-H460), prostate (PC-3), ovarian (OVCAR-3), colon (HT-29), leukemia (K-562); Human immortalized keratinocyte (HaCat); GI_{50} = 50% growth inhibition. *effective concentration higher than the highest tested concentration (250 µg/ml)*

Table 3. Compounds identified in the hexane and dichloromethane fractions of *Celtis iguanaea* leaves by GC/MS

RT (min)	Compound*	Molecular formula	Relative amount (%)	
			HEX	DCM
7.24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	30.25	5.49
7.53	Palmitic acid, methyl ester	$C_{17}H_{34}O_2$	10.97	-
8.30	Phytol	$C_{20}H_{40}O$	6.11	-
8.30	Stearic acid, methyl ester	$C_{19}H_{38}O_2$	5.09	-
14.09	Gamma-sitosterol	$C_{29}H_{50}O$	30.25	7.19

**By comparison of mass fragmentation profile with NIST library (version 8.0), similarity indices ≥ 90%. HEX = hexane fraction; DCM = dichloromethane fraction*

of caspase 3 in leukemia cells (U937) [36], while in in MDA-MB-231 cells (breast tumor), the apoptosis induction involved upregulation of bax/bcl-2 ratio, down regulation of IAP family and caspase activation [37]. Overall, phytosterols are reported to have multi-target action with immense anticancer potential modulating key signaling pathways that are implicated in various types of cancer [32,35,38]. These compounds interfere in different ways in the cell cycle and apoptosis pathways [39].

Based on this, our study demonstrated that the apolar fractions of *C. iguanaea* showed a strong anticancer potential against ovarian and colon cells, whose effects may be related to phytosterols found in these fractions and whose mechanisms of action have been widely studied and proven [40].

4. CONCLUSION

Hexane and dichloromethane fractions of *C. iguanaea* presented a strong antiproliferative effect against colon (HT-29) and ovarian (OVCAR-3) tumor cell lines. The biological effects observed appear to be partly related to the presence of gamma-sitosterol. Based on these findings, we predict that these fractions would be good candidates to search for novel bioactive components with antiproliferative activity.

CONSENT

Is not applicable

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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