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In vitro biological screening of the anticholinesterase and antiproliferative activities of medicinal plants belonging to Annonaceae

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Abstract

The aim of this research was to investigate the antiproliferative and anticholinesterase activities of 11 extracts from 5 Annonaceae species *in vitro*. Antiproliferative activity was assessed using 10 human cancer cell lines. Thin-layer chromatography and a microplate assay were used to screen the extracts for acetylcholinesterase (AchE) inhibitors using Ellman's reagent. The chemical compositions of the active extracts were investigated using high performance liquid chromatography. Eleven extracts obtained from five Annonaceae plant species were active and were particularly effective against the UA251, NCI-470 lung, HT-29, NCI/ADR, and K-562 cell lines with growth inhibition (GI_{50}) values of 0.04-0.06, 0.02-0.50, 0.01-0.12, 0.10-0.27, and 0.02-0.04 μg/mL, respectively. In addition, the *Annona crassiflora* and *A. coriacea* seed extracts were the most active among the tested extracts and the most effective against the tumor cell lines, with GI_{50} values below 8.90 μg/mL. The *A. cacans* extract displayed the lowest activity. Based on the microplate assay, the percent AchE inhibition of the extracts ranged from 12 to 52%, and the *A. coriacea* seed extract resulted in the greatest inhibition (52%). Caffeic acid, sinapic acid, and rutin were present at higher concentrations in the *A. crassiflora* seed samples. The *A. coriacea* seeds contained ferulic and sinapic acid. Overall, the results indicated that *A. crassiflora* and *A. coriacea* extracts have antiproliferative and anticholinesterase properties, which opens up new possibilities for alternative pharmacotherapy drugs.

Key words: *Annona*; *Duguetia*; Antiproliferative; Anticholinesterase activity

Introduction

Annonaceae is a large family of plants in the Cerrado region of Brazil, comprising approximately 27 genera and 290 species and including a large variety of exotic fruits. These plants are found in rural areas and are commonly consumed *in natura* by native people or used in the preparation of juice or ice cream. The fruits are known as 'araticum do cerrado' or 'marolo' (1). Many members of the Annonaceae family are used in folk medicine for antiparasitic or antitumoral treatment of intestinal diseases. Some species in the Annonaceae family have been intensely studied for the isolation and characterization of their diverse classes of compounds and pharmacological properties, including anticancer and anticholinesterase activities (2-5).

Our preliminary studies of the pharmacological properties of *Annona* species indicated that the methanolic extract and fractions of the leaves from *A. dioica* exert anti-inflammatory, hypoglycemic, antiproliferative, and antioxidant effects (6). The essential oil from the leaves of *A. sylvatica* exhibited anti-inflammatory and anticancer activities (7), and a methanolic extract and the ethyl acetate fraction (EAF) showed anti-mycobacterial activity (8). Furthermore, *Annona* species have potential as insecticides because they inhibit the development and reproduction of *Spodoptera frugiperda* (9).

The prevalence and use of medicinal plants among the Annonaceae family and the biological potential and significance of finding new potentially active agents have drawn us to study this family (especially the *Annona* and *Duguetia* species). The popular uses, parts of the plant used, and extraction yields of the plants that were

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selected for this study are reported in Table 1. However, more evidence is required to determine the antitumor effects and acetylcholinesterase (AchE) inhibitory properties of these species, which have not been investigated to date. The present study investigated the antiproliferative and anticholinesterase activities of 11 extracts from 5 Brazilian Annonaceae plants *in vitro*.

Material and Methods

Plant material

Vegetative samples of the 5 species were collected in Dourados (S 22° 08′ 25″, W 55° 08′ 17″), MS, Brazil. Botanical identification was conducted by Dr. Zefa Valdivina Pereira, Faculdade de Ciências Biológicas e Ambientais, Universidade Federal da Grande Dourados (FCBA, UFGD), Dourados, MS, Brazil. The specimens were deposited in the Herbarium of the FCBA, UFGD (Table 1) (10-16). In addition to the data from the literature, we investigated the main species from the Annonaceae family that were present in the Dourados region and used by the population.

Preparation of crude extracts

Annona crassiflora (L: leaves and S: seeds), *A. coriacea* (L, C: floral capitulum, and S), *A. sylvatica* (L and S), *A. cacans* (L), and *Duguetia furfuracea* (L, C and S) were air-dried and exhaustively extracted by maceration using methanol at room temperature. The solvent was evaporated under vacuum at 40°C and lyophilized to obtain the methanol extract of each sample studied.

In vitro antiproliferative activity assay

The human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI/ADR/RES (ovarian tumor expressing the phenotype of multiple drug resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), OVCAR-3 (ovarian), HT-29 (colon), K562 (leukemia), UA251 (glioma), and VERO (green monkey kidney cells) were kindly provided by the Frederick National Laboratory for Cancer Research, Frederick, MD, USA. Stock cultures were grown in 5 mL of RPMI 1640 (Gibco-BRL, Life Technologies, USA) supplemented with 5% fetal bovine serum. Penicillin and streptomycin (1 mL/L, at a ratio of 1000 μg/mL:1000 IU/mL) were added to the experimental cultures. Cells in 96-well plates (100 μL cells/well) were exposed to each extract in dimethyl sulfoxide (DMSO; 0.25, 2.5, 25, and 250 mg/mL) and 5% CO_2 at 37°C for 48 h. The final concentration of DMSO did not affect cell viability.

Next, the cells were fixed with a trichloroacetic acid solution (50%, v/v), and cell proliferation was determined via spectrophotometric quantification (540 nm, Molecular Devices Versa Max microplate reader, USA) of the cellular protein content using a sulforhodamine B assay (17). Doxorubicin (0.025-25 μg/mL) was used as a positive control, and averages were calculated from absorbance. Cell proliferation was determined using the equation $100 \times [(T–To)/C_0–To]$, where T is the absorbance of the treated cell, To is the absorbance of the control cells at the beginning of the incubation, and C_0 is the absorbance of the control. These values were determined from nonlinear regression analysis using the Origin software, version 7.5 (USA). The results were subtracted from 100% to obtain the percent growth inhibition. The samples were considered active when growth inhibition was greater than 50% (GI_{50}). A cytostatic effect (TGI) was observed when $T \geqslant To$, and a cytocidal effect (LC_{50}) was observed when $T < To$. The experiments were performed in triplicate.

Measurement of AchE activity

The solutions used were as follows: 50 mM Tris/HCl,

Table 1. Selected plant species from Annonaceae evaluated for antiproliferative and acetylcholinesterase inhibitory activities.

Plant species (Voucher)	Vernacular name	Ethnomedical use	Part of plant tested[1]	Extraction yield (% w/w)
Annona crassiflora Mart. (DDMS 4599)	''araticum do cerrado'' or ''marola''	The oil from the seeds is used against snake bites and in folk medicine, the leaf and seed infusion is used against diarrhea, antitumor, and for the treatment of Chagas' disease (1,10)	L, S	L (42%) S (16.8%)
A. coriacea Mart. (DDMS 186)	''marola'', ''araticum'' or ''araticum-liso''	Seeds and leaves are used against chronic diarrhea (11), antimalarial (12), rheumatism (13), anti-helmintic (14), and leishmaniasis (15)	L, S, C	L (52.6%) S (13.5%) C (5.1%)
A. sylvatica St.-Hil (DDMS 4600)	''araticum da mata''	Leaves are used for ulcers caused by syphilis, muscle spasms, angina, diarrhea, or as a febrifuge (16)	L, S	L (36.4%) S (8.0%)
Cacans Mart. (DDMS 4885)	''araticum cagão''	Not reported	L	L (40.8%)
Duguetia furfuracea A.St.-Hil (DDMS 166)	''araticum seco'' or ''araticum miúdo''	Leaves are used as a parasiticide and against rheumatism (12)	L, S, C	L (30.0%) S (8.5%) C (6.6%)

