Tropical Journal of Pharmaceutical Research April 2016; 15 (4): 793-798
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v15i4.18

Original Research Article

Phytochemical screening and evaluation of cytotoxicity of stem bark extracts of *Anaxagorea dolichocarpa* and *Duguetia chrysocarpa* (Annonaceae)

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Received: 14 March 2015 *Revised accepted: 6 February 2016*

Abstract

Purpose: *To investigate the phytochemistry and cytotoxic activity of stem bark extracts from Anaxagorea dolichocarpa and Duguetia chrysocarpa - two species of the Annonaceae family.*
Methods: *The crude ethanol bark extracts (EtOH) of the plants were obtained by maceration. The crude extracts were suspended in a mixture of methanol (MeOH) and water (H_2O) (proportion 3:7 v/v) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in ascending order of polarity to obtain the respective fractions. The extracts were evaluated on thin layer chromatography (TLC) plates of silica gel to highlight the main groups of secondary metabolites. Cytotoxicity was tested against human tumor cell lines - OVCAR-8 (ovarian), SF-295 (brain) and HCT-116 (colon) - using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.*
Results: *The screening results demonstrated that all the extracts were positive for the presence of flavonoids and tannins. The presence of alkaloids also was detected in some extracts. The hexane extract of A. dolichocarpa showed the strongest cytotoxicity against HCT-116 with cell growth inhibition of 89.02 %.*
Conclusion: *The findings demonstrate for the first time the cytotoxic activity of the extracts of A. dolichocarpa and D. chrysocarpa, thus providing some evidence that plants of the Annonaceae family are a source of active secondary metabolites with cytotoxic activity.*

Keywords: *Annonaceae, Anaxagorea dolichocarpa, Duguetia chrysocarpa, Anti-cancer agents, Ovarian, brain and colon cell lines*

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INTRODUCTION

The Annonaceae family, comprised of tropical and subtropical species with about 135 genera and more than 2500 species, is widely distributed in South and Central America, Africa, Asia and Australia [1]. It is known for its edible fruits and the medicinal properties of several of its species. In Brazil, there are 26 genera with about 260 species [2]. Previous chemical and pharmacological investigations of some species of this family revealed the presence of important

bioactive compounds, exhibiting pharmacological activities such as antimicrobial, antioxidant, insecticidal and antiparasitic properties against *Leishmania* sp., *Plasmodium falciparum*, and *Trypanosoma cruzi* as well as cytotoxicity against tumor cell lines. These activities generally are attributed to alkaloids, essential oils and acetogenins [3].

Plants of the Annonaceae family have been researched including the isolation and characterization of diverse classes of substances having pharmacological activities, especially with regard to alkaloids and acetogenins. Annonaceae is a known rich source of aporphinic alkaloids and Annonaceous acetogenins. Annonaceous acetogenins are long chain fatty acid derivatives isolated, until 2008, exclusively from plants belonging to the Annonaceae family. These natural products exhibit a broad range of biological properties, such as cytotoxic, immunosuppressive, pesticidal, antiparasitic and antimicrobial activities, and their potential to inhibit cells that are multiple drug-resistant has attracted increasing interest [4].

The genus Anaxagorea comprises approximately 30 species distributed in Central America and South America. Plants of this genus have previously yielded aporphinic alkaloids, fatty acids, polyprenols, cyanogenic glucosydes, neolignans and steroid [5]. The species *A. dolichocarpa* has a wide geographical distribution, being the neotropical species of Annonaceae most common and well distributed. In Brazil, it occurs in the states of Amapá, Amazonas, Acre, Rondônia, Goiás, Maranhão, Paraíba, Pernambuco, Bahia and Rio de Janeiro [6]. Chemical studies involving this species reported the isolation of two aporphinic alkaloids anaxagoreine and asimilobine [7] as well as volatile components of the essential oil extracted from the fruits [8,9]. In study realized by our research group, the phytochemical investigation of *A. dolichocarpa* led to isolation of three azaphenanthrene alkaloids (eupolauramine, sampangine and imbiline). Eupolauramine and sampangine showed concentration-dependent antitumoral activity in leukemic cells K562 with IC_{50} of 18.97 and 10.95 µg/ml, respectively [10].

