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Antiproliferative effect of *Amburana cearensis* seed extracts on human cancer cell lines

Aline Aires Aguiar[1*], Ernane Gerre Pereira Bastos[2], Ilsamar Mendes Soares[1], Ivana Grivicich[2], Jéssica Machado Miri[2], Sérgio Donizeti Ascêncio[1] and Raimundo Wagner de Souza Aguiar[1]

[1]Federal University of Tocantins (UFT), Palmas, Tocantins, Brazil.
[2]Lutheran University of Brazil (ULBRA), Canoas, RS, Brazil.

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Amburana cearensis **A. C. Smith (Fabaceae) is a plant of Brazil, popularly known as umburana or cumaru, and is widely used in folk medicine. The population uses its bark and seeds against many pathologies including cancer. The aim of this study was to examine the antiproliferative effect of *A. cearensis* extracts on human tumor cell lines of HT-29 colon adenocarcinoma and HepG2 hepatocellular carcinoma, as well as on normal mouse fibroblasts L929. This study consists of *in vitro* tests with extracts obtained from *A. cearensis* seeds with solvents of increasing polarity against neoplastic and normal cell lines. Knowing that *A. cearensis* extracts are rich in coumarins and have high antioxidant activity, aspects related to antineoplastic activity and cytotoxicity of four different extracts was evaluated. *A. cearensis* extracts were analyzed in cancer cells using the MTT assay. Cytotoxicity data demonstrated that methanolic extract (MEA) has activity against HT-29 cell line (IC_{50} 18.8 ± 0.4 µg mL^{-1}) while normal fibroblasts L929 and HepG2 cells were not affected by the extract. The crude seed extracts of *A. cearensis* did not demonstrate a cytotoxic effect against the cancer cell used in this study; however, the MEA extract can also be promising for this purpose.**

Key words: *Amburana cearensis,* cytotoxicity, antiproliferative, tumor cell, coumarin.

INTRODUCTION

The carcinogenesis process results from changes that accumulate progressively in the genetic material (DNA) of a normal cell. This process is related to exposure to chemical, physical and/or biological carcinogens present in the environment, to the lifestyle of a given individual or population, and to genetic and epigenetic factors (Parmigiani and Camargo, 2010; Vineis et al., 2010). Cancer is an epidemic disease that affects all regions, races and socioeconomic classes worldwide (Linsalata and Russo, 2008).

Colorectal cancer (CCR) is the second most important cause of cancer death in North America and Western Europe (Jemal et al., 2008) and it has a higher incidence in western countries (Linsalata and Russo, 2008). Diet is an important factor in their prevention (Block et al., 1992). In Brazil, according to INCA - 2018 (National Cancer Institute), the incidence varies according to the region evaluated, being higher in the south and southeast regions. Hepatocellular carcinoma (HCC) is the most common form of liver cancer, accounting for 85 to 90% of

*Corresponding author. E-mail: alineairesaguiar@hotmail.com.

all liver cancers (El-Serag and Rudolph, 2007). The HCC is associated to infection by the hepatitis virus and contact with carcinogens, such as aflatoxins (Idrees et al., 2009).

Plants have a long history of use in the treatment of cancer, serving as a source of therapeutic agents for many centuries and are used or served as the basis for the development of synthetic drugs (Cragg and Newman, 2005). Medicinal plants are traditionally used for the treatment of malignant neoplasms and have attracted considerable attention (Johnson et al., 2010). Because they exhibit pharmacological effects and may be potent chemotherapeutic agents (Hwang et al., 2007). Many studies have demonstrated that several secondary metabolites have antiproliferative activity, either by inducing apoptosis, or by their antioxidant capacity (Aguiar et al, 2017, Ezekiel et al., 2013; Feng et al., 2005). For example, polyphenols have been shown to inhibit cancer in various ways, acting as antioxidants, affecting signal transduction pathways, inducing apoptosis and inhibiting angiogenesis (Yang et al., 2009). Drugs such as vincristine and vinblastine, isolated from *Catharantus roseus* (Carvalhaes et al., 2002) are examples of plant metabolites used in chemotherapy. Currently, the chemistry of natural products is one of the main lines of research in the search for new anticancer agents (Silva et al., 2003). Sixty percent of antitumor drugs approved in the United States have a natural origin, and among these, many are secondary compounds of higher plant metabolism (Graham et al., 2000). The aim of the present study was to evaluate the *in vitro* antiproliferative activity of the *Amburana cearensis* seeds crude extract and fractions against three different cell lines: colorectal adenocarcinoma cells (HT-29), hepatocellular carcinoma cells (HepG2) and normal fibroblast cells (L929).

