Quim. Nova, Vol. 45, No. 7, 803-806, 2022 http://dx.doi.org/10.21577/0100-4042.20170885

Artigo

METABOLIC PROFILING AND CYTOTOXIC ACTIVITY OF METHANOL EXTRACTS FROM *Chamaecrista duckeana* (P. BEZERRA & A. FERN.) H. S. IRWIN & BARNEBY (LEGUMINOSAE, CAESALPINIOIDEAE)

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Recebido em 22/12/2021; aceito em 10/02/2022; publicado na web em 05/04/2022

The genus *Chamaecrista* comprises more than 330 species, with only a few studies on their chemical composition and biologic activities. In this study, the phytochemical profile of leaf, stems, and fruits extracts of the *C. duckeana* were examined by UPLC-ESI-HRMS analysis to determine possibly bioactive constituents. The antioxidant activity was carried out through in vitro assay, by the sequestration of the free radical DPPH. To evaluate the cytotoxic activity of the extracts, an MTT assay was used and the IC_{50} was determined against HL60 and RAJI cell lines. The metabolic profiles of the botanical parts are dominated by flavonoid class, highlighting isoflavonoids such as daidzin and ononin. All these compounds are reported for the first time in *C. duckeana*. The extracts presented antioxidant potential, and the activity of the stems extract was higher than the standard butylated hydroxytoluene. In the cytotoxic assay, only HL60 line (leukemia) had growth inhibition over 80%. The stems presented more expressive cytotoxicity with IC_{50} of 137.3 (104.6-180.1) and 106.8 (96.52-118.3) μmol. L^{-1} for HL60 and RAJI, respectively. In conclusion, the present work provides an in-depth knowledge about the chemical profile of *C. duckeana,* a species rich in bioactive secondary metabolites with cytotoxic activity.

Keywords: *Chamaecrista duckeana*; UPLC-ESI-HRMS; cytotoxicity; isoflavonoids.

INTRODUCTION

Since ancient times, natural products have been used to treat human ailments. After recent developments and technological advances, natural products have received great attention as a source of new drug candidates, since they have the potential to discover new supports for therapeutic targets for the treatment of various diseases, including cancer.[1] Flavonoid class compounds, frequently identified in plants, are proposed as potential chemotherapeutics since flavonoids have a dual role, acting as antioxidants under normal conditions and as powerful pro-oxidants in cancer cells.[2] Mass spectrometry (MS) is a widely used method for detection, identification, and structural elucidation of flavonoids which, associated with the Ultra Performance Liquid Chromatography (UPLC), is an efficient tool for flavonoid analysis.[3]

Chemotherapy remains to be one of the main approaches in the clinical treatment of acute leukemia. This treatment strategy still has some limitations, such as side effects and multidrug resistance, which limits the therapeutic efficacy. Therefore, drugs for leukemia with lower toxicity and higher effectiveness are expected. Natural products are a source of inspiration in medicinal chemistry for biological activity, including antileukemic potential.[4–7]

The genus *Chamaecrista* has a pantropical distribution, comprising more than 330 species which are mainly distributed across tropical America, with a few species native to Africa, Asia, and Australia. It is well represented in the Brazilian flora, mainly in rupestrian and riparian fields where 256 species occur, with 207 of them being endemic.[8] Species of this genus are widely used in traditional medicine in Africa, Asia, and Americas and they have been reported to be used for important pharmacological activities such as cholinesterase inhibitory activity, antitrypanosomal and anticonvulsant activity for *Chamaecrista mimosoides,*[9–11] and activity against bacterial strains of *Shigella sonnei* for *Chamaecrista desvauxii*.[12] *Chamaecrista diphylla, Chamaecrista repens* var. *multijuga, and Chamaecrista mimosoides* presented good antioxidant properties.[10,13–15]

Chamaecrista duckeana is popularly known as "palma-do-campo", and it is a sub-shrub species that can reach up to 1 m in height.[16] In the only chemical study of the species, the GC-MS analysis of the essential oil extracted from the roots of the Brazilian *C. duckeana,* reported the presence of methyl chavicol, methyl eugenol, and eugenol.[17] In this context, this work characterizes the chemical composition of *C. duckeana,* using dereplication methods by UPLC-ESI-HRMS, which is an efficient and rapid analytical tool to obtain the chemical fingerprinting of the sample.[18–20] Furthermore, the evaluation of the antioxidant and cytotoxic properties against tumor cells of these extracts was performed.

