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ORIGINAL ARTICLE

Leaf anatomical-histophytochemical study and evaluation os the cytotoxicity of *Trigynaea Oblongifolia schltdl* (Annonaceae)

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Abstract: Research has shown the antitumor potential of many species of the Annonaceae family. This is the first scientific investigation of the antitumor action of *Trigynaea oblongifolia* and the first anatomical-histophytochemical study of this species. This study aimed to investigate the cytotoxic effect of *T. oblongifolia* Schltdl (Annonaceae) extract on U937 leukemic cells and COLO-205 colorectal cells lines as well as phytochemical prospecting and antioxidant capacity of the extracts, leaf micromorphology, histoanatomy and histochemical analysis. Leaf micromorphology and histoanatomy were detailed and recorded by optical and transmission and scanning electron microscopy. The phytochemical analysis of the ethanolic extract of the *T. oblongifolia* leaves was performed and the chromatographic process, mobile phases, developers and specific reference standards were used for each of the groups of secondary metabolites. The species exhibited a dose-dependent viability and apoptotic effect on leukemic and colorectal cancer cells at all tested leaf extract concentrations. The histochemical results reinforce that this plant provides a new source for secondary metabolite classes: alkaloids, anthraquinones, coumarins, polyphenols, saponins, flavonoids and terpenes, which may be able to induce apoptotic death of cancer cells. The phenolic compounds present in the fractions of the sample contribute to its antioxidant capacity, as observed for other Annonaceae. Scientific studies can be directed to investigate the interference of different environments in which *T. oblongifolia* is adapted, the histochemical and anatomical foliar variations and the relation with the phytochemical bioactivity of this Annonaceae on U937 leukemic cells and COLO-205 colorectal cells as well as other human tumor cell lines. Keywords: Phytochemical, Cytotoxicity, Leukemia, Colorectal cancer.

INTRODUCTION

Leukemia is among the ten most common cancers in number of deaths worldwide and most of the currently used chemotherapy drugs for cancers are known to develop resistance and restrictions by dose-limiting side effects. Colorectal cancer is the fourth leading cause of cancer-related deaths worldwide [1-4]. Most of the currently used chemotherapy drugs for cancers are known to develop resistance and restrictions by dose-limiting side effects. On the other hand, plants are an important source of bioactive phytoproducts, which may serve as commercially significant products in themselves or which may provide prominent structures for the development of modified derivatives possessing enhanced activity and/or reduced toxicity in cancer treatment [5,6]. The *WHO Traditional Medicine Strategy 2014-2023* aims

to develop proactive practices and implement action plans that will strengthen the role that traditional and complementary medicine plays in keeping populations healthy [7].

Plant-derived bioactive compounds, especially polyphenols, have shown to reduce tumorigenesis, preventing metastasis and/or increasing chemotherapy and radiotherapy efficacy. They have been reported to interfere in the initiation, promotion and progression of cancer by modulating different enzymes and receptors in signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis, metastasis and reversal of multidrug resistance [8-16]. Both whole plant extracts and bioactive phytochemicals, particularly polyphenols, derived from several medicinal plants, have exhibited cytotoxic activities in leukemia [17-19] and colorectal neoplasias [14,19-21].

The Annonaceae family has approximately 110 genera and 2400 plant species [22] distributed mainly in tropical areas and exhibit potential source of drugs based on the use of some species in traditional medicine [23-25], many still with unknown biological activity [26]. Many species exhibit groups of secondary metabolites with highly significant pharmacological potential, such as iridoids, anthraquinones, triterpenes, alkaloids, flavonoids, phenolic derivatives and terpenoids, to name a few major ones [9,27,28] with molecular mechanisms of the biochemical pathways that trigger the cell death investigated [11,29]. The diversity of Brazilian biomes indicates great potential for promising scientific studies of numerous anonaceous plants, many recently discovered and endemic to Brazil [26, 30-32].

Research has shown the *in vivo* and *in vitro* antitumor potential of many species of the Annonaceae family [33-36]. Trigynaea (Tribe Bocageeae) is a genus with only eight species described, now adding a new species, Trigynaea (*T. flagelliflora*), recently discovered in Brazil, endemic to the Brazilian Atlantic Forest [31]. This is the first scientific investigation of the antitumor action of *Trigynaea oblongifolia* and the first anatomical-histophytochemical study of this genus. The present study was structured to investigate the anticarcinogenic potential of *T. oblongifolia* leaf extract on leukemic and colorectal tumor human cells, leaf micromorphology and anatomy and the preliminary leaf histo-phytochemical evaluation of this species.

MATERIAlS AND METHODS

Plant Material

The botanical material was collected in a region in the Atlantic Forest in the ombrophyla vegetation in red-yellow latosol on the Santa Rita farm located in the Rio Paraíba do Sul watershed, in the West Forest Zone of the State of Minas Gerais (20º 46' S and 42° 02' W). The annual temperature varies from 16 °C to 28 °C and the annual average rainfall is 2,268 mm.

Leaves of adult specimens of *Trigynaea oblongifolia Schltdl* (Annonaceae) collected with the aid of pruning shears were duly identified by researcher L.S. Leoni. Voucher specimen was deposited in the Guido Pabst - GFJP herbarium, Itaperuna - RJ, Brazil, registered under GFJP-943.

Optical Microscopy

Fragments of the leaves of the selected species were placed in aqueous solution containing 2.5% glutaraldehyde and 4.0% formaldehyde diluted in 0.05 M of sodium cacodylate buffer, pH~7.2, at room temperature for 2 h. After washing the fragments for 45 min in the same buffer, they were then placed in an aqueous solution containing 1% osmium tetroxide diluted in 0.05 M of sodium cacodylate buffer, pH~7.2, at room temperature for 1 hour

with the absence of light. After three 45-minute washes in the same buffer, the fragments were submitted to an ascending ketone series [50%, 70%, 90%, 100% (3x)] for 1 hour at each step for dehydration. Next, the fragments were infiltrated with epoxy resin (Epon Polibed™), using an increasing resin series in propanone. Polymerization of the resin was performed at 60 °C. Semi-thin sections (1 µm thick) were obtained with ultramicrotome glass cutters (Reichert Ultracut-S®). The staining was performed with a 1% toluidine blue aqueous solution, plus 0.1% of Borax. Permanent blades were assembled with Entellan® for optical digital documentation (Axioplan Zeiss Cannon Power Shot 14 mpixel; Oberkohen, Germany).

Transmission electron microscopy

Ultra-thin sections (~70 nm thick) were obtained using a diamond knife (Diatome®) in ultramicrotome (Leica Reichert Ultracut-S®, Germany), collected in 300 mesh copper grids. The staining was performed in saturated aqueous solution of uranyl acetate (40 min) followed by a washing in distilled water (5 min) in 1% lead citrate [37]. The images were obtained through transmission electron microscopy (Zeiss® EM900) under an accelerating voltage of 80 kV, with the aid of the iTEM (Olympus®) program.

Scanning electron microscopy

After dehydration, the samples were dried by the CO_2 critical point method (Bal-Tec® SCD-050 Sputter Coater, Liechtenstein). Dry leaf fragments were attached to appropriate stands with double-face carbon adhesive tape (3M) and carbon glue, covered by sputtering a layer of approximately 20 nm of gold (Bal-Tec® SCD-050 Sputter Coater, Liechtenstein). Samples were observed under a scanning electron microscope (DSM 962 and EVO 40-Zeiss®) at an accelerating voltage of 15 kV or 25 kV.

Histochemical analysis of the plant sample

Histochemical tests of freshly collected leaf material were performed using freehand vegetable cuttings. Acid and base compounds were evaluated from exposure of the plant sections for 10 sec to a toluidine blue 0.05% aqueous solution at pH 6.8 [38]. Alkaloids were evaluated by Dragendorff's reagent for 5 to 10 min and washed quickly in sodium nitrite, 5% and subsequently in water [39]. Total proteins were evaluated by Coomassie brilliant blue G, 0.25% (acetic acid, 5% for 30 min) and washed in 5% aqueous acetic acid three times for 5 min at each stage; semi-permanent slides were assembled with glycerin, 50%. Phenolic compounds were detected by an aqueous solution of ferric chloride (10%, 5 min); peptic substances were detected using an aqueous solution of ruthenium red (0.05%, 10 sec); acid intracellular compartments were evaluated by an aqueous solution of acridine orange (0.01%, 5 min) [40]. Lipids were evaluated by the sudan IV test, 0.03% [41].

Plant extract

T. oblongifolia leaves were submitted to forced ventilation drying, at a constant temperature of 40 °C, until stabilization of the weight, to obtain dry plant matter; 350 g of leaves were ground with a mortar and pestle in the presence of liquid nitrogen. The material with reduced particle size was deposited in an amber flask and extracted by maceration in 10% (w/v) methanol for seven days at room temperature. The period for exhaustive extraction was 21 days. Every seven days the fluid extractor was filtered and a new fluid extractor was added. The obtained extract was concentrated in a rotary evaporator and the drying process of the extracts was finalized

under direct ventilation (40 to 50 °C). Finally, the dried extract was verified and stored in an opaque plastic container in the presence of a desiccant agent.

Phytochemical prospecting of extracts

The phytochemical analysis of the methanolic extract of the *T. oblongifolia* leaves was performed in thin layer chromatography [42]. The following groups of secondary metabolites were evaluated: Alkaloid, Anthraquinones, Cardiotonics, Coumarins, Flavonoids, Polyphenols (Tannins), Saponin, and Triterpene. In the chromatographic process, mobile phases, developers and specific reference standards were used for each of the groups of secondary metabolites (Table 1).

Evaluation of the antioxidant activity of the extract

The antioxidant capacity of the extract was determined using the stable radical method 2,2-diphenyl-1-picrylhydrazyl (DPPH), and is based on the free radical reduction DPPH [43]. The solution prepared daily was stored away from the light and kept at 4°C until use. The final concentrations of the sample were 3, 6, 12, 18 and 24 µg/mL. The samples were diluted in methanol at five different concentrations: 30, 60, 120, 180, 240 µg/mL. 300 µL of each of the five concentrations was added in 2.7 mL of a DPPH methanolic solution (60 µM). The control was prepared from 2.7 mL of the methanolic solution of DPPH and 300 µL of methanol. A blank test was performed with the extract, fractions and controls to reduce the contribution of the color to the absorbance. The absorption of the samples was measured in quartz cuvettes at the following times: 0, 15, 30 and 45 min (515 nm). The antioxidant activity of the sample, in percentage of DPPH (Absorbance, Ab) intake, was obtained by the formula [Ab control - (Ab sample - Ab white) / Ab control] x 100.

Evaluation of the extract effect on the viability of the cell lines

U937 leukemia human cells of lymphoid origin (ATCC® CRL1593.2™) and COLO-205 colorectal adenocarcinoma human cells (ATCC® CCL222™) were cultured in D-MEM F12 medium (Gibco®, BRL) supplemented with 20 µg/mL of gentamicin (Gibco®, BRL) and 10% of fetal bovine serum (Gibco®, BRL). Cultures were replicated every 2 days and kept in an incubator (Forma Scientific® Inc., model 3159) at 37 ° C, with 5% of CO2 and controlled humidity.

Cell viability was assessed from the assays with 3-(4.5-dimethyl-2-thiazole) 2.5-diphenyl-2-H-bromide of tetrazolium, MTT [44] and from release levels of the lactic dehydrogenase enzyme, LDH [45]. Cells were plated in a volume of 100 µL/well ($1x10^6$ cells /mL) in 96-well plates and exposed to the extracts of *T. oblongifolia* at the final concentrations of 50, 100, 200 and 400 µg/mL and kept in an incubator at 37 °C, with 5% CO_2 and controlled humidity. After 24 hours under cell culture with the extracts, cell viability assays were performed. For this, 10 µL of MTT (Sigma®) in a 100 µL culture was added for further spectrophotometric reading (Thermo Scientific Multiskan-Ex®) at 570 nm.

To evaluate LDH release (Doles® Kit, Brazil), 50 µM of the supernatant was transferred to another 96-well plate containing 100 µM of solution A (250 µM of ferric alum solution + 400 µM of substrate) and kept in an incubator for 3 min. Afterwards, 100 µM of solution B (225 µM FMS / NAD + 4275 µM solution of distilled water) were added and kept in an incubator for 7 min for subsequent spectrophotometric readings at 492 nm (Thermo Scientific Multiskan-Ex®).

As negative controls, cells were incubated with DMEM F12 medium (Gibco®, BRL) supplemented with 20 μg/mL of gentamicin (Gibco®, BRL) and 10% fetal bovine serum (Gibco®, BRL). For the assays with the extracts, the cells were incubated with 1% DMSO (Sigma®). The experiments were carried out in triplicates.

Evaluation of apoptosis through fluorescence microscopy

The leukemic cells U937 and carcinoma colorectal cells COLO-205 exposed at 12, 24 and 36 hours with different concentrations of the extract were stained with 10 μg/mL of acridine orange solution (Sigma®) and 10 μg/mL of ethidium bromide (Sigma®). In random fluorescence microscopy (Axioplan Zeiss®) fields, ~300 cells were evaluated for apoptosis and necrosis. Duplicates were made for each condition and the experiments were repeated at least twice.

Statistical analysis

The results were expressed as mean ± standard deviation, analyzed independently and evaluated by one-way ANOVA followed by the Tukey test (GraphPad 5.0 Software, USA). All experiment samples were made into triplicates and repeated at least three times. Statistically significant differences were defined for $p<0.05$, $p<0.01$ and $p<0.001$.

Table 1. Phytochemical prospection of the ethanolic extract of the *T. oblongifolia* leaves.

Metabolit group	Mobile phase	Developer	Standart	Reference
Alkaloids	Ethyl metanol-water acatate (100:14:10)	Dragendorff's Reagent	Quinine	[39]
Anthraquinones	Ethyl metanol-water acatate (100:17:13)	5% KOH solution in ethanol	1,8-dihydroxy-anthraquinone	[42]
Cardiotonics	Ethyl metanol-water acatate (81:11:8)	Kedde's Reagent	Digitoxin	[40]
Coumarins	Acetic acid-toluene-diethyl ether-water (50:25:25:5)	5% KOH solution in ethanol	Benzopyrone	[39]
Flavonoids	Ethylacetate-acetic acid-acetic acid-water (55:5:5:1)	5% KOH solution in ethanol	Routine	[42]
Polyphenols (Tannins)	Toluene-butanol-acetic acid (80:40:16)	Barton's Ragent	Pirogalic acid	[41]
Saponin	Chloroform-glacial acetic acid-methanol-water (15:8:3:2)	Sulfuric anisaldehyde	18-β-glycyretin	[41]

RESULTS AND DISCUSSION

Foliar micromorphology

T. oblongifolia displayed regular abaxial surface with the presence of many stomata and multicellular tectonic trichomes. The anomocytic stomata are located below the level of the epidermis. Both surfaces are smooth with rare designs on the epicuticular wax (Figure 1).

Foliar anatomy

Anatomically, the leaf blade of *T. oblongifolia* presented an adaxial epidermis with a single layer of regular isodiametric cells with a straight to slightly convex outer periclinal wall. The abaxial epidermis showed smaller cells, with irregular contour and concave wall. The palisade parenchyma consists of two to three cell layers, rich in chloroplasts. The lacunar parenchyma consists of 6 to 8 layers of isodiametric cells with small intercellular spaces (Figure 2).

Transmission electron microscopy

Under transmission electron microscopy, it was possible to observe that the outer periclinal wall is divided into a basal polysaccharide layer, with cuticular extracts divided into arborescent (closest to the polysaccharide layer) and reticulated (further above the extract). There is a thin layer of cuticle. The protoplast observation of the mesophilic cells revealed large central vacuoles, cytoplasm occupying a relatively small volume as well as peripheral and abundant chloroplasts with an accumulation of lipids (Figure 3).

Leaf histochemistry

Histochemical tests were performed and the results were evaluated qualitatively according to the intensity of the resulting reactive staining (Figure 4; Table 2). Histochemical tests revealed a strong marking of proteins, phenolic compounds and alkaloids in the palisade parenchyma and spongy parenchyma, while pectin and lipids were more intensified in both leaf epidermises of *T. oblongifolia*.

The leaf epidermis exhibits reactive staining much more acid compartments, pectins and lipids than other sites on the leaf, but without reactive epidermal detection of proteins and phenolic compounds (Table 2).

Phytochemical prospecting

Foliar phytochemical analysis, associated with the phytochemical prospection of foliar methanolic extracts of *T. oblongifolia*, reveals the presence of alkaloids, anthraquinones, coumarins, flavonoids, polyphenols (tannins) and saponins, attributing recognized antitumor effects [9,11,15,46,47] including leukemic cells and colorectal cancer [8,18,19,48]. Cardiotonics and triterpenes compounds not were detected, however, they have already been identified in other genera such as *A. muricata* [49]. With similar results, alkaloids, coumarins, tannins, anthraquinone and flavonoids have also been found in preliminary phytochemical screening of extracts from other annonaceae such *Uvaria longipes*, *Dasymaschalon sp.* and *Marsypopetalum modestum*, but not for saponins, differently, absent in extracts of these plant [50].

Phytochemical diversity is related to several plant ecophysiological functions including protection against biotic and abiotic environmental stresses [51-54] in addition to the extractor solvent technique used in the experimental assay and the part of the plant phytochemically analyzed. as found for *Annona muricata* [55].

Determination of the antioxidant activity of the extract

The antioxidant activity of the *T. oblongifolia* leaf extract was evaluated (Table 3).

The phenolic compounds present in the fractions of the sample contribute to its antioxidant capacity. In *Annona muricata*, the strong antioxidant effect is highly correlated mainly to phytochemicals such as flavonoids and phenolic compounds. However, several other antioxidants compounds have been identified, such as tannins, saponins, glycoside, and acetogenins in this species [49] and other Annonaceae [27,56,57].

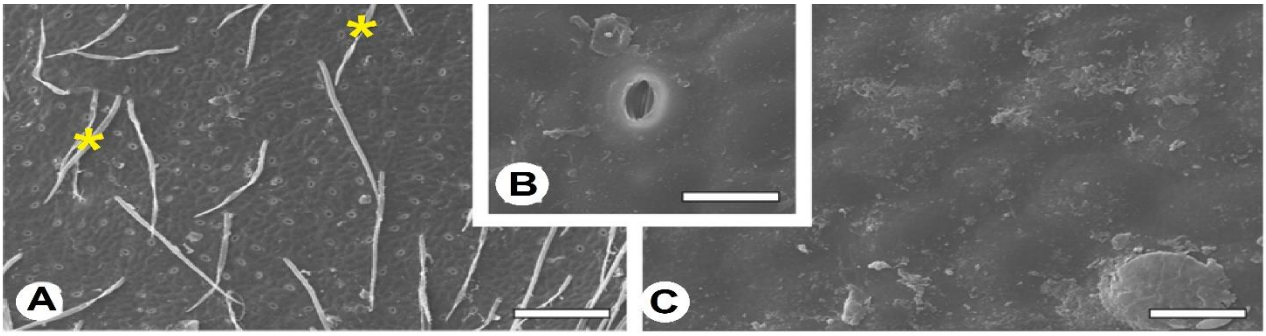

Figure 1. Scanning electron microscopy of the *T. oblongifolia* leaf in frontal view of the abaxial epidermis with presence of multicellular tectonic trichomes (asterisks) and stomata (A), with detail of an anomocytic stomata present on the abaxial surface (B) and smooth wax covering the adaxial surface (C). Bars: A: 400 µm; BC: 20 µm.