The genus Duguetia consists of approximately 80 known species native to tropical America. Few chemical data are available on this genus, despite the considerable number of species. Chemical study realized by our group with species of this genus showed the isolation of alkaloids and a new cinnamate derivative from *D. gardneriana* [11]. The chemical composition and antimicrobial activity of the leaf essential oils of *D. gardneriana* and *D. moricandiana* also were evaluated [12]. Discretamine, an alkaloid isolated from *D. moricandiana*, demonstrated antinociceptive activity in experimental models [13]. The ethanol extract from the fruits of *D. chrysocarpa* was evaluated for its antinociceptive activity and produced a significant antinociceptive effect. The phytochemical investigation yielded the isolation of the benzenoid derivative 3-methoxy-4-ethoxy benzoic [14].

In a previous study, we evaluated the phenolic quantification and antioxidant activity of *Anaxagorea dolichocarpa* and *Duguetia chrysocarpa* [15]. The aim of the present work is to investigate the chemical composition and cytotoxicity of these species.

EXPERIMENTAL

Plant material

The stem barks of *Anaxagorea dolichocarpa* Sprague & Sandwith were collected in the city of Santa Rita, State of Paraíba, Brazil, in February 2006. A voucher specimen was deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), of the Federal University of Paraiba, with the code Agra & Góes 5543. The stem barks of *Duguetia chrysocarpa* Maas were collected in Santa Rita, State of Paraiba, Brazil, in January 2004. A voucher specimen (no. Agra 5538) was also deposited at the Herbarium Prof. Lauro Pires Xavier (JPB), of the Federal University of Paraiba.

Preparation of extracts

The stem bark of *A. dolichocarpa* (2000 g) and *D. chrysocarpa* (2000 g), dried and pulverized, were subjected to maceration with ethanol (EtOH) 95 % for 72 h. The EtOH solution was concentrated under vacuum yielding 64 and 107 g of crude ethanolic extract of *A. dolichocarpa* (Ad-EtOH) and *D. chrysocarpa* (Dc-EtOH), respectively. The extracts were suspended in a mixture of methanol (MeOH) and water (H_2O) (proportion 3:7 v/v) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in crescent order of polarity to obtain the respective extracts.

Qualitative analysis of phytochemicals

The extracts were evaluated on thin layer plates of silica gel 60 F254 aluminum supports, applied

with a micropipette and eluted in different solvent systems as described by Wagner and Bladt [16] and Sobrinho *et al* [17], seeking to highlight the main groups of secondary metabolism (Table 1).

Cell lines and culture

Human tumor cell lines, including OVCAR-8 (ovarian), SF-295 (brain) and HCT-116 (colon) were obtained from the National Cancer Institute (Bethesda, MD, USA). All cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37 ^{o}C with 5 % CO_2.

Determination of cytotoxicity

All extracts were tested for cytotoxic activity against three tumor cell lines. For all experiments, cells were plated in 96-well plates: OVCAR-8 (0.1×10^6 cells/ml), SF-295 (0.1×10^5 cells/ml) and HCT-116 (0.7×10^6 cells/ml). After 24 h, all extracts (50 µg/ml) dissolved in 1 % DMSO was added to each well using a high-throughput screening system (Biomek 3000 – Beckman Coulter, Inc. Fullerton, CA, USA), and the cultures were incubated for 72 h. Control groups received the same amount of DMSO. The general viability of cultured cells was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product [18]. At the end of the incubation, the plates were centrifuged and the medium was replaced with fresh medium (150 µl) containing MTT (0.5 mg/ml). Three hours later, the plates were centrifuged, the MTT formazan product was dissolved in 150 µl DMSO, and the absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The extract effect was quantified as the percentage of the control absorbance of the reduced dye at 595 nm. All absorbance values were converted into cell growth inhibition (GI) values, using Eq 1.

$$GI\ (\%) = 100 - \{(T/C)100\} \quad (1)$$

where C was the absorbance for negative control and T was the absorbance in the presence of the tested extracts.

Statistical analysis

Data are presented as growth inhibition (GI, %). Each sample was tested in triplicate in two independent experiments. Values were computed using GraphPad Prism® 5.0 program. Statistically significant differences between groups were computed by Student's t-test. Values were considered significantly different $p < 0.05$.