EXPERIMENTAL

Chemicals and reagents

Solvent methanol (J. T. Baker, USA) was of chromatographic grade. The formic acid (Synth, Brazil) was of analytical grade (ACS). Ultrapure water was obtained using a Millipore water purification system (Millipore, USA). BHT, quercetin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma (USA).

Plant material collection

Leaves, stems, and fruits of *C. duckeana* (P. Bezerra & A. Fern.) H. S. Irwin & Barneby were collected in September 2018, in Massapê, Ceará, Brazil. *C. duckeana* was authenticated by the

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Federal University of Ceará Herbarium Prisco Bezerra (Ceara State, Brazil) as number 61466. The plant is registered in the National Genetic Heritage Management System (SISGEN) under the number AB06D11. After collection, leaves, stems, and fruits were kept in an oven at 40 °C until drying (from 12 to 24 h, depending on the collected material).

Plant material extraction

The dried and grounded samples were extracted with ultrasound assistance (UAE) in a ratio of 1 g of plant material to 30 mL of methanol three times, and then dried on a rotary evaporator. The dried extracts were weighed and stored at -18 °C until analysis.

Sample preparation for UPLC-ESI-HRMS analysis

The extract solutions were prepared at concentrations of 1 mg mL^{-1} for UPLC-ESI-HRMS analysis and filtered on Chromafil®Xtra RC-20/25 membranes (0.20 μm pores) before analysis.

UPLC-ESI-HRMS analysis settings

The chromatography was performed on a 100×2.1 mm Waters® ACQUITY UPLC® 1.8 μm HSS T3 column in an ACQUITY Ultra Performance LC™ system equipped with a QTOF mass spectrometer LC-MS/MS platform. The column was kept at 40 °C. Injection volume was 0.8 μL. The flow rate was set at 0.580 μL min^{-1} and gradient elution was carried out with a binary system consisting of [A] 0.1% aqueous formic acid (Synth, Brazil) and [B] 0.1% formic acid (Synth, Brazil) in methanol (J. T. Baker, USA). The gradient elution was programmed as follows: 5–95% (B) from 0 to 4.31 min, 100% (B) from 4.31 to 5.75 min. Data was collected in centroid mode, with a lock spray frequency of 10 s, and the data was averaged over 10 scans. Calibration with sodium formate (reference mass 860.8467 uma) was performed in positive mode within an *m/z* range between 100 to 1200 in positive ionization mode. MassLynx software (version 4.1, Waters) was used for data acquisition and processing. Experimental *m/z* and MS/MS spectra obtained at both higher and lower energy were used for compound matching across UNIFI platform from Waters Corporation, a database with more than 6000 compounds.

Cytotoxic assay

Cell lines culture

The human tumor cell lines SNB19 (central nervous system), HCT116 (human colon), PC3 (prostate), HL60, and RAJI (leukemia) were gently provided by the National Cancer Institute (Bethesda, MD, USA). The cells were maintained as monolayer cultures in appropriate media. The RPMI 1640 was supplemented with 10% fetal bovine serum (FBS), 1% antibiotic at 37 °C in a humidified atmosphere of 5% CO_2.

Determination of cytotoxicity

The cytotoxic effect of the samples, fruits, stems and leaves were determined by MTT assay.[21] The three extracts were first tested against four human cancer cell lines: SNB19 (central nervous system), HCT116 (human colon), PC3 (prostate), and HL60 (leukemia). In this round of cytotoxicity evaluation, cell growth inhibition was estimated for all cell lines treated with the different extracts at a single concentration (100.0 μg mL^{-1}) for 72 hrs to determine the percentage of cell growth inhibition (GI%). In the second round, the half-maximal inhibitory concentration (IC_{50}) against HL60 and RAJI cell lines was determined. The cells were plated at concentrations of 0.3 x 10^6 cells mL^{-1}. After the incubation, the supernatant was replaced by a fresh medium containing MTT (100 μL). Three hours later, the formazan product was dissolved in 100 μL of DMSO, and the absorbance was measured at 595 nm (DTX-880 Multimode Detector, Beckman Coulter®). The samples were diluted in DMSO to stock concentrations of 20 mg mL^{-1}. To perform the single concentration test, all samples were tested on a concentration curve that ranged from 3.13 to 200 μg mL^{-1}.

Statistical analysis

Single concentration experiments were analyzed according to the mean ± standard deviation (SD) of the percentage of cell growth inhibition rom three experiments carried out in triplicate, using the GraphPadPrism 6 program.

Antioxidant assay

The antioxidant activity was determined using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method, according to Yepes *et al.*[22] In a test tube, 3.9 mL of a 6.5×10^{-5} mol L^{-1} methanol solution of DPPH was mixed with 0.1 mL of each methanolic extract solution. After 60 min, the absorbance was read using a spectrophotometer at 515 nm at concentrations of 10,000, 5,000, 1,000, 500, 100, 50, 10 and 5 ppm of the samples. The inhibition percentage (IP) was calculated in relation to the UV absorption of the initial DPPH solution by the equation: IP (%) = [(AbsDPPH – AbsSAMPLE)/AbsDPPH] × 100. Linear regression analysis of the inhibition percentage of the various concentrations was used to find a linear equation to obtain the IC_{50}, the effective concentration of the sample that inhibits 50% of the DPPH radical.

RESULTS AND DISCUSSION

UPLC-HRMS-ESI (+) and HRMS/MS analysis

Table 1S presents the chemical composition of leaf, stem, and fruit extracts analyzed by UPLC-ESI-HRMS regarding their molecular formula, retention times (Rt), exact mass detected (positive ionization mode), as well as the MS/MS fragment ions and bibliographic references used to confirm characterization. The analysis showed that flavonoids were the most prevalent constituent, representing 79% of the components identified in the extracts. The main components were kaempferol-3-O-β-D-glucopyranoside (**7**, *m/z* 449.1080) at Rt 1.98 min and eupalinolide A (**20**, *m/z* 501.1543) at Rt 2.70 min, present in extracts of fruits and stems. Analysis of *C. duckeana* samples provided the tentative identification of 21 compounds (Figure 1S). Putatively identified compounds correspond to seventeen flavonoids (**1-3, 5-10, 12-13, 15-19, 21**), including glycosides of flavones, flavonols, flavononols, catechins, acylated flavonol glycosides and isoflavonoids, one anthraquinone (**11**), one sesquiterpenoid lactone (**20**), one thiazinedione (**14**), and one semi-quinone chalcone (**4**).

Fabaceae (Leguminosae) represent the third largest plant family, with 770 genera and approximately 19,500 species distributed among the subfamilies. In Brazil is the largest plant family with wide distribution and an estimated 2,834 species. Flavonoids quercetin and kaempferol have awide distribution in this family, however, others occur in a limited number, including isoflavones, catechins and terpenoids.[23,24] Therefore, it was interest to observe the presence of daidzin (6, *m/z* 417.1173) and ononin (19, *m/z* 431.1324) in the stems of *C duckeana*, once these metabolites are almost exclusively found in the Papilionoideae (Fabaceae) subfamily. They are associated with many biological activities, including the treatment of osteoporosis,

cardiovascular diseases, menopausal symptoms, and cancer prevention. Studies indicate antioxidant and estrogenic activities in addition to antifungal and insecticidal properties.[24-26]