L: leaves; S: seeds; C: floral capitulum. [1]Methanol was used as the solvent.

pH 8; 50 mM Tris/HCl, pH 8, containing 0.1% BSA; 50 mM Tris/HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$; 3 mM 5,5′-dithiobis-(-2-nitrobenzoic acid) (DTNB or Ellman's reagent), 15 mM acetylcholine iodide (ATCI), 1 mM Ellman's reagent, and 1 mM ACTI. Lyophilized enzyme AchE was dissolved in the Tris/HCl buffer solution, pH 8, to obtain a 1000 U/mL stock solution. This solution was allowed to rest for 20 min and then stirred for 10-15 min to obtain a homogeneous solution. For subsequent dilutions, the Tris/HCl buffer solution at pH 8 and containing 0.1% BSA was used.

Microplate assay

The enzyme hydrolyzed the acetylcholine substrate and generated thiocholine, which reacted with the Ellman's reagent to produce 2-nitrobenzoate-5-mercaptothiocoline and yellow-colored 5-thio-2-nitrobenzoate anion, which can be detected at 405 nm (18). Twenty-five microliters of 15 mM ATCI, 125 μL of 3 mM Ellman's reagent, and 50 μL of Tris/HCl, pH 8, containing 0.1% BSA were added to the 96-well plates. Next, 25 μL of the extract to be analyzed was dissolved in methanol and diluted 10 times with Tris/HCl, pH 8. The absorbance was read 5 times every 13 s. Next, 25 μL of the enzyme (0.22 U/mL) was added, and the absorbance was measured 8 times every 13 s. The speed of the reaction was calculated. Possible increases in the absorbance rate due to spontaneous hydrolysis were corrected by subtracting the speed of the reaction before the enzyme was added from the speed of the reaction after the enzyme was added. Next, the inhibition percentage was calculated by comparing the speeds of the sample reactions to a blank (10% methanol, Tris/HCl, pH 8).

Thin-layer chromatography (TLC) assay

Aliquots (1.5-2.5 μL) of each extract were spotted on silica gel plates (60 F254, 0.2 mm, Merck, USA) and developed in chloroform/methanol (8:2 v/v) solvent. The plate was then sprayed with 1 mM DTNB and 1 mM ACTI solution, dried for 3-5 min, and then 3 U/mL of the enzyme solution was sprayed on the plate. Within 10 min, a yellow color appeared, and white spots were observed where the inhibiting effect of the enzyme was present. The coloration disappeared between 20 and 30 min.

LC analysis of the standards and samples

The extracts and standards were analyzed in an analytical LC (Varian 210) system with a ternary solvent delivery system equipped with an autosampler and a photodiode array detector (PDA) monitored at $\lambda = 200$-800 nm. The LC column was a C-18 column (4.6 mm × 25 cm; particle size, 5 μm; Luna, Phenomenex, USA) with a pre-column (3 mm × 2.5 cm) containing the same packing material. The injection volume was 20 μL and the flow rate 1.0 mL/min at 22°C. The column was eluted with sodium acetate (2 mM; Solvent A) and acetonitrile (Solvent B). The following solvent gradient program was used: 10% Solvent B in 10 min, 15% in 5 min, 100% in 15 min.

