



Cytotoxic, antitumor and leukocyte migration activities of resveratrol and sitosterol present in the hidroalcoholic extract of *Cissus sicyoides* L., Vitaceae, leaves

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RESUMO: *Cissus sicyoides* pertence à família das Vitaceae. É conhecido popularmente como “insulina, cipó-puca, bejuco caro, puci, anil trepador”. Esta planta apresenta efeito vasoconstritor e atividade antibacteriana. No Brasil, *C. sicyoides* foi avaliado pelas suas propriedades anticonvulsivante e anti-diabética. Estudos fitoquímicos identificaram e isolaram a partir de suas partes aéreas o sitosterol e o resveratrol compostos que são apontados por apresentar atividade antitumoral. O objetivo deste estudo foi investigar as atividades citotóxica e antitumoral do extrato hidroalcoólico *Cissus sicyoides* bem como a sua capacidade de recrutar leucócitos para os tecidos lesados. *Cissus sicyoides* não demonstrou atividade citotóxica, mas apresentou uma inibição do crescimento tumoral frente aos tumores testados. O extrato teve um forte efeito quimiotático 24 h após o tratamento. O extrato hidroalcoólico de *Cissus sicyoides* apresentou atividade antitumoral, relacionada ao recrutamento de linfócitos T para o local da lesão sugerindo que esta atividade esteve relacionada à ativação da linhagem linfóide.

Unitermos: *Cissus sicyoides*, atividade antitumoral, migração de leucócitos, ativação da linhagem linfóide.

ABSTRACT: *Cissus sicyoides* L. belongs to the Vitaceae family. It is popularly known as “insulina, cipo-pucá, bejuco caro, puci, anil trepador”. A vasoconstrictor effect and an antibacterial activity have also been allocated to it. In Brazil, *C. sicyoides* was evaluated for its anticonvulsant and anti-diabetic properties. Phytochemistry studies identified and isolated sitosterol and resveratrol compounds from its aerial parts which are pointed out as having antitumor activities. The goal of this study was to investigate the cytotoxic and antitumor activities of *Cissus sicyoides* hydroalcoholic extract as well as its ability to repair leukocytes cells to injured tissue. *Cissus sicyoides* did not demonstrate cytotoxic activity but showed an inhibition of tumor growth in face of the tumors tested. The extract had a strong chemotactic effect on the 24 h period after treatment. The hydroalcoholic extract of *Cissus sicyoides* presented antitumor activity which was prompted by T lymphocytes recruitment to the local lesion and it suggests a new pathway to antitumor activity by activation of lymphoid lineage.

Keywords: *Cissus sicyoides*, antitumor activity, leukocyte migration, activation of lymphoid lineage.

INTRODUCTION

Cissus sicyoides L. pertaining to the Vitaceae family is made up of about one hundred sixty five genus and one thousand three hundred seventy species, which are distributed throughout the tropics, mainly in Brazil and

the Caribbean. It is popularly known as “insulina, cipo-pucá, bejuco de porra, bejuco caro, puci, anil trepador” and its origins from the Dominican Republic (Beltrame et al., 2001). It has also demonstrated a vasoconstrictor effect on guinea-pig aorta rings (Pepato et al., 2003) and an antibacterial activity (Garcia et al., 1999). In Brazil, *C.*

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sicyoides was evaluated for its anticonvulsant property, where it is used against epilepsy and cytotoxic activity (Elisabetsky et al., 1995a, b, 1999). Used as an infusion of tea, the treatment induced an increase in the amount of chromosomal damage in bone marrow cells without altering the cell division cycle. (Saénz et al., 2000). The central antinociceptive effect of *C. sicyoides* on mice, and the action of dry leaves extract in pregnant rats and offspring, also showed postal development. (Almeida et al., 2006a, b, 2007). Phytochemistry studies identified and isolated from the aerial parts of *C. sicyoides* a new coumarin glycoside 5,6,7,8-tetrahydrocoumarin 5 β -xylopyranoside, which was obtained together with known coumarin sabandin: two flavonoids kaempferol 3-rhamnoside and quercetin 3-rhamnoside and two steroids, among them sitosterol, which is pointed out as inducing apoptosis, together with TNF- α and antitumor activity (Park et al., 2007; Beltrame et al., 2002). Leaves of the genus *Cissus* contain sterols, quinones and phenolic compounds. Anthocyanins, saponins and flavonoids are also found in the plant's leaves and fruit (Beltrame et al., 2002). Phytochemistry studies demonstrated that *C. sicyoides* has hydroxystilbene resveratrol in its leaves, another compound responsible for antitumor activity. Resveratrol has pleiotropic effects, altering many different signalling pathways (nuclear factor- κ B, Rb-E2F, p53, phosphatidylinositol 3-kinase/Akt, and mitosis-activated protein kinase pathways), leading to suppression of tumours cell proliferation, adhesion, invasion and metastasis, reduced signs of inflammation and angiogenesis, and induction of apoptosis and differentiation (Bharat et al., 2004). However, there are no references on antitumor activity of *Cissus sicyoides*. The inflammatory process consists of a sequence of events that affect vascularity (Vasodilatation increased permeability and expression of adhesion molecules) (Bhoola et al., 1992). The leukocytes dynamics on the blood, which are attracted to the focus inflammatory tissue, are important because these cells travel to the tissues from the peripheral blood and carry out a protective functions such as phagocytes by neutrophils and monocytes, release of cytokines by T helper lymphocytes (CD4+) and cytotoxicity by T lymphocyte cytotoxic (CD8+) (Baumann & Gaudie, 1994). The goal of this study was to investigate the cytotoxic and antitumor activities of *Cissus sicyoides* hidroalcoholic extract as well as studying their ability to repair leukocytes cells to the injured tissue.

MATERIAL AND METHODS

Botanic materials and extract preparation

Aerial parts of plant material *Cissus sicyoides* L., Vitaceae, were collected in the Várzia District at Rua Maria Jaboatão nº 115, