PharmacologyOnLine December 30, 2012
Archives • 2012 • vol.3 • 63 - 69

CYTOTOXIC AND ANTIOXIDANT ACTIVITY OF *PERESKIA ACULEATA* MILLER

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Abstract

Pereskia aculeata Miller (Cactaceae) is a climbing cactus native to South America and naturally distributed from south to northeast of Brazil, where the leaves are very appreciated as a vegetable in traditional cuisine. Leaves of *P. aculeata* are also used as anti-inflammatory, emollients and in skin wound healing in Brazilian folk medicine. Some other species of *Pereskia* genus are reported to be used as natural remedies against cancer and other diseases. The aim of this study was to perform a phytochemical characterization by thin-layer chromatography on chromatoplates sprayed by reagents for detection of the major classes of chemical constituents, and evaluate the cytotoxic activity of methanol crude extract of *P. aculeata* leaves and fractions against MCF-7 and HL60 cell lines by MTT assay. In addition, thin-layer chromatography DPPH bioautography was accomplished to investigate antioxidant compounds. The results showed that some fractions inhibited MCF-7 and HL60 cell proliferation, and that the most of antioxidant compounds found in *P. aculeata* leaves are phenol compounds. No activity was found against normal cells. These findings are interesting, since the leaves of this plant are commonly used as a food and there are no reports of toxicity for this vegetable.

Keywords: *Pereskia aculeata*, antioxidant, cytotoxicity, phenols

This paper was presented by the author at the XXI Congress of Italo-Latin American Society of Ethnomedicine (SILAE), Paestum, Italy, 25-29 September 2012

Introduction

Pereskia aculeata Miller (Cactaceae) is a climbing cactus native to South America and naturally distributed from south to northeast of Brazil, where the leaves are very appreciated as a vegetable in traditional cuisine [1, 2, 3].

The leaves of *P. aculeata* contain high levels of proteins (25.5% m/m) when compared to other plants commonly used as human food, such as lettuce (1.3% m/m), kale (1.6% m/m), common corn (7.6-10.0% m/m) and bean (18-20% m/m) [4]. This vegetable is also known as "meat of the poor" in many Brazilian poor communities, where the leaves are considered as the main source of proteins [5, 6]. The leaves also contain high levels of minerals, dietary fiber and vitamins A, C and folic acid [6].

There are reports that leaves of *P. aculeata* are also used in Brazilian folk medicine as anti-inflammatory, emollients and in skin wound healing [7, 8]. In addition, some species of *Pereskia* genus are reported to be used as natural remedies against cancer and other diseases [9, 10]. Cancer is characterized by uncontrolled cell proliferation and it is the second leading cause of death worldwide, and the first in developed countries. Some estimates indicate that cancer mortality will continue to increase, reaching 9 million deaths in 2015 and 11.4 million deaths in 2030 worldwide [11, 12]

Nurestri et al. (2009) reported significant cytotoxic activity of *P. grandifolia* leaves against nasopharyngeal epidermoid carcinoma cells (KB cells) and human breast adenocarcinoma cells (MCF-7) [13]. In addition, other studies have reported significant cytotoxic activity of *P. bleo* against breast carcinoma cells (T47-D cells) and KB cells, however it was not cytotoxic against normal cells [14, 15].

There are only few studies about *P. aculeata* phytochemicals and their therapeutic potential. So, the aims of this study were to report the phytochemical characterization of *P. aculeata* leaves, their antioxidant activity by TLC DPPH bioautography and cytotoxic activity by MTT assay.

Methods

Plant material

Leaves of *P. aculeata* were collected in Juiz de Fora (MG, Brazil) in August 2010, in the morning. The plant was identified and a voucher specimen (No. 57539) was deposited for future evidence at Herbarium Leopoldo Krieger of Federal University of Juiz de Fora.

The leaves were air-dried in a well-ventilated place at room temperature (25 °C) for fifteen days. Once dried, the material (approximately 1 kg) was powdered using a knife mill and then extracted by maceration with methanol until exhaustion. The extract was concentrated on a rotary evaporator to obtain the crude methanol extract (ME -140 g), which was dissolved in water/methanol (8:2 v/v) and then fractioned with different solvents in order of increasing polarity, to obtain the hexane (HEF - 38g), dichloromethane (DCF - 12g), ethyl acetate (EAF - 1g) and butanol (BUF - 7g) fractions.

This process resulted also in a remaining hydromethanolic fraction (HMF - 12g) and in a precipitate (PPT - 37g), which were stored in a refrigerator at 4°C.