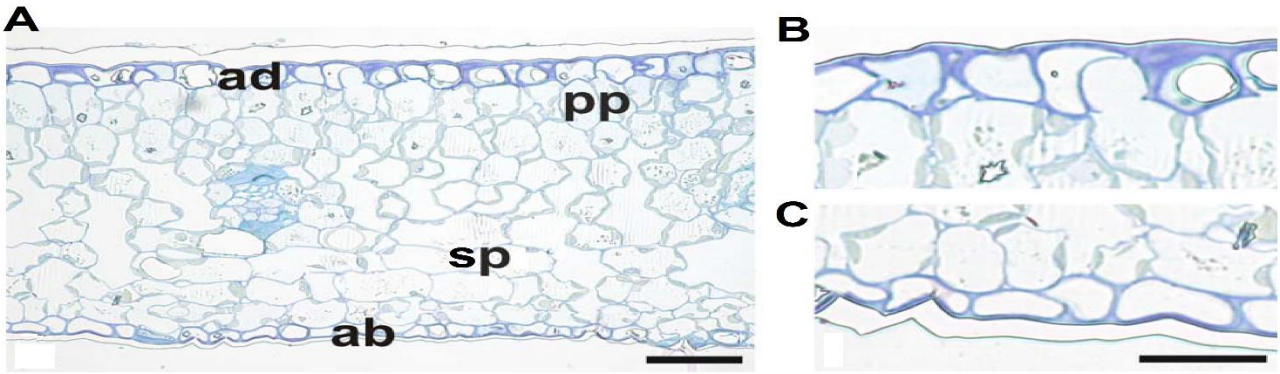

Figure 2. Leaf blade of *T. oblongifolia* in transverse section under optical microscopy (A), with detail of uniserial adaxial epidermis (B) and abaxial epidermis (C). Abbreviations: ad, adaxial surface; ab, abaxial surface; pp, palisade parenchyma; sp, spongy parenchyma. Bars: A: 50 µm; B, C: 25 µm.

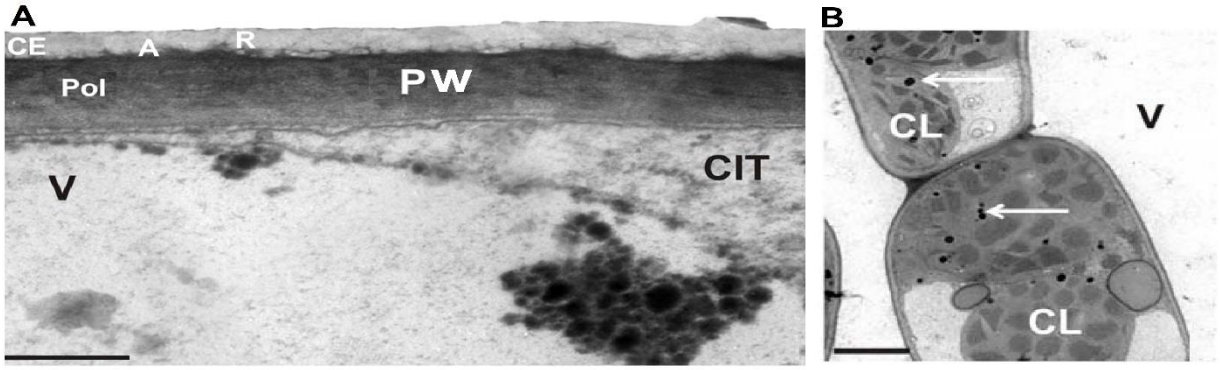

Figure 3. Transmission electron microscopy of *T. oblongifolia* leaf cell. A. Detail of the outer periclinal wall (PW) showing organization of the basal layer of polysaccharides (Pol) and cuticular extracts (CE) divided into arborescent (A) and reticulated (R); a large vacuole (V) and cytosol (CIT) are shown; B. Spongy parenchyma cell showing a large vacuole (**V**), a thin cell wall and chloroplasts (CL) with accumulation of lipids (white arrow). Bars: 2 µm.

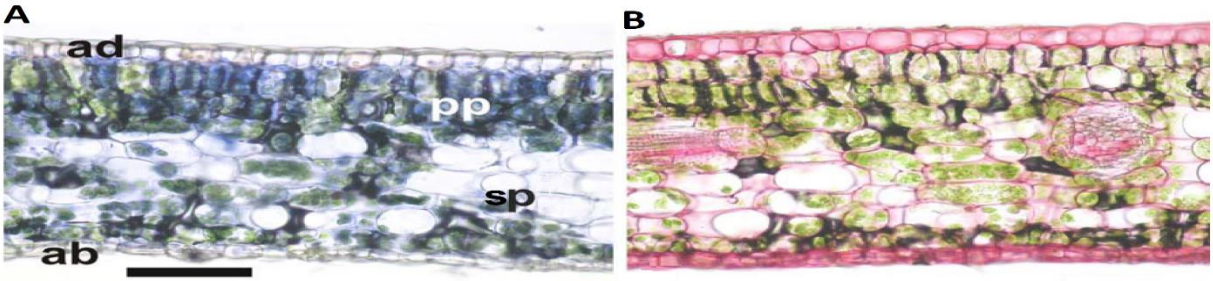

Figure 4. Optical microscopy of the leaf sections of *T. oblongifolia* submitted to histochemical tests of Comassie Brilliant Blue (A) identifying total proteins in blue color, and ruthenium red identifying pectins in light purple color (B) in leaf cells. Numerous chloroplasts can be observed (in green). Abbreviations: ad - adaxial surface; ab - abaxial surface; pp - palisade parenchyma; sp - Spongy parenchyma. Bars: 50 µm.

Table 2. Histochemistry of the T. oblongifolia leaf blade.

Leaf Mesophyll					
Reagente	Target Compound	Adaxial Epidermis	Palisade Parenchyma	Spongy Parenchyma	Abaxial Epidermis
Coomassie Blue	Proteins	-	++	-	-
Ferric Chloride	Phenolic Compounds	-	++	++	-
Dragendorff	Alkaloids	-	++	++	+
Acridine orange	Acid Compartments	+++	++	++	+++
Ruthenium Red	Pectins	+++	-	-	+++
Sudam IV	Lipids	+++	+	-	+++

Legend: +++ Very strong marking; ++ Moderately strong marking; + Poor marking; - Unmarked

Table 3. Percentage of the antioxidant activity of the *T. oblongifolia* foliar methanolic extract by the DPPH method, under final concentrations of 3, 6, 12, 18 and 24 µg/mL, for 30 min. Mean ± standard deviation obtained by triplication.

Extract Concentration (ug/mL)				
3	6	12	18	24
14.4 ± 3.8	16.3 ± 0.1	19.3 ± 0.6	24.4 ± 0.5	32.3 ± 0.8

Effect of extract on cell viability

The cytotoxic effect of the *T. oblongifolia* extract on the U937 leukemic and COLO-205 colorectal human cell lines was evaluated. All concentrations tested showed significant inhibitory effect in a dose-dependent relationship on cell viability, with over 80% inhibition at a concentration of 400 μg/mL after 24 h of incubation (Figure 5).

There is a greater sensitivity of leukemic cells to treatments. The determination of lethal dose 50 (LD50) was > 400 μg/mL after 12 h of culture, 110.8 ± 1.70 μg/mL after 24 h and 58.42 ± 1.04 μg/mL after 36 h of exposure to the U937 cells, and LD50> 400 μg/mL after 12 h of culture, 146.4 ± 1.99 μg/mL after 24h and 143.3 ± 1.87 μg/mL after 36 h of exposure to the cell extract for the COLO-205 colorectal cells. The phytochemical compounds present in the fractions of the sample contribute to its effect on cell viability, as observed for other annonaceae [26,36,58].

A similar result was obtained in colorectal tumor cells after 24 h of exposure to the same concentrations of the leaf extract of *Annona squamosa* [59] e *A. reticulata* [60]. From another perspective, alkaloid compounds present in *Dasymaschalon rostratum* demonstrated weak cytotoxicity against some human cancer cell lines, among them, colorectal cancer cells COLO-205 [61]. The cytotoxic effect observed in our study was better than that found for colorectal tumor cells and leukemic cells exposed to extracts of *A. squamosa* [59,62] and *A. muricata* [63], species most commonly studied [26], showing here genus specificities in the Annonaceae family. Other assays find that leukemic line cells (K562, U937, and RAJI) were less affected by the annonaceae extracts of *Uvaria longipes*, *Dasymaschalon sp.* and *Marsypopetalum modestum* compared with cervical carcinoma and human hepatocellular carcinoma [50] indicating that the cytotoxicity induced by the plant extracts occurs in a cell-type specific manner.

This is the first investigative study on the antitumor bioactivity of leaf extract in the genus *Trigyneae*, which somewhat limits the safe inference of the results obtained. On the other hand, it will contribute as a source of primary data for future scientific studies involving this anonnaceae species. Further studies at our research center will be conducted in order to understand the chemical composition of fractionated bioactive substances present in the plant extract and their effects on oncogenesis.

Quantification of Lactate Dehydrogenase

As indicative of cell membrane damage and a key feature of cells undergoing apoptosis, necrosis, and other forms of cellular damage, the activity of the cytosolic enzyme Lactate Dehydrogenase (LDH) was evaluated. The results were presented as fold increase of the control cells (Figure 6). Statistically significant results were shown for all tested concentrations of *T. oblongifolia* foliar extract in a dose-dependent effect.

Initially, there was a similar behavior among the cells exposed to the treatments. When exposed to 200 μg/mL of the extract, the both cells released about 1.5 times more LHD than the control (0 μg/mL) but under 400 μg/mL of the extract, U937 leukemic cells release more than twice LHD than the control.

Evaluation of apoptosis induction and cellular necrosis

To investigate the antitumor activity of the *T. oblongifolia* foliar extract on U937 leukemic cells and COLO-205 colorectal cells, cell death was evaluated by fluorescence microscopy. All concentrations tested showed a significant dose and time-dependent apoptotic effect, with more than 90% apoptosis of U937

leukemic cells achieved in 24 h under 100 μg/mL of culture extract (Figure 7). There was a rate of less than 3% of necrosis in U937 cells only at the concentration of 400 μg/mL under 36 h of culture. The highest necrosis value (50%) in COLO-205 cells was reached at the concentration of 400 μg/mL under 24 h of culture (data not shown). The phytochemical compounds present in the fractions of the sample contribute to its apoptotic capacity, as observed for other Annonaceae [36,58].

The apoptotic effect observed here was better than that found for colorectal tumor cells after 24 h of exposure to higher concentrations of leaf extracts of *Annona squamosa* [59]. *A. cherimola* seed extracts inhibit the proliferation of different leukemic cell lines inducing apoptosis by upregulating the expression of pro-apoptotic proteins which control both intrinsic and extrinsic pathways of apoptosis [64].

A class of phytochemicals not included in our screening was acetogenins. Annonaceous acetogenins (ACGs) constitute a series of polyketides found almost exclusively in plants of the Annonaceae family with bioactivity and mechanisms of action of scientific interest [65,66]. The mechanism of cytotoxic action of ACGs consisted mainly in the inhibition of mitochondrial complex I electron transport resulting in inhibition of cellular respiration. The mechanism of selective cytotoxicity against cancer cells is explained by the metabolic specificity of tumor cells [67].

Our expectation is to isolate and elucidate unknown ACGs structures in the extract of *T. oblongifolia* and evaluate its bioactivity on leukemic and colorectal cells, and other tumor types. And then, perform *in vivo* tests. There are promising results associating ACGs with the suppression of human colon cancer cell lines SW620 and RKO growth *in vivo* through downregulation of anti-apoptotic family proteins frequently overexpressed in human cancers [68].

Phytochemical studies of different annonaceae have drawn scientific interest [27,69,70] being the first anatomical histophytochemical study of *T. oblongifolia*, especially involving tumor cells. Its cytotoxic effects on U937 leukemic and COLO-205 colorectal cells indicate a potential alternative for treatment of this type of cancer. This plant provides a new source for classes of secondary metabolites: alkaloid, anthraquinones, coumarins, polyphenols, saponins, flavonoids, acetogenins and terpenes, which might be capable of inducing cancer cell apoptotic death [10,15,18,36,46,47]. Scientific studies can be directed to investigate the interference of different environments in which *T. oblongifolia* is adapted, the histochemical and anatomical foliar variations, and other plant structures, and the relation with the phytochemical bioactivity of this annonaceae and the different plant parts on U937 leukemic cells and COLO-205 colorectal cells as well as another human tumor cell lines.

Tests with crude extracts of plant organs provide good indications of the plant's bioactivity and the prospect of isolation of promising biocompounds for the development of new future antitumor drugs. However, there is a limitation of predicting whether the isolation of a certain. *T. oblongifolia* biocompounds will present interesting experimental results if there is synergy between the components of the leaf extract. Another limitation is to safely predict which chemical class most affected the viability of human leukemia and colorectal tumor cells. The experimental use of two cell types of cancers so different also brought some limitation in defining the comparative tests with a physiologically compatible cell control, since

one cancer exhibits blood characteristics and the other cancer with solid tumor characteristics. In addition, tests with extracts using different tumor cells also limit a safe interpretation of the results in relation to the possible mechanism of action, since they are different tumorigenic biochemical pathways for each type of cancer.

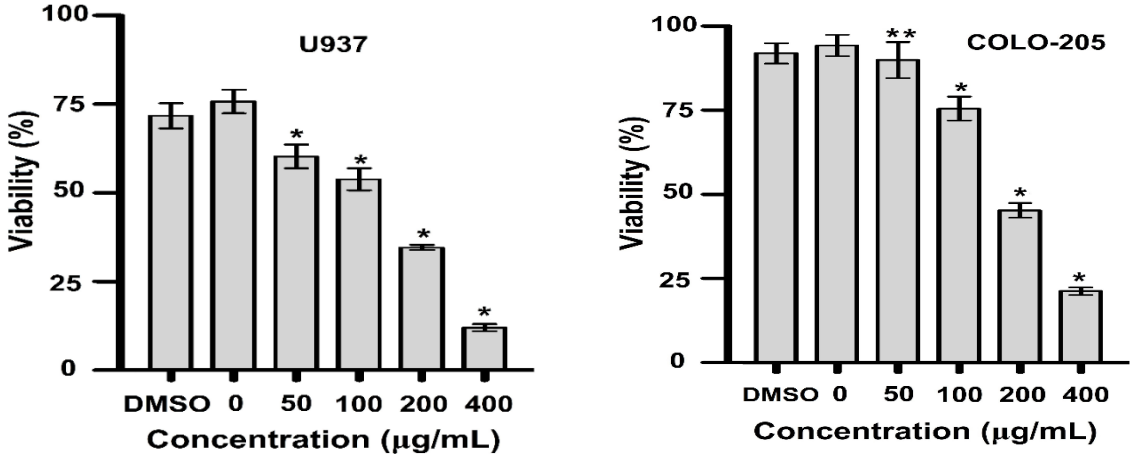

Figure 5. Cytotoxic effect of *T. oblongifolia* extracts on U937 leukemic and COLO-205 colorectal human cell lines after 24 h of incubation. The evaluation was done through use of MTT colorimetric microassay; n = 3; *P < 0.05, **P > 0.05. ANOVA, One-way Tukey.