MATERIALS AND METHODS

This study was carried out in Natural Products Research Laboratory (LPPN) of Federal University of Tocantins (UFT), Palmas, Tocantins, Brazil and in Laboratory of Cancer Biology - Lutheran University of Brazil (ULBRA), Canoas, RS, Brazil.

Plant

The seeds of *A. cearensis* were obtained from popular commerce in the city of Palmas, Tocantins, Brazil, and their authenticity recognized by EMBRAPA Herbarium, voucher specimen (CPAP 5948). The seeds were dried in drying oven at 45°C and powdered using knives mill (Start FT 50 - Fortinox). The powder obtained was stored in glass bottles and kept at room temperature and sheltered from light and moisture (Al-Marby et al., 2016).

Seed extract preparation

To prepare the extracts, 15 g of pulverized seeds were extracted for 4 h using the Soxhlet apparatus with different solvents, *n*-hexane, methanol, ethanol 80% and water, from the least to the highest polarity according to the methodology of Soares et al. (2017). All solutions obtained had the solvents removed on a rotary evaporator at -600 mm Hg (Fisaton 804) and 45°C. They were then dried in the exhaust hood and stored in an amber bottle and kept at 4°C. The hexanic, methanolic, 80% ethanolic and water extracts were respectively named as, HEA, methanolic extract (MEA), EEA and AEA.

Phytochemical screening

The plant material was subjected to qualitative chemical screening for the identification of the major classes of active constituents. The phytochemical profile of *A. cearensis* seeds was determined according to methodology described by Matos (2009). The method consists in colorimetric reactions for qualitative detection of flavonoids, tannins, alkaloids, saponins and coumarins.

Cell lines used

The human colon adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (Rockville, MD, USA), and the mouse normal fibroblast cell line L929 and hepatocellular carcinoma cell line HepG2 were purchased from the RJCB Collection (Rio de Janeiro, RJ, Brazil).

Cytotoxic assay

Cell cultures were maintained in 25 cm^2 culture flasks with Dulbecco's Modified Eagle's medium culture medium (DMEM) containing 10% heat-inactivated fetal bovine serum (v/v) and antibiotic (1% penicillin/streptomycin) at 37°C in an atmosphere of 5% CO_2 and humidity of at least 95%. For the experiments, cell cultures were inoculated into 96-well microplates at a density of 5×10^4 cells/well/100 µL in triplicates. After stabilization for 24 h, the cultures were treated with serial concentrations (0 to 100 µg/ml) of the compounds. Cytotoxicity was assessed using the MTT colorimetric assay ((3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide)). Cultures in triplicates were exposed for 24 h to the compounds. After the treatments, the cells were incubated with 100 µL of MTT solution (1 mg/mL MTT) in fetal bovine serum free culture medium without phenol-red at 37°C for 4 h. After incubation, the supernatant was carefully removed, and the violet formazan crystals were solubilized in 200 µl of Dimethyl sulfoxide (DMSO) and quantified in a microplate reader (Multiskan, UNISCIENCE) at 540 nm optical density. The 10% DMSO was used as positive control. Each experiment was performed 3 times independently.

For compounds that exhibited cytotoxicity according to ISO10993-5 guide (ISO, 2009) (that is, cell viability less than 70%), the IC_{50} value (amount of compound required to inhibit growth/induce death of 50% of cells) was determined. IC_{50} values of 20 to 30 µg/mL were considered by the National Cancer Institute (USA) to be of interest to crude extracts with anticancer potential (Suffness and Pezzuto, 1990).