Phytochemical characterization

The phytochemical characterization of ME and its fractions was performed by thin-layer chromatography on chromatoplates coated with silica gel 60 F 254 which were sprayed by the following reagents: $FeCl_3$ 1% (phenols, condensed and hydrolysable tannins), KOH 5% (coumarins, anthraquinones, and anthrones), NP/PEG or $AlCl_3$ 1% (flavonoids), Dragendorff reagent (alkaloids), and Liebermann-Burchard reagent (steroids and triterpenes), and also visualized under 365 nm UV light or visible light [16].

For better visualization of phenols, tannins, alkaloids, steroids and triterpenes the chromatoplates were heated to 100 °C. Ten µL of each sample were applied on chromatoplates for elution with dichloromethane/methanol 9:1 (v/v) for ME, he-

xane/ethyl acetate 7:3 (v/v) for HEF, hexane/ethyl acetate 5:5 (v/v) for DCF, ethyl acetate/methanol 8.5:1.5 (v/v) for EAF and 2-propanol/ammonia solution 30%/water 5:4:1 (v/v) for HMF and PPT. In addition, saponins were detected by the foam test as described by Matos (1997) [17].

TLC antioxidant bioautography

The free radical–scavenging activity of the samples was detected on the basis of their ability to react with stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The TLC plates were developed as described above and allowed to air-dry for 30 minutes followed by spray with a 2.54 mM DPPH methanol solution for derivatization. Spots with the DPPH scavenging activity were observed as white yellow bands on a purple background [18].

Amount of phenol compounds and flavonoids

The amount of phenol compounds in the samples was determined by the Folin-Denis method [19]. Tannic acid was used as the standard for the calibration curve. One mL of the samples resuspended in ethanol (0.5 mg/mL) was mixed thoroughly with the Folin-Denis reagent (1mL) and Na_2CO_3 2% in 0.1N NaOH (8mL). After a 60-minute incubation period at 30 °C, the absorbance was read against a blank at 730 nm. All determinations were performed in triplicate. Total content of phenol compounds was expressed in mg/g plant extracts, in tannic acid equivalents.

The amount of flavonoids was determined as described by Miliauskas et al. (2004), with slight modifications [20]. Rutin was used as the standard for the calibration curve. One mL of plant samples was resuspended in methanol (0.5 mg/mL) and mixed with 2% aluminum trichloride in ethanol (1mL) and 1 drop of acetic acid. Then, it was diluted with ethanol to 25 mL and allowed to stand for 40 minutes at 20 °C. The absorbance was measured at 415 nm. The blank was prepared from the plant samples (1mL) and 1 drop of acetic acid diluted to 25 mL in ethanol. All determinations were performed in triplicate. Total amount of flavonoids was expressed in mg/g plant extracts, in rutin equivalents.

Evaluation of cytotoxic effect against human tumor cell lines

This assay is based on the ability of viable cells to metabolically reduce the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, and allows to evaluate both proliferation and cell viability. Human promyelocytic leukemia and human breast adenocarcinoma cells (HL60 and MCF-7 cell lines, respectively) were used at a concentration of 50,000 and 40,000 cells / well (96 well plates), respectively. All cells were stored for 24 h at 37 °C and 5% CO_2 for stabilizing. After the stabilization, the cells were incubated with ME 100µg/mL, HEF, DCF, EAF, HMF or PPT 50µg/mL, etoposide 10µM (reference drug) or DMSO 0.05% (vehicle) for 48h in 5% CO_2 atmosphere and 100% humidity at 37 °C. Four hours before the end of incubation period, 20µL of a MTT solution (2.5 mg/mL) were added to each well (final volume was 200µL). After the remaining 4 h of incubation, the supernatant was carefully removed under a vacuum. To each well 200µL of 0.04 HCl in isopropanol were added. After the solubilization of formazan crystals, the plates were read at 595 nm. The assay was performed in triplicate [21].

Cytotoxicity against mammalian cells

Murine peritoneal macrophages were used for this assay. Briefly, inflammatory macrophages were obtained from BALB/c mice previously inoculated intraperitoneal with 2mL of 3% thioglycollate medium. Adherent macrophages ($2x10^6$ cells per well/ 96 well plates) were incubated with the extract and fractions (concentration ranging from 6.25 to 100 µg/ml), in duplicate, for 72 hours at 37°C in 5% CO_2. The viability of the macrophages was determined with the MTT assay and was confirmed by comparing the control group morphology via light microscopy.