RESULTS

Phytochemical profile of extracts

Preliminary analysis by TLC demonstrated that all extracts were positive for the presence of flavonoids and tannins. All extracts of *D. chrysocarpa* were positive for the presence of alkaloids. The hexane, chloroform and ethyl acetate extracts of *D. chrysocarpa* also showed positive reaction for the presence of anthracene derivatives, coumarins, mono and diterpenes.

Table 1: Elution systems and reagents used to characterize the main secondary metabolites of the extracts of *Anaxagorea dolichocarpa* and *Duguetia chrysocarpa* by thin layer chromatography

Phytochemical	Elution system	Standard	Reagent
Alkaloids	Toluene: ethyl acetate: diethylamine (70:20:10, v/v)	Yohimbine	Dragendorff reagent
Anthracene derivatives	Ethyl acetate: methanol: water (100:13.5:10, v/v)	Aloin	10 % ethanolic KOH reagent
Coumarins	Toluene: ethyl ether: (1:1 saturated with acetic acid 10 %, v/v)	Scopoletin	10 % ethanolic KOH reagent
Flavonoids and tannins	Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26, v/v)	Quercetin	NEU reagent
Lignans	Chloroform: methanol: water (70:30:4, v/v)	Flaxseed extract	Vanillin sulfuric reagent
Mono and diterpenes	Toluene: ethyl acetate (93:7, v/v)	Thymol and carvacrol	Vanillin sulfuric reagent
Naphthoquinones	Toluene: formic acid (99:1, v/v)	Lapachol	10 % ethanolic KOH reagent
Triterpenes and steroids	Toluene: chloroform: ethanol (40:40:10, v/v)	Lupeol and β-Sitosterol	Liebermann-Burchard reagent

Extracts of *A. dolichocarpa* were negative for the presence of anthracene derivatives, lignans and naphtoquinones. Extracts of *D. chrysocarpa* were negative for the presence of naphtoquinones. The presence of compounds in the extracts ranged to low presence (+) to strong presence (+++). Some classes of secondary metabolites were not detected in extracts. These results are presented in Tables 2 and 3.

Cytotoxicity

The extracts obtained from the stem barks of *A. dolichocarpa* and *D. chrysocarpa* did not demonstrate cytotoxic activity at tested concentration, except for hexane extract of *A. dolichocarpa*. In the test with tumor cells, the extracts showed, in general, low (1 – 50 %) to moderate (51 – 75 %) cell growth inhibition, with values ranging from -5.38 to 89.02 % (Table 4).

DISCUSSION

As expected, phenols were found in all extracts, confirming the previous study with the two plants [15]. Among the phenolic compounds, flavonoids and tannins occurred more frequently.

Among the medical properties attributed to plants and a gamut of varieties encountered in tropical countries, among them Brazil, there is innumerable research with the objective of identifying efficient activities against cancer,

Table 2: Phytochemical profile of extracts of *Anaxagorea dolichocarpa* (Ad)

Phytochemical	Ad-EtOH	Ad-Hex	Ad-$CHCl_3$	Ad-AcOEt
Alkaloids	+++	++	+	-
Anthracene derivatives	-	-	-	-
Coumarins	++	+	-	+++
Flavonoids and tannins	+++	+	+++	++
Lignans	-	-	-	-
Mono and diterpenes	++	+	-	-
Naphthoquinones	-	-	-	-
Triterpenes and steroids	+++	+	+++	-

(-) not detected; (+) low presence; (++) moderate presence; (+++) strong presence. Ad-EtOH (crude ethanol extract), Ad-Hex (hexane extract), Ad-$CHCl_3$ (chloroform extract), Ad-AcOEt (ethyl acetate extract)

Table 3: Phytochemical profile of extracts of *Duguetia chrysocarpa* (Dc)

Phytochemical	Dc-EtOH	Dc-Hex	Dc-$CHCl_3$	Dc-AcOEt
Alkaloids	+	++	++	++
Anthracene derivatives	-	+	+	+
Coumarins	-	+++	++	+
Flavonoids and tannins	+++	++	++	+
Lignans	++	++	-	-
Mono and diterpenes	-	+++	++	+
Naphthoquinones	-	-	-	-
Triterpenes and steroids	+	+	+	-

