In vitro cytotoxicity of lignans isolated from *Ficus citrifolia* P.Miller (Moraceae) on C6 glioma cell line

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Matairesinol and arctigenin, two dibenzylbutyrolactonelignans, were isolated by chromatographic analysis of the CH₂Cl₂ extract from the leaves of *Ficus citrifolia*. Structural identification was performed using typical spectroscopic methods, NMR ¹H and NMR ¹³C, in addition to DEPT, NOE-diff, HETCOR, COSY ¹H x ¹H and HMBC, confirmed by literature comparison. Furthermore, to date, no reports have described the phytochemical investigation of *Ficus citrifolia*. Previous studies have suggested that dibenzylbutyrolactonelignans exert a preventive effect against different type of cancers such as breast, prostate and colon. In this study, we analyzed the cytotoxic effects of matairesinol and arctigenin and the induction of apoptosis of C6 rat glioma cells. These compounds produced a dose-dependent reduction in the viability of C6 cells, as determined by an MTT assay and were not cytotoxic against astrocyte cell cultures from rat cerebral tissue. For ultrastructural analysis, C6 cells were incubated with 50 µM and 250 µM of arctigenin and matairesinol. Treated cells presented altered morphology, condensation of nuclear chromatin into irregular clumps, swollen mitochondria, and disorganized internal-membrane cristae. However, the nuclear and plasma membranes remained intact. These morphological changes are characteristic of apoptotic cellular death. In addition, these treatments did not significantly affect astrocyte viability. Thus, these results suggest that both dibenzylbutyrolactonelignans have significant anti-proliferative and apoptotic effects on glioma cells and may contribute as selective agents against brain cancer.

**Keywords:** *Ficus citrifolia*; Moraceae; dibenzylbutyrolactonelignan; matairesinol; arctigenin; cytotoxicity, glioma cells (C6).
INTRODUCTION

Ethnopharmacological studies have identified many natural products with significant biological activity that could benefit novel drug discovery and development. It is reasonable to consider that a survey of ethnomedical uses of a plant may provide useful clues for drug discovery. Phytochemical studies are the basis for drug development and are sometimes associated with use for a particular illness, in this case glioblastoma, and could have an important socioeconomic value (Paavilainen, 2005). The genus Ficus, better known as the fig, has been cultivated for over 11,000 years, possibly pre dating cereal grains (Kislev et al., 2006). Ficus consists of approximately 800 species and is one of approximately 40 genera of the mulberry family, Moraceae (Carauta, 1989; Woodland, 1997). The species of the genus are distributed in the tropical and subtropical regions in the world, and 64 are referred to Brazil (Van den Berg, 1982). Plants of this genus are used by the population of the Amazon region to combat numerous maladies, and the leaves and latex are the most commonly used parts (Van den Berg, 1982). This work describes the first phytochemical study of the species Ficus citrifolia (Moraceae). Three previously known dibenzylbutyrolactonelignans were isolated from the dichloromethane extract of leaves: matairesinol (1), arctigenin (2) and arctiin (3).

These substances have been isolated and purified using conventional chromatographic techniques, and structural identification was performed using spectroscopic methods, including NMR $^1$H and NMR $^{13}$C, in addition to DEPT, NOE-diff, HETCOR, COSY $^1$H x $^1$H and HMBC. The structures were also confirmed by comparison with literature data. Previous studies suggest that dibenzylbutyrolactonelignans have a preventive effect against hormone-dependent cancers such as breast, prostate and colon (Adlercreutz, 2002) and are potent cytostatic agents against human leukemia cells HL-60 (Hirano et al., 1994); exhibit antiviral activity, being potent inhibitors of HIV type-1 Integrase (Eich et al., 1996; Yang et al.,1996); act as phytoestrogens (Kato et al., 1998; Meagher et al, 1999) and exhibit immunoregulatory (Cho et al., 1999); neuroprotective (Jang et al., 2001; Jang et al., 2002), antioxidative (Willfö r et al., 2003; Jin et al., 2005), anti-inflammatory (Cho et al., 2002; Jin et al., 2005), anti-asthmatic (Lee et al., 2011) and hypolipidemic properties (Charlton, 1998; Ward, 1999). Therefore, in the present study, we have investigated the effect of matairesinol (1) and arctigenin (2) on the viability of C6 rat glioma cells, primary tumors of the brain that are difficult to treat due to common recurrence (Jovčevska et al., 2013). These data were compared with astrocytes from the cortex of rats used as a control.