Erazua[27] evaluated anticancer activities of eleven flavonoids: luteolin, apigenin, chrysin, quercetin, galangin, hesperetin, naringenin, taxifolin, daidzein, kaempferol, and genistein. These flavonoids were tested against leukemia cell line using quantum chemical method through density functional theory (DFT) and molecular docking approach. These flavonoids were docked against leukemia cell line (PDB: 1AOl) and the correlation between the calculated descriptors and their binding affinity with leukemia cell line was examined. The results showed that taxifolin had the highest inhibition efficiency against leukemia cell line among the studied compounds, while daidzein had the least inhibition efficiency. These results reinforce flavonoids as potential therapeutic agents.

Kaempferol-3-O-β-D-glucopyranoside (**7**) was isolated from *Wedelia chinensis* and this compound displayed moderate inhibitory effects on LPS-stimulated NO production. The values were comparable with that of resveratrol which has been extensively studied by previous researchers. In addition, the compound did not display any obvious cytotoxicity.[28] Kaempferol-3-O-β-D-glucopyranoside is a natural tetrahydroxyflavone that can be isolated from the bark of *Harungana madagascariensis*. In a recent study, it is listed as a potent cytotoxic compound that can be used for cancer chemotherapy as an anticancer agent.[29] The presence of this compound in extracts of fruits and stems of *C. duckeana* can be an important indicator of cytotoxicity, being able to partially justify the results found in this study.

Procyanidin B2 (**21**, *m/z* 579.1504) at Rt 2.75 min was previously reported in 40 samples of *Chamaecrista nictitans* collected in different sites of the Central Valley and the Pacific coast of Costa Rica.[18] To the best of our knowledge, it is reported for the first time for *C. duckeana*. Procyanidin B2 shows a wide anticancer activity in various human cancer cells. In gastric cancer, for example, this compound exerts anti-proliferative and apoptotic effects and induces autophagy; therefore, it may be developed as a potential therapeutic drug for this cancer.[30]

Antioxidant assay

The values of the antioxidant activities of the studied extracts are informed by median inhibitory concentrations (IC_{50}), as shown in Table 1. The highest antioxidant activity obtained was from stem extracts, with an IC_{50} value of 165.71. Stems presented a higher antioxidant activity in comparison to the standard butylated hydroxytoluene (BHT, IC_{50} = 175.18 μg mL^{-1}). All extracts presented lower antioxidant activity in comparison to standard rutin (IC_{50} = 133.01 μg mL^{-1}) or quercetin (IC_{50} = 57.07 μg mL^{-1}). In the past two decades, a great number of flavonoid derivatives - belonging to the class of chalcones, flavones, flavanones, isoflavones, and other complex structures - has been studied for their potential as antioxidant agents.[31] Comparing the results of the antioxidant activity with the data obtained in the UPLC-ESI-HRMS analysis, it was possible to verify that the predominance of flavonoids in the extracts may be associated with a greater antioxidant capacity. Stems had a higher number of flavonoids compounds, nine in total, and they had the highest antioxidant activity.

Table 1. Antioxidant activity of methanol extracts from *C. duckeana* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method

Methanolic Extracts	IC_{50} ± SD (μg mL^{-1})
Leaves	283.48 ± 4.19
Stems	165.71 ± 6.94
Fruits	261.08 ± 2.53
BHT[a,b]	175.18 ± 6.41
Rutin	133.01 ± 3.11
Quercetin	57.07 ± 0.39

[a]butylated hydroxytoluene; [b]standard synthetic antioxidant; IC_{50}: median inhibitory concentration; SD: standard deviation.

Cytotoxic assay

The growth inhibition percentage (GI%) of all cells treated with each extract is described in Table 2. The samples exhibited cell growth inhibition only against HL60 (leukemia), with a GI% ranging from 86.62% to 89.32%. The three extracts showed a GI% below 58.17% against SNB19 (central nervous system), HCT116 (human colon), and PC3 (prostate). Only HL60 (leukemia) had a growth inhibition higher than 80%.