The extracts concentration of 25 µg/mL was used as a parameter to evaluate the cytotoxic potential of the compound. The extracts concentration of 12.5 µg/ml (half the previous dose) was also analyzed in order to see if reducing the dose would also have cytotoxicity.

Gas chromatography-mass spectrometry (GC-MS) analysis

Qualitative analyses were performed through GC-MS using the

Table 1. Phytochemical profile of *A. cearensis* extracts.

Extract	Saponins	Alkaloids	Flavonoids	Coumarins	Tannins
CEA	+		+	+	
HEA			+	+	+
MEA		+	+	+	+
EEA			+	+	
AEA		+	+	+	+

+ indicates the presence of the compound.
Crude extract (CEA); hexanic extract (HEA), methanolic extract (MEA), 80% ethanol extract (EEA) and water extract (AEA).

Shimadzu GC-2010 model equipped with selective detector for the mass Model QP2010Plus, with the equipment operated under the following conditions: fused silica capillary column RTX-5MS (30 m × 0.25 mm × 0.25 µm film thickness), with the following schedule of temperature in the column: 60 to 240°C (3°C min-1), temperature of the injector 220°C, helium gas carrier, injection with rate of split (1:100) with injected volume of 1 µL of a solution 1:1000 in hexane. For the mass spectrometer (MS), the following conditions were used: impact energy of 70 V and temperature of the source of ions and the interface 200°C. A homologous series of *n*-alkanes (C_9H_{20} … $C_{26}H_{54}$) were injected under the same conditions as samples. The constituents were identified by comparing their spectra of masses with those from the databases from the Nist and Wiley 229 libraries and by comparing between their rates of retention calculated using those reported in the literature (Adams, 2007). The quantification of the levels of the compounds, expressed as a percentage based on the standardization of areas, was obtained by using a gaseous chromatograph equipped with a detector flame (DIC), using a diagnostic Shimadzu GC-2010, in the following experimental conditions: a capillary column RTX-5MS (30 m × 0.25 mm × 0.25 µm film thickness); temperature of the injector 220°C; temperature of the DIC 300°C; programing the column: initial temperature of 60°C with a heating rate of 3°C min^{-1} up to 240°C, then increasing to a heating rate of 10°C min^{-1} up to 300°C and remaining at this temperature for 10 min; nitrogen drag gas (1.18 mL min^{-1}); rate of split 1:50; pressure in the column of 115 kPa, and injected volume of 1 µL, diluted in hexane (1:100 v/v). The calculated retention index was performed according to Mühlen (2009).

Statistics

All the experiments were conducted in triplicates. The differences in means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. $P \leq 0.05$ was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Phytochemical analyses

Phytochemical analyses of *A. cearensis* seeds indicated the presence of saponins, alkaloids, flavonoids, coumarins and tannins (Table 1). Lopes (2003) have shown that secondary metabolites are frequently associated with cytotoxic potential that is associated with increased oxidative damage. This data was also reported by Rattmann et al. (2005) in *C. roseus*, which has alkaloids as vinblastine and vincristine, that are cytotoxic.

Cytotoxic assay

Evaluation of cell proliferation was performed using the MTT assay, where it is reduced to formazan purple crystals by living cell mitochondria. Increased numbers of cells are detected by increased metabolism of MTT, and decrease in the number of cells is reflected by decreased metabolism of the same (Seoane et al., 2010). *A. cearensis* seeds crude extract (CEA) and fractions (HEA, MEA, EEA and AEA) were assayed against three different cells: HT-29; HepG2 and L929. The cytotoxicity was measured and the IC_{50} values were determined. The HEA and AEA extracts showed cytotoxic action against L929 and HepG2 cells at concentration 25 µg mL^{-1} (Figure 1). Meanwhile, HEA, MEA and AEA extracts showed cytotoxic action against HT-29 cells at concentration 25 µg mL^{-1} (Figure 1). At concentration of 12.5 µg mL^{-1} the extract AEA showed cytotoxicity against L929 and HT-29 cells (Figure 2).

Toxic concentrations of the 3 extracts that reduce the viability of HT-29, HepG2 and L929 cells by 50% (IC50) were determined. The HEA, MEA and AEA have demonstrated to be active in the HT-29 cell line (IC_{50} 46.2 ± 0.2, 18.8 ± 0.4 and 41.7 ± 4.6 µg mL^{-1}, respectively) (Table 2). Moreover, the MEA demonstrates a great antiproliferative effect in this cell line.