Linearity. The content estimation of the standards [caffeic acid (98%), *p*-coumaric acid (98%), ferulic acid (99%), sinapic acid (98%), luteolin (98%), quercetin (99%), rutin (98%), kaempferol (98%), and apigenin (95%) purchased from Sigma Aldrich, USA] in the samples (methanolic extract of *A. crassiflora* and *A. coriacea* seeds) was performed using external calibration. Next, 20 μL aliquots of the dilutions were analyzed via LC. Each determination was conducted 5 times. For the standard, the corresponding chromatogram was obtained and a graph was constructed from the mean of the chromatogram areas plotted against the standards at a concentration of 1-50 μg/mL. A linear least-squares regression of the peak areas was performed as a function of the concentrations to determine the correlation coefficients. Equation parameters (slope and intercept) of the standard curve were used to obtain the concentration values for the samples.

Results

Plant material – methanolic extract

Extracts from the lyophilized leaves (L) and seeds (S) of *A. crassiflora*, *A. coriacea*, *A. sylvatica*, *A. cacans*, and *Duguetia furfuracea* showed high yields (L: efficiency values of 30.0-52.6%; S: efficiency values of 10.2-16.8%; Table 1) when methanol was used as the extractor liquid. The yield was calculated using the total dry mass of the plant extract (w/w).

Antiproliferative activity assay

The antiproliferative properties of the 11 extracts of Annonaceae were assessed in 10 human cancer cell lines, with the chemotherapeutic drug doxorubicin as positive control. The response parameter (GI_{50}) was calculated for each tested extract cell line, and the results are summarized in Table 2. The GI_{50} values (growth inhibitory activity) refer to the drug concentrations that resulted in a 50% reduction in the cellular growth relative to the untreated control cells. The TGI (cytostatic activity) and LC_{50} (cytotoxic activity) value parameters refer to the drug concentrations for total growth inhibition and for killing 50% of the cells, respectively (as shown in Table 3).

All evaluated seed extracts exhibited broad antiproliferative effects (Table 2). The *A. coriacea* extract showed GI_{50} values ranging from 0.02-3.83 μg/mL against all of the tested human tumor cell lines, showing effective growth inhibition against specific cell lines, including UA251 ($GI_{50} = 0.04$ μg/μL), HT-29 ($GI_{50} = 0.03$ μg/mL), NCI-H460 ($GI_{50} = 0.02$ μg/mL), and K-562 ($GI_{50} = 0.02$ μg/mL). In addition, *A. crassiflora* possessed potent antitumor activity against all of the 10 human tumor

Table 2. *In vitro* antitumor activity (GI_{50} in μg/mL) of the methanolic extracts from the Annonaceae plants against the tumor cell lines.

Extract	Cancer cell line									
	UA251	UACC-62	MCF-7	NCI-H460	786-0	HT-29	NCI/ADR/RES	OVCAR-3	K562	VERO
Doxorubicin	5.13	0.06	0.14	0.05	0.22	2.20	1.68	0.30	0.89	1.38
A. crassiflora										
L	5.09	28.12	24.95	3.86	>100	2.49	11.61	44.83	26.02	14.14
S	0.06	3.78	3.54	0.04	5.33	0.01	0.25	8.90	3.25	0.05
A. coriacea										
L	>100	1.76	5.58	0.02	37.69	0.08	0.10	36.47	21.11	0.10
S	0.04	2.92	2.63	0.02	3.74	0.03	0.25	3.83	0.02	0.12
C	1.80	29.32	4.43	0.50	25.41	0.12	0.27	15.98	0.04	6.33
A. sylvatica										
L	0.05	26.41	29.85	0.06	69.14	0.01	1.94	36.47	5.30	41.60
S	0.10	32.27	29.68	0.02	0.06	0.02	0.06	36.68	0.02	1.87
A. cacans										
L	49.47	32.27	57.97	78.63	63.97	50.82	59.90	49.18	33.55	51.28
D. furfuracea										
L	34.36	27.22	60.62	48.16	79.81	78.63	37.80	77.92	58.49	56.09
S	0.04	54.60	62.28	0.09	–	0.02	1.71	–	2.34	2.27
C	32.27	34.04	42.23	44.69	86.52	23.79	49.03	54.77	0.07	>100