Statistical analysis

The results of the evaluation of cytotoxic effect against human tumor cell lines were expressed as mean of cell proliferation ± S.E.M. and One-way ANOVA followed by Newman-Keuls test were used for statistical analysis with the software GraphPad Prism 4. $p < 0.05$ was considered significant. For cytotoxicity against mammalian cells, the IC_{50} values were carried out at 5% significance level ($p < 0.05$, CI 95%), calculated using a nonlinear regression curve, by using GraFit Version 5 software.

Results

The phytochemical characterization is shown in Table 1.

see Table 1.

The TLC bioautography showed the presence of antioxidant compounds, most of them were phenols, as shown in Figure 1. The dichloromethane fraction showed higher content of flavonoids and phenol compounds (Table 2).

see Fig. 1

see Table 2.

Some of the samples showed cytotoxic activity against MCF-7 tumor cells, such as HEF, DCF, EAF and PPT, which inhibited between 25% and 30 % of cell proliferation. Etoposide, used as reference drug, reduced the cell proliferation by 40% (Figure 2). PPT was the most active fraction against HL60 cells, inhibiting 44% of cell proliferation, but other samples also reduced the cell proliferation when compared to vehicle (Figure 3). Besides, ME and its fractions did not show cytotoxicity against macrophages with $IC_{50} > 100\mu g/mL$ (data not shown).

see Fig. 2

see Fig. 3

Discussion

All chemical constituents investigated were detected in at least one of the samples, excepted triterpenes. Despite saponins were found only in DCF, they could not be detected in ME, probably for being in lower concentration. Steroids, chemical constituents already reported in this species [22], were also detected in most samples. It is emphasized the presence of phenols , flavonoids and alkaloids in ME and in almost all fractions.

Some alkaloids are used clinically as chemotherapeutic agents, such as camptothecin and vinca alkaloids vincristine and vimblastine [23]. Many isoquinoline, quinoline and indole alkaloids have been reported to induce apoptosis in HL60 cells [24]. Flavonoids are phenol compounds widely distributed in plants and several biological activities of plants are related to these chemical constituents. The *in vitro* and *in vivo* antitumor activities of various flavonoids are well documented. Some flavonoids are reported to be cytotoxic against HL60 cells, but not against normal cells [25]. Several studies suggested that many flavonoids in plants commonly used as food are capable to reduce the proliferation of some tumor cells. In addition, the lower incidence of colon, prostate and breast cancer in Asian countries, where vegetables, fruits and teas consumption is much higher than in Western countries, raises the question whether these foods are exhibiting chemopreventive effects [26].

Moreoever, HEF, DCF, EAF and PPT showed cytotoxicity against MCF-7 cells and all samples were active against HL60 cells. In studies about other *Pereskia* species, the ethyl acetate fractions showed remarkable cytotoxic activity [13, 14, 15]. In this study, however, none of the samples showed strong cytotoxicity. It was not found any cytotoxicity against non-tumor cells, suggesting a possible selective activity against HL60 and MCF-7 tumor cells. These findings are interesting, since leaves of *P. aculeata* are widely used as a food in Brazil and there are no reports of toxicity.

The TLC DPPH bioautography and $FeCl_3$ spray

reagent showed that most of the antioxidant compounds in *P. aculeata* leaves are phenols. Phenol compounds, in many cases, are capable to reduce the oxidative stress, which is associated to cancer development and other diseases [27, 28, 29]. Thus, it is possible that these chemical constituents are among the bioactive compounds responsible for cytotoxic activity found for ME and its fractions, especially for DCF, which showed higher amount of total phenols and flavonoids.

The present study on the leaves of *P. aculeata* encourages further studies to identify its bioactive compounds.

Acknowledgments

This work was supported by the grant from Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG CEX APQ 01137-09) and Coordenacão de Aperfeicoamento de Profissional de Ensino Superior (CAPES). The authors are grateful to Dr Daniela Zappi for the botanical identification of the species and to Delfino Antônio Campos for technical assistance.