Table 2. Cytotoxic effect of extracts from *C. duckeana* at single concentration (100.0 μg mL^{-1}) against four human tumor cell lines after 72 h of incubation using MTT assay

Sample	SNB19	HCT116	PC3	HL60
Fruits	28.10 ± 4.62	4.16 ± 8.53	30.44 ± 0.74	88.28 ± 3.57
Leaves	27.31 ± 0.89	58.17 ± 0.70	38.35 ± 4.60	89.32 ± 3.07
Stems	15.38 ± 1.37	28.67 ± 4.81	31.55 ± 1.94	86.62 ± 3.66

Tumor cell lines: SNB19 (central nervous system); HCT116 (human colon); PC3 (prostate); HL60 (leukemia).

In a second round of MTT assays, with extract-treated cells, the half-maximal inhibitory concentration (IC_{50}) was estimated in comparison to untreated cells. Two tumor cell lines, HL60 and RAJI, were tested at 72 h of incubation (Table 3). Extract of stems displayed a cytotoxic effect against two tumor cell lines, with IC_{50} values of 137.3 (104.6-180.1) and 106.8 (96.52-118.3) μmol L^{-1} for the HL60 and the RAJI, respectively. Among the tumor cell lines used, HL60 cells were more sensitive to extract of fruits treatment, showing IC_{50} values of 133.4 (114.2-155.7) μmol L^{-1}. Sample of leaves did not show cytotoxic activity at a concentration of 200 μg mL^{-1} in any of the tested cell lines.

Table 3. Cytotoxic activity of extracts on cancer cell lines after 72 h

Sample	HL60	RAJI
	IC_{50} (Confidence interval)	
Fruits	133.4 (114.2 -155.7)	> 200
Leaves	>200	>200
Stems	137.3 (104.6-180.1)	106.8 (96.52-118.3)

For human cells, data is presented as IC50 values and 95% confidence intervals; Tumor cell lines; HL60 and RAJI = leukemia.

The sesquiterpene eupalinolide A (**20**, *m/z* 501.1543) isolated from *Psiadia punctulata* has been reported in the literature for its potent cytotoxicity. This compound was tested for cytotoxicity against lung cancer cell A549, gastric gland cancer cell BGC-823, liver cancer cell SMMC-7721 and leukemia cell HL60 tumour cell. The results showed the potent cytotoxicity of sesquiterpene lactone Eupalinolide A against A-549, BGC-823, and HL60 tumor cell lines.[32] Eupalinolide A has been related as a natural anti-inflammatory agent and this compound also presented potent cytotoxicity against NB4 and K562 leukemia cell lines using the MTT method.[33] In our results, this compound is present in extracts of fruits and stems of *C. duckeana* and can be associated with the cytotoxic activity of extracts.

CONCLUSIONS

The UPLC-ESI-HRMS techniques led to putatively identified of 21 compounds of several classes of metabolites including flavonoid, anthraquinone, sesquiterpenoid lactone, thiazinedione, and semi-quinone chalcone. Of the flavonoid classes present, two are isoflavonoids considered rare for the Leguminosae family. To the best of our knowledge, the cytotoxicity of the crude extracts of *C. duckeana* towards cancer lines is being reported herein for the first time, and cytotoxic capacity of *C. duckeana* against cancer cell lines HL60 and RAJI was identified. These extracts are potential natural products that deserve more investigation to develop novel anticancer agents. In conclusion, results demonstrate that *C. duckeana* is a species rich in bioactive secondary metabolites with antioxidant capacity and cytotoxic activity.

SUPPLEMENTARY MATERIAL

Information about structural representation and chromatographic data of compounds annotated in *C. duckeana* extracts by UPLC-ESI-HRMS is given in the supplementary material.

ACKNOWLEDGMENTS

The authors are extremely grateful to INCT BioNat (CNPq 465637/2014-0/FAPESP 2014/50926-0, CIBFar/CEPID-FAPESP (2013/07600-3) and CAPES for all the financial support provided in the conduct of this research.

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