L: leaves; S: seeds; C: floral capitulum; UA251 (glioma); UACC-62 (melanoma); MCF-7 (breast); NCI-H460 (lung); 786-0 (renal); HT-29 (colon); NCI-ADR/RES (ovarian expressing the phenotype of multiple drug resistance); OVCAR-3 (ovarian); K562 (leukemia); VERO (monkey kidney normal cells); –: not presented.

cell lines assayed with a GI_{50} of less than 10 μg/mL, which showed GI_{50} values within the 0.01 to 8.90 μg/mL range (Table 2). The highest growth inhibitory activities occurred toward UA251 (GI_{50} = 0.06 μg/mL), VERO (GI_{50} = 0.05 μg/mL), NCI-H460 (GI_{50} = 0.04 μg/mL), and HT-29 (GI_{50} = 0.01 μg/mL) cells (Table 2). *A. sylvatica* extract showed inhibitory activity against the NCI/ADR/RES, 786-0 (GI_{50} = 0.06 μg/mL), NCI-H460, HT-29, and K-562 (GI_{50} = 0.02 μg/mL) cell lines (Table 2). The *D. furfuracea* extract presented the highest growth inhibitory activity toward UA251 (GI_{50} = 0.04 μg/mL), NCI-H460 (GI_{50} = 0.09 μg/mL), and HT-29 (GI_{50} = 0.02 μg/mL) cell lines (Table 2).

In addition, the *A. coriacea* extract showed potent activity (mean TGI value = 8.19 μg/mL) with very similar effects on UA251 (TGI = 4.59 μg/mL), UACC-62 (TGI = 8.01 μg/mL), MCF-7 (TGI = 9.00 μg/mL), NCI-H460 (TGI = 2.66 μg/mL), 786-0 (TGI = 7.17 μg/mL), HT-29 (TGI = 5.92 μg/mL), OVCAR-3 (TGI = 10.12 μg/mL), K562 (TGI = 4.69 μg/mL), and VERO (TGI = 5.91 μg/mL) cell lines (Table 3). Furthermore, *A. coriacea* exhibited cytotoxic activity with LC_{50} levels of 13.64-34.67 μg/mL against the 6 cell lines shown in Table 3. Another extract that presented weak antiproliferative activity was the *A. crassiflora* extract (mean TGI value = 28.16 μg/mL), with a selective effect on HT-29 (TGI = 0.10 μg/mL) and NCI/ADR/RES (TGI = 6.56 μg/mL, LC_{50} = 24.29 μg/mL) cell lines (Table 3).

For the extracts obtained from the floral capitulum, *D. furfuracea* (GI_{50} = 0.07 μg/mL) and *A. coriacea* (GI_{50} = 0.04 μg/mL) were particularly effective against the K-562 cell line (Table 2). In addition, *A. coriacea* also showed moderate activity against the HT-29 (GI_{50} = 0.12 μg/mL), NCI/ADR (GI_{50} = 0.27 μg/mL), and NCI-460 (GI_{50} = 0.50 μg/mL) cell lines (Table 2).

Regarding the extracts obtained from the leaves, *A. coriacea* and *A. sylvatica* demonstrated high activities against specific cell lines toward NCI-H460 (GI_{50} = 0.02 μg/mL; GI_{50} = 0.06 μg/mL) and HT-29 (GI_{50} = 0.08 μg/mL; GI_{50} = 0.01 μg/mL), respectively (Table 2). In addition, *A. sylvatica* demonstrated high activity against UA251 (GI_{50} = 0.05 μg/mL), and *A. coriaceae* demonstrated high activity against NCI/ADR/RES and VERO (GI_{50} = 0.10 μg/mL) cell lines (Table 2). Extracts from *A. crassiflora*, *A. cacans*, and *D. furfuracea* did not show activity against the cell lines.