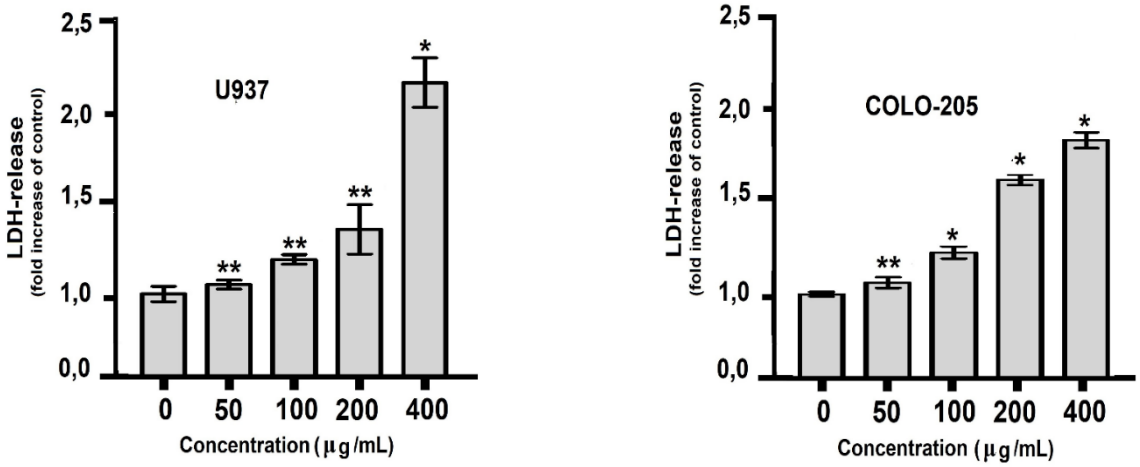

Figure 6. Evaluation of LDH release by U937 leukemic and COLO-205 colorectal human cell lines after 24 h of incubation exposed to the T. oblongifolia leaf extract. The results were presented as fold increase of the control cells (0); n = 3; *P < 0.05; **P > 0.05. ANOVA One-way Tukey.

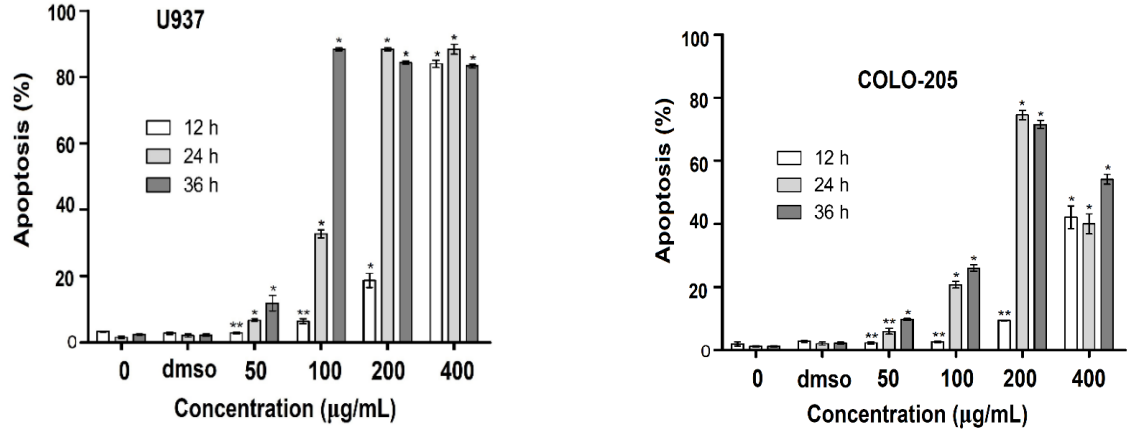

Figure 7. Percentage of apoptosis of U937 leukemic and COLO-205 colorectal human cells exposed to different concentrations of *T. oblongifolia* foliar extract at 12, 24 and 36 h of culture; n = 2; *P < 0.05, **P > 0.05. ANOVA, One-way Tukey.

CONCLUSION

The extracts from *T. oblongifolia* leaves increased the percentage of the death in the leukemic and colorectal cancer cells lines, with leukemic cells exhibiting the greatest antitumor effects in all trials. This plant provides a new source for classes of secondary metabolites with apoptotic antitumor properties: alkaloid, anthraquinones, coumarins, polyphenols, saponins, flavonoids and terpenes. This study represents the first report on the *in vitro* cytotoxicity, anatomy and histophytochemistry of the genus *Trigyneae*, which somewhat limits the inference and scientific interrelationships of the results obtained for the species. In addition, the use of different extracting solvents or chromatographic fractions of the extract can provide differentiated experimental results. On the other hand, it will contribute as a source of primary data for future scientific studies involving this anonnaceae species. *T. oblongifolia* should be further examined in different test systems to understand its potential cytotoxicity properties and biochemical pathways that interfere with tumor cell lethality.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

CONTRIBUTIONS OF THE AUTHORS

WMP conducted execution and experimental work, acquisition, analysis and interpretation of data and the preparation of the academic thesis that generated this article. MMK and TTO intervened as advisor and co-supervisor respectively throughout the elaboration and execution of the project and scientific activities; they also contributed with the provision of reagents and resources for the feasibility of research and critical revision of the intellectual content. MAF updated the literature review, drafted the manuscript and standardized its preparation for the feasibility of publication in a scientific journal, with the support of MRC and LMC, supervising all written material and translated into English under careful critical revision of the content.

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