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	Classes of chemical constituents [a]										
Samples	Ph	Ct	Ht	C	Atq	Atn	F	A	St	Tr	S
ME	+	+	+	+	+	+	+	+	+	-	-
HEF	+	-	-	-	-	-	+	+	+	-	-
DCF	+	+	-	+	-	+	+	+	+	-	+
EAF	+	+	-	+	+	+	+	+	+	-	-
HMF	+	-	+	+	-	+	+	+	-	-	-
PPT	+	-	-	+	-	+	+	-	-	-	-

Table 1: Phytochemical characterization of the crude methanol extract and fractions of *Pereskia aculeate* leaves

[a] Chemical constituents: Ph - Phenols; Ct – condensed tannins; Ht – hydrolysable tannins; C - coumarins; Atq - anthraquinones; Atn - anthrones; F - flavonoids; A - alkaloids; St - steroids; T - triterpenes; S - saponins. + means presence and - means absence.

Samples: methanol extract (ME), hexane fraction (HEF), dichloromethane fraction (DCF), ethyl acetate fraction (EAF), hydromethanolic fraction (HMF) and precipitate (PPT)

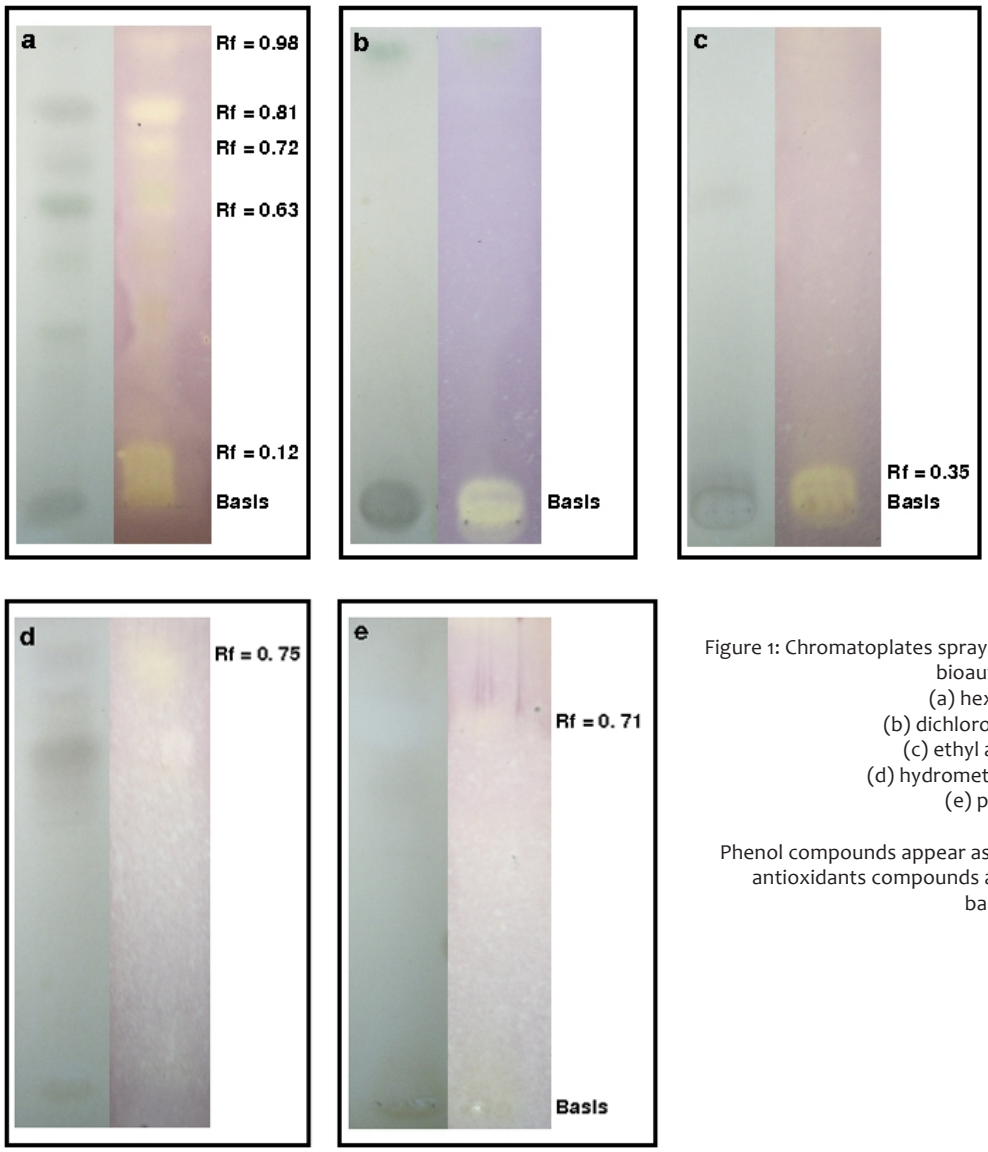

Figure 1: Chromatoplates sprayed with $FeCl_3$ spray reagent and DPPH bioautography for
(a) hexane fraction,
(b) dichloromethane fraction,
(c) ethyl acetate fraction,
(d) hydromethanolic fraction and
(e) precipitated.