MATERIALS AND METHODS

Reagents

MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide), penicillin, streptomycin, trypsin and EDTA were obtained from Sigma Chemicals (St. Louis, MO), CH$_2$Cl$_2$,MeOH, Silica gel, HCl, isopropanol purchased from MERCK, glutaraldehyde type II, paraformaldehyde, sucrose, sodium cacodylate, osmium tetroxide, uranyl acetate, acetone and Epon resin were purchased from Electron Microscopy Sciences (EMS). DMEM F-12 and fetal bovine serum were obtained from Gibco.

General experimental procedures

NMR spectra were recorded in CDCl$_3$ on a Varian Mercury spectrometer 300 (300 MHz for $^1$H and 75 MHz for $^{13}$C NMR) using tetramethylsilane (TMS) as internal standard. Splitting patterns are described as singlets (s), doublets (d), double doublets (dd), and multiplets (m). Proton and carbon shifts are reported in δ units (ppm) relative to TMS. Column chromatography was performed with Silica gel 60 H (70-230 mesh/ Merck), TLC was performed with Silica gel GF$_{254}$ (Merck) and preparative TLC was performed with Silica gel PP-254. Cytotoxic activity was determined by spectrophotometry (SP-2000 uv-Spectrum). Cells were analyzed using transmission electron microscopy (TEM - Zeiss LEO 906E).

Plant material

Leaves of Ficus citrifolia (Moraceae) were collected in the forest of EMBRAPA, Pará State, Brazil and identified by the Botanic Department of EMBRAPA. A voucher specimen (n° 164987) has been deposited in the Herbarium of the CPATU-EMBRAPA, Pará State, Brazil.

Extraction and isolation

Dried leaves (3.1 kg) of Ficus citrifolia were powdered and successively extracted with n-C$_6$H$_{14}$, CH$_2$Cl$_2$ and MeOH for 72 h at room temperature. Solvent was evaporated in a vacuum to give crude extracts. The CH$_2$Cl$_2$ extract (20.0 g) was subjected to column chromatography over silica gel 60 H (70-230 mesh/Merk) using n-C$_6$H$_{14}$, EtOAc and MeOH as eluents, containing gradually increased amounts. This yielded 56 fractions that were recombined based on thin layer chromatographic analysis. Fractions 58-63 were purified by preparative TLC [Silica gel PP-254, n-C$_6$H$_{14}$, EtOAc-MeOH (7:2.5:0.5)] in two consecutive elutions. The chromatography techniques applied resulted in the isolation 49.2 mg of the dibenzylbutyrolactonelignan,
Famatresinol (1). Fractions 28-29 (3 g) were rechromatographed in a silica gel 60 H (70-230 mesh/Merck) column using n-C8H16, EtOAc and MeOH. After analysis of all 120 fractions, fractions 48-53 (306.7 mg) were rechromatographed using CH2Cl2 and MeOH as eluents; eluting with gradually increased amounts of solvent resulted in the isolation of 52.3 mg of the dibenzylbutyrolactone lignan, arctigenin (2). Fractions 39-43 were rechromatographed using a silica gel 60 H (70-230 mesh/Merck) column. The column was eluted with n-C8H16, CH2Cl2 and MeOH, resulting in the isolation of 18.2 mg of the dibenzylbutyrolactone lignan, arctiin (3).

Cell cultures and drug treatment

The rat C6 glioma cell line was originally derived from N-nitrosomethylurea-induced rat brain tumors (Jacobs et al., 2011; Chesler et al., 2012). C6 cells were seeded in 24-well culture plates at 5 x 10⁴ cells per well and maintained in Dulbecco’s modified Eagle medium (DMEM F-12) supplemented with 10 % fetal bovine serum (FBS) (2.5 mL/L penicillin and 2.5 µL of streptomycin). Incubation was carried out at 37 ºC in a humidified atmosphere of 5 % CO2 and 95 % air. After 24 h of incubation, the medium was discarded and replaced with medium containing different concentrations (50, 100, 250 µM) of matairesinol and arctigenin dissolved in 0.1 % DMSO. Control cells were treated only with 0.1 % DMSO and incubated at 37 ºC in a humidified atmosphere of 5 % CO2 for 72 h. After that, the cells were washed with phosphate buffered saline (PBS) and incubated with 0.5 µL MTT (0.5 mg/mL) and 500 µL PBS for 4 h. The formazan crystals were solubilized with 50 µL of 0.04N HCl in isopropanol, and the plates were measured using a spectrophotometer at 570 nm. Each experiment was repeated three times in quadruplicate samples.