AchE activity assay

The results from the extracts tested for AchE inhibition are presented in Table 4. The extracts were tested at concentrations of 1.0-2.0 mg/mL. Some extracts were not soluble in methanol, which limited the analysis in the microplate. Based on the microplate assay, the percentage of AchE inhibition by the extracts varied from 12 to 52%, with the seed extract of *A. coriacea* showing the greatest inhibition (52%).

Table 3. Cytostatic (TGI, μg/mL) and cytocidal effects (LC_{50}, μg/mL; data in parentheses) for methanolic extracts from the Annonaceae.

	UA251	UACC-62	MCF-7	NCI-H460	786-0	HT-29	NCI/ADR/RES	OVCAR-3	K562	VERO	TGI mean value
Doxorubicin	23.98 (–)	0.86 (–)	23.98 (–)	–	1.54 (–)	7.31 (21.49)	23.95 (–)	1.73 (–)	–	11.42 (–)	11.84
A. crassiflora											
L	186.87 (–)	63.59 (>100)	63.78 (>100)	28.12 (>100)	–	9.11 (49.47)	66.31 (>100)	145.56 (–)	167.77 (–)	–	91.39
S	55.59 (–)	11.75 (45.23)	25.56 (78.63)	16.03 (–)	57.79 (>100)	0.10 (50.82)	6.56 (24.29)	37.69 (95.48)	–	42.35 (>100)	28.16
A. coriacea											
L	–	188.43 (–)	134.87 (–)	165.62 (–)	72.31 (>100)	–	145.90 (–)	133.65 (–)	–	47.16 (>100)	125.99
S	4.59 (17.22)	8.01 (21.75)	9.00 (–)	2.66 (13.64)	7.17 (14.18)	5.9 (72.53)	23.79 (–)	10.12 (34.67)	4.69 (–)	5.91 (18.62)	8.19
C	55.44 (>100)	80.29 (>100)	43.12 (>100)	–	56.60 (>100)	–	–	77.23 (–)	44.83 (>100)	–	59.58
A. sylvatica											
L	19.24 (–)	59.37 (>100)	–	–	–	0.36 (>100)	–	84.22 (>100)	–	91.03 (>100)	50.84
S	26.97 (90.48)	68.53 (>100)	145.86 (–)	4.88 (–)	65.92 (>100)	3.86 (–)	58.66 (–)	89.94 (>100)	74.06 (–)	48.31 (>100)	58.70
A. cacans											
L	167.86 (–)	156.67 (–)	–	–	189.56 (–)	–	–	–	–	168.98 (–)	170.77
D. furfuracea											
L	143.88 (–)	–	179.90 (–)	256.56 (–)	–	–	–	–	–	244.78 (–)	206.28
S	167.43 (–)	–	–	289.56 (–)	–	11.20 (–)	287.98 (–)	–	–	–	189.04
C	78.16 (>100)	75.18 (>100)		–	–	72.31 (>100)	–	156.54 (–)	57.28 (–)	–	87.89

L: leaves; S: seeds; C: capitulum floral; UA251 (glioma); UACC-62 (melanoma); MCF-7 (breast); NCI-H460 (lung); 786-0 (renal); HT-29 (colon); NCI/ADR/RES (ovarian tumor expressing the phenotype of multiple drug resistance); OVCAR-3 (ovarian); K562 (leukemia); VERO (monkey kidney normal cells); –: not presented.

LC analysis

The most active extracts of the *A. coriacea* and *A. crassiflora* seeds were investigated using chemical characterization. Identification of the compounds using a PDA detector over a spectral range of 200-800 nm did not reveal any interference in the retention time of the samples in LC when using the developed elution method. Standards were easily identified and quantified based on their absorption spectra in the UV region and their retention time. The standards that were found in the extracts were unambiguously identified by performing co-injection experiments, in which aliquots of the extracts and standards were mixed and diluted to a known volume and analyzed using LC.