National Cancer Institute (USA) considers extracts with IC_{50} values of 20 to 30 µg mL^{-1} good candidates to anticancer compounds (Suffness and Pezzuto, 1990). Based on this, the MEA extract has been shown to be the most promising extract of *A. cearensis* seeds. In addition, this extract has no cytotoxic action against L929 cells from mice, inferring that it is not cytotoxic against non-cancerous cells. To the best of our knowledge, this is the first study to examine the effect of *A. cearensis* seeds in cancer cell lines.

CG-MS analysis

Many compounds were identified in *A. cearensis* HEA,

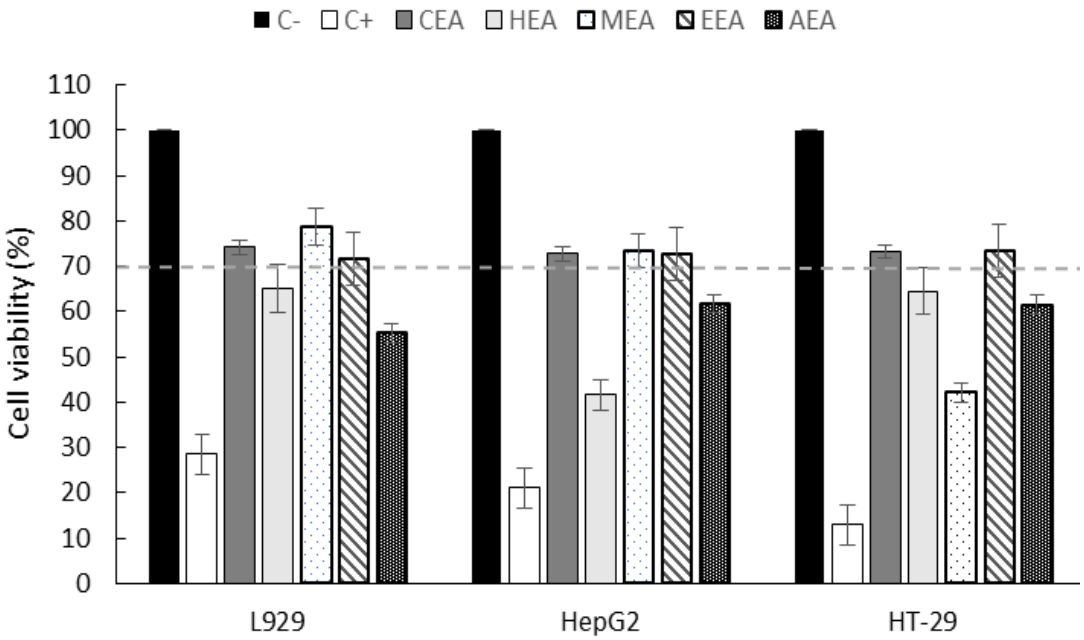

Figure 1. Cell viability expressed as a percentage (mean ± standard deviation), with the untreated control (C-) considered as 100% viability. Extract concentration (25 µg mL^{-1}); (C+) = positive control (10% DMSO).

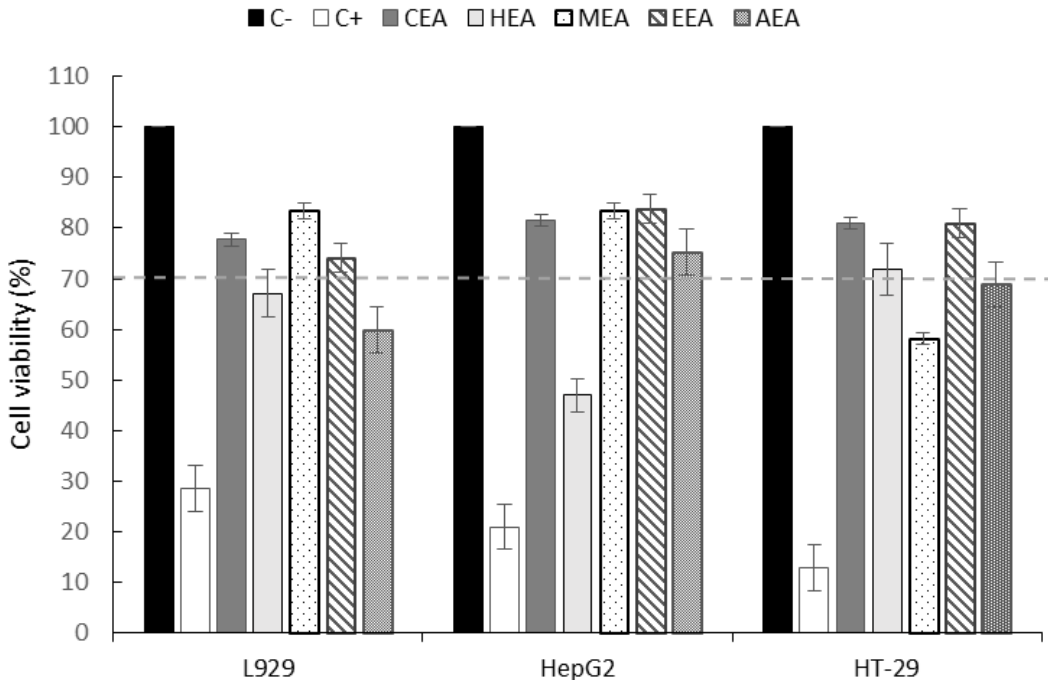

Figure 2. Cell viability expressed as a percentage (mean ± standard deviation), with the untreated control (C-) considered as 100% viability. Extract concentration (12.5 µg mL^{-1}); (C+) = positive control (10% DMSO).

MEA and AEA extracts (Table 3), such as 2,3-dihydro-benzofuran, catechol, hydrocoumarin, coumarin; the predominant molecule is coumarin with 14.41% of area for HEA extract (Figure 3, Table 4), 55.62% of area for MEA (Figure 5, Table 6) and 62.75% for AEA extract (Figure 4, Table 5). Recent data suggests that coumarin

Table 2. IC_{50} values of *A. cearensis* extracts (µg mL^{-1}: mean ± SD, *n* = 6) that presented viability less than 70%.

Extract	Cell lines (IC_{50})		
	HT-29	HepG2	L929
HEA	46.2 ± 0.2	87.4 ± 8.5	65.0 ± 6.1
MEA	18.8 ± 0.4	ND	ND
AEA	41.7 ± 4.6	44.2 ± 1.9	59.1 ± 3.7

Hexanic extract (HEA), methanolic extract (MEA) and water extract (AEA).
ND: Values could not be determined in the concentrations tested.

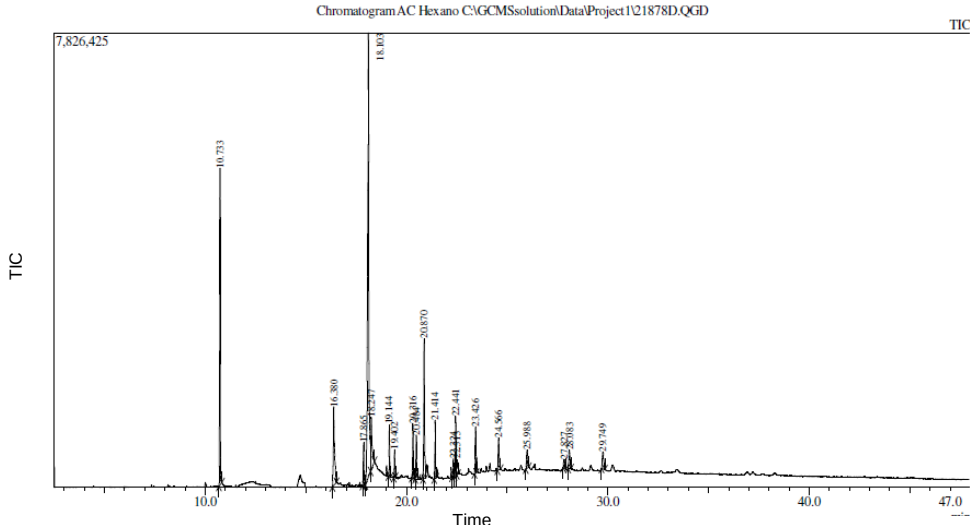

Figure 3. CG-MS of HEA extract of *A. cearensis* seeds.