Ultrastructural analysis of C6 glioma cells by TEM

Ultrastructural analysis of C6 cells was performed as previously described (Rodrigues et al., 2011). Briefly, C6 cells were incubated with 50 µM and 250 µM of arctigenin and matairesinol for 72 h at 37 ºC. After that, the controls and treated cultures were washed in PBS and fixed with 2.5 % glutaraldehyde and 4 % freshly prepared formaldehyde in a buffer solution containing 60 mM Pipes, 20 mM Heps, 10 mM ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 70 mM KCl and 5 mM MgCl2, pH 6.9, for 1 h at room temperature. Subsequently, cells were washed in the same buffer and post-fixed in a solution containing 1 % osmium tetroxide, 0.8 % ferrocyanide, and 5 mM calcium chloride for 1 h. Cells were washed, dehydrated in graded acetone and embedded in Epon ®. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss LEO 906E TEM.

Statistical analysis

Data were presented as the mean ± standard deviation (SD), and One-way ANOVA followed by the Bonferroni post-hoc test compared the differences between experimental groups. P-values <0.05 were considered statistically significant. All statistical analyses were performed using the application BioEstat 4.0.

RESULTS

Structure elucidation

The structures of compounds 1, 2 and 3 (Figure 1) were elucidated by 1H- 13C NMR, HETCOR, 1H-1H COSY, DEPT, NOE-diff and HMBC, and their spectral data were compared with published data (Rahman et al., 1990). Here, we phytochemically investigated the dichloromethane extract of leaves from Ficus citrifolia by chemical characterization of three lignans present: matairesinol, arctigenin, and arctiin. These three compounds are dibenzylbutyrolactone-type lignans that were isolated for the first time in the genus Ficus. The chemical profile of Ficus citrifolia shares similarity with members belonging to the Moraceae family, indicating that this study has contributed to a better understanding of the genus beyond the knowledge of the chemical composition of these species.
Cytotoxicity of matairesinol and arctigenin to C6 glioma cells and rat cortical astrocytes

Viability of rat C6 glioma cells was assessed using an MTT assay after incubation with matairesinol and arctigenin at different concentrations (50 µM, 100 µM, and 250 µM) for 72 h as shown in Fig 2. The lignans matairesinol and arctigenin reduced glioma cell viability in a dose-dependent manner (IC₅₀ = 125 µM for matairesinol and IC₅₀ = 50 µM for arctigenin).

Under the same conditions, we studied whether the effect of drug exposure is time-dependent at a drug concentration of 250 µM during treatment intervals of 18 h, 36 h, 54 h and 72 h as shown in Fig 3. Cells treated with matairesinol showed a 24 % reduction in cell viability after 18 h, a 41 % reduction in viability after 36 h, a 69 ± 3.6 % reduction in viability at 54 h, and an 81 ± 1.8 % reduction in viability after 72 h. Cells treated with arctigenin showed a 23 % ± 6.0 reduction in viability after 18 h, a 60 ± 6.0 % reduction in viability after 36 h, a 79 ± 3.0 % reduction in viability at 54 h, and an 85 ± 1.0 % reduction in viability after 72 h.

Astrocyte cell cultures from rat cerebral tissue were used as positive controls to evaluate the cytotoxicity of matairesinol and arctigenin at the same concentrations used in previous experiments. It was possible to compare the effects produced by matairesinol (Figure 2) and arctigenin (Figure 3) in C6 rat glioma cells and
Figure 3. Cell viability of C6 cells treated with matairesinol and arctigenin at different time intervals. Cytotoxic effects were evaluated using an MTT assay following 72 h treatment with 250 µM lignan. * p <0.05 compared to control (ANOVA, followed by the Bonferroni post-test).

Figure 4. Cell viability of rat astrocytes treated with matairesinol and arctigenin at different concentrations. Cytotoxic effects were evaluated using an MTT assay following 72 h treatment with 50, 100 and 250 µM lignan. * p <0.05 compared to control and # p <0.05 compared to astrocytes treated in the same concentration (ANOVA, followed by the Bonferroni post-test).

astrocytes from rats (Figure 4). Astrocyte cells used as controls revealed resistance to matairesinol and arctigenin treatment.