The calibration curves were determined by linear regression using LC. The linearity of the standards was assessed using 5 concentration ranges. The average standard errors for the peak areas of the replicated injections (n = 5) were less than 1%, showing good repeatability of the calibration curve. The respective coefficients of determination (r^2) were 0.9994 for caffeic acid and *p*-coumaric acid, 0.992 for ferulic acid and sinapic acid, and 0.9996 for luteolin, kaempferol, quercetin, rutin, and apigenin. Caffeic acid (retention time (*rt*) = 1.73 min; 302 μg/mL), sinapic acid (*rt* = 3.75 min; 248 μg/mL), and rutin (*rt* = 4.98 min; 493 μg/mL) were present in higher concentrations in the sample of *A. crassiflora* seeds. The *p*-coumaric acid (*rt* = 2.56 min; 106 μg/mL) and ferulic acid (*rt* = 2.98 min; 176 μg/mL) showed smaller concentrations. The *A. coriacea* seeds contained ferulic acid (*rt* = 2.99 min; 197 μg/mL) and sinapic acid (*rt* = 3.75 min; 256 μg/mL; Figure 1A and B).

Discussion

Extract yield is an important factor for evaluating the extraction capacity of a solvent and the extraction

Table 4. Acetylcholinesterase (AchE) inhibition by the methanolic extracts from some Annonaceae plants in the microplate assay (% inhibition) and thin-layer chromatography (TLC).

Plant	Concentration extracts (mg/mL)	TLC	Microplate assay (% AchE inhibition)
A. crassiflora			
L	2.0	+	22
S	1.5	+	45
A. coriacea			
L	2.0	+	33
S	1.5	+	52
C	1.0	–	0
A. sylvatica			
L	2.0	+	12
S	1.3	–	0
A. cacans			
L	2.0	–	0
D. furfuracea			
L	2.0	+	25
S	1.5	+	46
C	1.3	–	0
Donepezil			87

L: leaves; S: seeds; C: floral capitulum; +: inhibited; –: not inhibited.

method. The leaf extracts from all species performed well when subjected to extraction with alcohol (methanol). The low extraction yield of the extracts that were obtained from the floral capitulum of *A. coriacea* and *D. furfuracea* resulted from the solvent extractor (methanol). These vegetal samples are preferentially extracted in methanol-water, most likely by more polar substances.

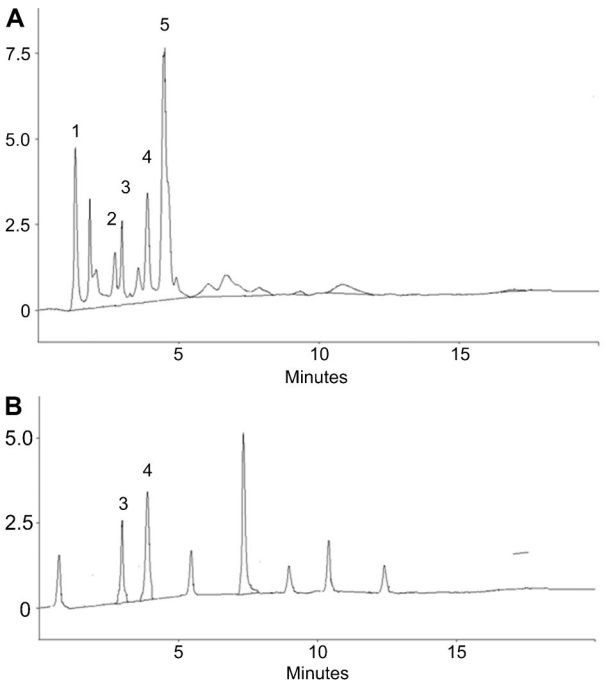

Figure 1. Chromatogram of *A. crassiflora* (*A*) and *A. coriacea* (*B*) seed extracts. 1: caffeic acid; 2: p-coumaric acid; 3: ferulic acid; 4: sinapic acid; 5: rutin.

When analyzing the bioassay results, an interesting antiproliferative effect was observed for the majority of methanolic extracts that were obtained from the 5 Annonaceae plants. The antiproliferative effect was particularly effective against UA251, NCI-H460, HT-29, NCI/ADR/RES, and K-562 cell lines. The extracts that were obtained from *A. cacans* and *D. furfuracea* leaves did not reveal antiproliferative activity.

Our previous studies have shown that the essential oils of *A. sylvatica* leaves exhibited antiproliferative activity, with GI_{50} values of between 36.04 and 45.37 μg/mL. However, at the highest concentrations, cytostatic activity and cytotoxic effects were observed for all of the cell lines (7).

In an earlier work, the *in vitro* antitumor activities of *A. crassiflora* and *D. furfuracea* were reported. The cytotoxicity of the extracts that were tested against human colon carcinoma, leukemia, brain, and melanoma tumor cell lines at 50 μg/mL demonstrated substantial antiproliferative effects, with at least 85% inhibition of cell proliferation in one or more of the cell lines (19). Coriadenin and gigantetronenin were isolated from *A. coriacea* roots and showed potent cytotoxicity against the VERO and KB cell lines (20,21). This species is popularly known as marola, and is known for its acetogenin (22-26) and alkaloid

(3,27-31) content with cytotoxic potential.

Our studies revealed that the extracts obtained from *A. coriacea* seeds displayed the most significant anti-proliferative activities toward all of the tested cell lines. Thus, these extracts are considered promising candidates for future *in vivo* studies. In addition, the *A. crassiflora* extract that was obtained from the seeds demonstrated a potent antiproliferative effect. These extracts are rich in phenolic acids, especially caffeic acid, sinapic acid, ferulic acid and flavonoid, and exhibited significant anticancer activities that could be attributed, at least in part, to their phenolic acid content. Several studies have demonstrated the anticancer and anticholinesterase potentials of these compounds (32-34).

Using the criteria of the American National Cancer Institute for considering a promising crude extract for further purification, the IC_{50} value must be less than 30 μg/mL (35). The extracts selected in our study showed significant activity ($IC_{50} \leq 0.10$ μg/mL) and were considered promising for further bioassay-guided fractionation.

In addition, the tests performed on these extracts were only performed in TLC and showed negative results. Although the TLC assay is a qualitative method, it can reliably indicate whether the compounds have positive activities. The use of this TLC method along with the quantitative microplate assay provides useful information for further sample purification. Extracts with inhibition values of more than 50% are considered suitable candidates for future research.

The presence of phenolic compounds and tannins in the methanolic extracts may result in false positives owing to the promotion of denaturation of the AchE enzyme. Therefore, it must be remembered that the *in vitro* tests can only assist in optimization and selection, and cannot be used to replace *in vivo* tests. AchE occurs mainly in the central and peripheral nervous systems and at the parasympathetic and neuromuscular junctions. The *A. coriacea* seed extract can be considered a promising candidate for future research regarding the activity of AchE in different brain structures (such as the cerebral cortex, hippocampus, cerebellum, and striate cortex), because earlier data were not found in the literature regarding the activity of this plant enzyme.

Briefly, our results demonstrate that methanolic extracts of Annonaceae can inhibit the proliferation of cancer tumor cells, especially the methanolic extract of *A. crassiflora* and *A. coriacea* seeds, which were the most active among the tested extracts (resulting in GI_{50} values of less than 8.90 μg/mL against all of the tumor cell lines). However, the methanolic extracts of *A. coriacea* seeds were the most effective for reducing tumor cell growth at the observed GI_{50}, TGI, and LC_{50} levels, and inhibited AchE activity according to the TLC test and microplate assay. Future *in vivo* studies should be conducted with toxicity studies to determine whether this plant induces changes in the functionality of the cholinergic system, especially in brain structures.

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