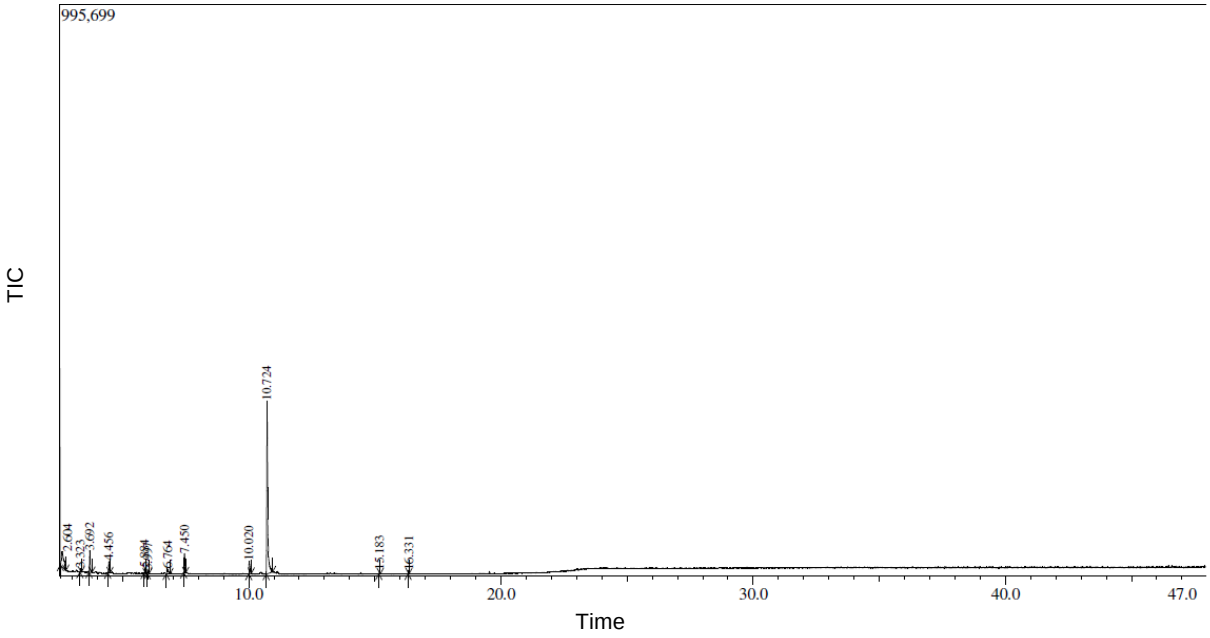

Figure 4. CG-MS of AEA extract of *A. cearensis* seeds.