Phenol compounds appear as dark spots on gray background and antioxidants compounds appear as yellow spots on purple background

Sample	Total phenols as tannic acid equivalent (mg/g)[a]	Flavonoids as rutine equivalent (mg/g)[a]
ME	17.27 ± 3.94	13.10 ± 2.16
HEF	23.54 ± 4.15	27.75 ± 2.54
DCF	49.11 ± 3.30	54.58 ± 4.73
EAF	3.95 ± 1.66	18.53 ± 0.94
HMF	2.45 ± 0.81	10.40 ± 2.36
PPT	10.97 ± 0.57	8.33 ± 0.72

Table 2: Phenol and flavonoids contents of methanol crude extract and fractions of *Pereskia aculeate* leaves
[a] Sample concentrations ± standard deviation (triplicate) for methanol crude extract (ME), hexane fraction (HEF), dichloromethane fraction (DCF), ethyl acetate fraction (EAF), hydromethanolic fraction (HMF) and precipitate (PPT)

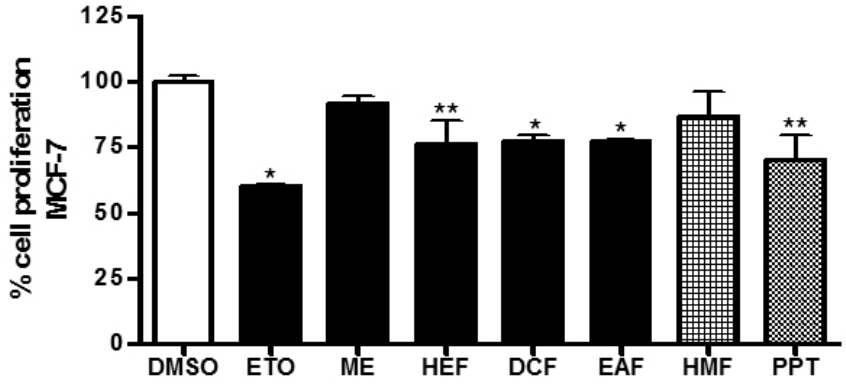

Figure 2: Cytotoxic activity of methanol extract (ME), hexane fraction (HEF), dichloromethane fraction (DCF), ethyl acetate fraction (EAF), hydromethanolic fraction (HMF) and precipitate (PPT) against MCF-7 cells. DMSO (dimethyl sulphoxide) was used as vehicle and etoposide (ETO) as reference drug. *$p < 0.01$ and **$p < 0.05$ *vs* vehicle.

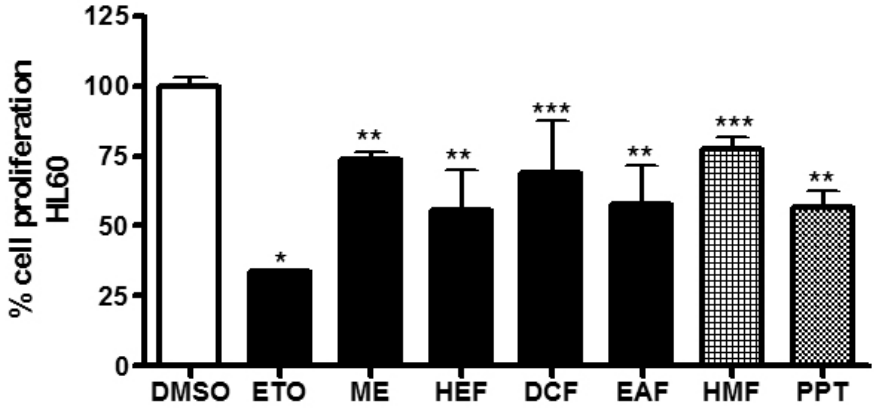

Figure 3: Cytotoxic activity of methanol extract (ME), hexane fraction (HEF), dichloromethane fraction (DCF), ethyl acetate fraction (EAF), hydromethanolic fraction (HMF) and precipitate (PPT) against HL60 cells. DMSO (dimethyl sulphoxide) was used as vehicle and etoposide (ETO) as reference drug. *$p < 0.001$, **$p < 0.01$ and *** $p < 0.05$ *vs* vehicle