Ultrastructural analysis of C6 glioma cells treated with matairesinol and arctigenin

Ultrastructural analysis showed significant morphological changes in drug-treated cells when compared to untreated cells. Untreated cells are shown in Figure 5A and exhibited well-preserved ultrastructural morphology and elongated shapes. In this case, glioma cells exposed to UV were used as positive controls (Figure 5B) and exhibited rounded shapes. Cells treated with 50 µM and 250 µM of arctigenin (Figures 5C and D, respectively) exhibited rounded shapes; the nucleus showed irregular chromatin condensation, and swollen and disorganized mitochondrial internal membranes were frequently observed (Figures C-D – inset, arrowhead). Cells treated with 50 µM of matairesinol also exhibited rounded shapes similar to what was observed in UV-exposed cells (Figure 5E), and intense disorganized mitochondrial internal membranes were also seen (Figure 5E – inset, arrowhead). However, when cells were treated with 250 µM of matairesinol, few cells maintained their elongated shape (Figure 5F) but with several autophagic vacuoles (Figure 5F – inset, arrow).
DISCUSSION

Phytochemical investigation of the dichloromethane extract of the leaves of *Ficus citrifolia*, was chemically characterized by the presence of three lignans, matairesinol, arctigenin and arctiin. These three compounds are lignans type dibenzylbutyrolactone and were isolated for the first time in the genus *Ficus*. The chemical profile of *Ficus citrifolia* share similarity with members belonging to the family Moraceae, indicating that this study has contributed to better understanding of the genus, beyond the knowledge of the chemical composition of this species.

Compounds obtained from plants offer perspectives toward the discovery of new active and promising compounds with anticancer activity (Zhang and Liu, 2015; El-Mesallamy et al., 2016). Arctigenin and matairesinol have shown a vast range of biological properties...
(Adlercreutz, 2002; Jin et al., 2005). Previous studies have shown that arctigenin protects neural and neuroblastoma cells when treated with H89 via down regulation of the CREB pathway (Zhang et al., 2013). In addition, it is possible that arctigenin and matairesinol as phytoestrogens produce a cytotoxic effect against glioma cells that can be explained by the direct interaction with estrogen receptors in these cells. Similarly, other studies have shown evidence of a possible protective role of female sex hormones against glioma cells (Kabat et al., 2010) and it has recently been shown effects of arctigenin on glioma cells (Maimaitili et al., 2017). However, to date, no reports have described morphological alterations in glioma cells that occur in response to matairesinol and arctigenin treatment.

In the present report, we investigated the effect of arctigenin and matairesinol on proliferation and cellular viability of rat glioma cells culture (C6 cells). We also evaluated the citotoxicity of these compounds against astrocytes cell cultures from rat cerebral tissue. Results showed that 50 µM of arctigenin and 125 µM matairesinol reduced approximately 50% on C6 cells proliferation. Thus, the results showed that arctigenin was more potent than matairesinol regarding of cytotoxicity against glioma cells. Similar concentration of these molecules was not cytotoxic against astrocytes cell culture used as positive control. In contrast to glioma cells, primary rat astrocytes revealed a striking resistance in the same conditions of treatment against arctigenin and matairesinol.

One of the novel findings of the present study is the morphological alteration of cells induced by these lignans. Electron microscopy techniques can be a useful tool in drug studies for the identification of cell surface and ultrastructural alterations, as well as specific organelle targets on the glioma cells. Indeed, identification of morphological changes can help elucidate the mechanism of drug action (Rodrigues et al., 2011). Treatment of C6 cells with 50 µM or 250 µM of arctigenin and matairesinol induced changes in cell morphology and irregular chromatin condensation. For the last ten years, many studies have shown that induction of apoptosis using medicinal plants may provide efficient and therapeutic strategies to suppress cancer cell growth (Formisano et al., 2012; Seo et al., 2011; Solak et al., 2014). On the basis of these considerations, arctigenin and matairesinol demonstrated a cytotoxic effect on C6 cells, inducing cellular death by apoptosis. Apoptosis is a specialized mode of cell death finely regulated at the molecular level and conserved throughout evolution (Seo et al., 2011). Apoptotic cells usually exhibit similar morphological changes including cell shrinkage, nuclear chromatin condensation and DNA fragmentation. In order to maintain homeostasis of multicellular organisms, a strategic intracellular program is initiated, ensuring the fate of unwanted cells. Interference with this program has been implicated in many diseases, including cancer and autoimmune diseases. Therefore, in the search for anticancer drugs, it is very important to find a compound with the ability to induce cellular death by apoptosis. Arctigenin and Matairesinol have significant effects on proliferation and death of C6 glioma cells and may contribute as selective agents against brain cancer. Furthermore, these lignans extracted from Ficus citrifolia show potential for chemotherapeutic and/or chemo preventive usage, representing a promising natural alternative source against brain cancer.

CONCLUSION

In conclusion, data presented in this study demonstrate that Ficus citrifolia represents an attractive source of secondary metabolites, specifically lignans such as arctigenin and matairesinol, which effectively promote anticancer activity. These substances induce apoptosis and ultrastructural alterations in glioma cells but do not affect rat astrocytes used as controls.

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