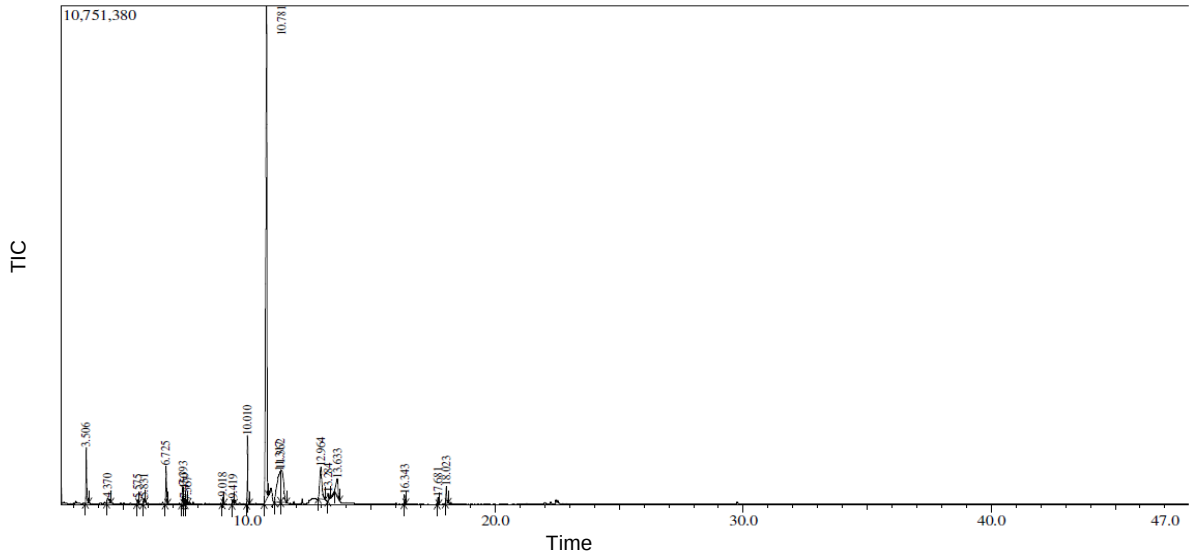

Figure 5. CG-MS of MEA extract of *A. cearensis* seeds.

Table 3. Chemical constituents of *A. cearensis* seed extracts in GC-MS analysis.

Compound	Extracts		
	HEA	MEA	AEA
l-(+)-Ascorbic acid 2,6-dihexadecanoate		+	
2,3-dihydro-Benzofuran	+	+	
1,6-anhydro-beta-D-Glucopyranose		+	+
Catechol		+	+
Coumarin	+	+	+
D-Allose		+	
Decanal		+	+
2-Hydroxy-gamma-butyrolactone		+	
Hydrocoumarin		+	+
Oleic Acid	+	+	
1,3,5-Triazine-2,4,6-triamine		+	

Hexanic extract (HEA), methanolic extract (MEA) and water extract (AEA).
+: Presence of the compound in extract.

may exert significant antineoplastic effects in several systemic malignancies and of clinical benefit in mammary malignancies (Kapoor, 2013). Coumarin also attenuates the extent of extremity lymphedema following breast cancer surgery and radiation therapy. In a study, almost 12.5% decline in the volume of upper extremity lymphedema was noticed. As a result, quality of life is significantly improved, and attenuation of tumor growth has been seen in pulmonary malignancies (Liu et al., 2012).

Choi et al. (2015) have demonstrated that the 2,3 dihydrobenzofuran molecule is cytotoxic for following cancer cell lines: HCT-15 (colon), NUGC-3 (gastric), and NCI-H23 (lung). In addition, this compound also inhibited NF-κB transcriptional activity. The GC-MS analysis has shown this compound in MEA extract (Table 3). Arulmurugan and Kavitha (2013) have shown that the molecule 1,3,5-Triazine-2,4,6-triamine has potent cytotoxicity against HT-29 and MCF-7 (breast cancer) cells. MEA extract has also this molecule, corroborating the cytotoxicity potential of this extract showed in this study. Purushothaman et al. (2013) demonstrated that *Hibiscus rosa-sinenis* extract is rich in 1,6-anhydro-beta-D-glucopyranose as in MEA extract, and in their study, it induces a significant reduction of tumor multiplicity and tumor volume in a defined experimental rat mammary carcinogenesis model mammary carcinoma in rats.

D-Allose, also present in MEA extract, is a rare

Table 4. Chemical composition of HEA extract of *A. cearensis*.

Peak #	Name	RT	Area (%)
1	Coumarin	10.733	14.41
2	*n*-Hexadecanoic acid	16.380	6.28
3	Cyclooctasiloxane, hexadecamethyl-	17.865	1.98
4	Oleic Acid	18.103	40.07
5	Octadecanoic acid	18.247	4.44
6	Cyclononasiloxane, octadecamethyl-	19.144	2.21
7	Glycidyl palmitate	19.402	1.06
8	Cyclodecasiloxane, eicosamethyl-	20.316	2.51
9	Oleoyl chloride	20.484	2.07
10	Glycidyl oleate	20.870	7.33
11	Tetracosamethyl-cyclododecasiloxane	21.414	2.63
12	Petroselinic acid, TMS derivative	22.324	0.84
13	Tetracosamethyl-cyclododecasiloxane	22.441	3.10
14	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	22.513	0.82
15	Tetracosamethyl-cyclododecasiloxane	23.426	2.45
16	Tetracosamethyl-cyclododecasiloxane	24.566	2.26
17	Tetracosamethyl-cyclododecasiloxane	25.988	1.47
18	Tetracosamethyl-cyclododecasiloxane	27.827	0.82
19	Heptacosanal	28.083	1.55
20	gamma.-Sitosterol	29.749	1.69

RT = Retention time.

Table 5. Chemical composition of AEA extract of *A. cearensis*.

Peak#	Name	RT	Area (%)
1	2-Hydroxyisocaproic acid, methyl ether, methyl ester	2.604	13.34
2	4-Ethylbenzoic acid, tridec-2-ynyl ester	3.323	0.69
3	2-Cyclopenten-1-one, 2-hydroxy-	3.692	5.46
4	Decane, 6-ethyl-2-methyl-	4.456	3.15
5	3-Pentanone, 2-methyl-	5.884	1.18
6	Oxalic acid, butyl propyl ester	5.997	0.61
7	N,N-Dibenzylethylenediamine diacetate	6.764	2.69
8	Decanal	7.450	4.05
9	1H-2-Benzopyran-1-one, 3,4-dihydro-	10.020	4.94
10	Coumarin	10.724	62.75
11	2,2'-Bifuran, 2,2',5,5'-tetrahydro-	15.183	0.49
12	Butanoic acid, 2-hydroxy-2-methyl-, methyl ester	16.331	0.65

RT = Retention time

monosaccharide known to exert anti-proliferative effects on cancer cells. The effects of D-Allose on the cellular membranes of hormone-refractory prostate cancer cell line (DU145), prostate cancer cell (LNCaP), and normal prostate epithelial cells (PrEC) were shown by Jeong et al. (2011).

Recently, the interest in phenolics has increased due to its protective role, through the ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease and cancer (Fernandes et al., 2010). In this line, it was demonstrated that the presence of phenolic compounds in different plants has antitumor effect (El-Hawary et al., 2012; Bhatt et al., 2013; Hasibuan et al., 2013).

The natural products are frequently associated to anticancer properties, inferring that bioactive compounds present in *A. cearensis* seeds extracts might be responsible for their cytotoxic activity against the cells

Table 6. Chemical composition of MEA extract of *A. cearensis*.

Peak#	Component	RT	Area (%)
1	1,2-Cyclopentanedione	3.506	3.50
2	2-Hydroxy-gamma-butyrolactone	4.370	0.82
3	1,3,5-Triazine-2,4,6-triamine	5.575	0.29
4	2-methoxy-Phenol	5.831	0.31
5	2,3-dihydro-Benzofuran	6.725	2.36
6	Catechol	7.393	1.49
7	Decanal	7.450	0.35
8	1,4:3,6-Dianhydro-alpha.-d-glucopyranose	7.567	3.89
9	1-(2-hydroxy-5-methylphenyl)-Ethanone	9.018	1.74
10	4-hydroxy-Benzenemethanol	9.419	2.58
11	Hydrocoumarin	10.010	4.29
12	Coumarin	10.781	55.62
13	D-Allose	11.317	9.20
14	1,6-anhydro-beta-D-Glucopyranose	11.362	7.78
15	Ethyl .alpha.-d-glucopyranoside	12.964	6.25
16	Silane	13.284	2.28
17	4-O-Methylmannose	13.633	3.31
18	l-(+)-Ascorbic acid 2,6-dihexadecanoate	16.343	0.54
19	6-Octadecenoic acid, methyl ester	17.681	0.39
20	Oleic Acid	18.023	1.22

RT = Retention time.

studied in this work.

Conclusion

This study was the first to investigate the antiproliferative properties of *A. cearensis* seeds on cancer cells. It was verified that the MEA extract has active principles with relevant antiproliferative potential. The results obtained are promising for a chemical and medicinal point of view, and stimulate the continuity of this study for the search of compounds with potential antiproliferative activity, with the aim to discover new and effective anticancer therapeutic agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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