(-) not detected; (+) low presence; (++) moderate presence; (+++) strong presence. Dc-EtOH (crude ethanol extract), Dc-Hex (hexane extract), Dc-$CHCl_3$ (chloroform extract), Dc-AcOEt (ethyl acetate extract)

Table 4: Cell proliferation inhibition (%) of extracts of *Anaxagorea dolichocarpa* and *Duguetia chrysocarpa* determined by MTT assay after 72 h of incubation at a concentration of 50 µg/mL

Extract	Cell proliferation inhibition (%)		
	OVCAR-8	***SF-295***	***HCT-116***
A. dolichocarpa			
Ad-EtOH	53.94	65.49	50.19
Ad-Hex	44.31	62.68	89.02
Ad-$CHCl_3$	52.51	58.82	67.15
Ad-AcOEt	-5.38	0.09	3.40
D. chrysocarpa			
Dc-EtOH	49.65	43.95	60.16
Dc-Hex	18.39	34.84	15.61
Dc-$CHCl_3$	42.24	63.17	59.35
Dc-AcOEt	13.01	30.99	9.76

Tumor cell lines: OVCAR-8 (ovarian), SF-295 (brain), HCT-116 (colon)

from which several studies are being made to find active substances with anti-cancer activity [19].

In our continuing search for new biologically active extracts and compounds from the Annonaceae family, as well as novel plant antitumor agents, the present study was designed to investigate the cytotoxic activity of extracts obtained from *A. dolichocarpa* and *D. chrysocarpa*.

The cytotoxicity of the extracts was tested against OVCAR-8 (ovarian), SF-295 (brain) and HCT-116 (colon) human tumor cell lines using the thiazolyl blue test (MTT) assay. MTT assay is a well-characterized colorimetric assay that is based on the enzymatic reduction of the tetrazolium salt MTT in living, metabolically active cells, but not in dead cells. It has been largely used to determine cytostatic/cytotoxic potential of medicinal agents in screening programs [20]. The cytotoxicity of the extracts was tested at a concentration of 50 µg/ml. Those extracts that caused more than 75 % cell growth inhibition in any cell line were considered active.

The study of natural products represents the most successful strategy for discovering new drugs for anticancer therapy [21] and the potential of extracts and chemical constituents of the plants from the Annonaceae family to inhibit tumor cells has attracted increasing interest.

The hexane extract of *A. dolichocarpa* was found to show the strongest cytotoxicity against cell culture of human colon tumor (HCT-116) with percentage of cell growth inhibition of 89.02 %. From the hexane extract of this plant were obtained the alkaloids sampangine and imbiline 1. Sampangine showed antitumoral activity against leukemic cells [10], suggesting that the antitumor activity of the hexane extract could be related to the presence of this active metabolite.

The cytotoxicity of extracts and essential oils of other species of Annonaceae were previously investigated. Extracts obtained from *Guatteria blepharophylla* and *Guatteria hispida* could be considered promising for anti-cancer drug development because its extracts presented values of IC_{50} below of 30 µg/ml. Essential oils obtained from *G. blepharophylla* and *G. hispida* also presented strong cytotoxic activity. Extracts of *Annona pickelli* and *Annona salzmannii* did not show any expressive antiproliferative effect. In these species, it seems therefore that associations of compounds or the minor compounds are responsible for their cytotoxic activity [22]. The presence of acetogenins identified as the major constituents of some Annonaceae species could explain the anti-cancer activity observed for *Annona crassiflora* and *Xylopia aromatica* [23].

The moderate activity of the other extracts of *A. dolichocarpa* and *D. chrysocarpa* with activity ranging 50 to 75 % can also be considered interesting, taking into account that the cell lines are tumor cells. These results encourage further phytochemical studies to characterize the active molecules in the extracts.

CONCLUSION

The findings of this work demonstrate, for the first time, the cytotoxic activity of the stem bark extracts of *A. dolichocarpa* and *D. chrysocarpa*. These extracts possess moderate to high cytotoxic activity against some human tumor cell lines. The results also provide some evidence that plants of the Annonaceae family are a rich source of active secondary metabolites with cytotoxic activity, and thus can guide future phytochemical studies in the search for novel anticancer agents.

ACKNOWLEDGEMENT

This work was supported by grants from the following Brazilian government agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP).

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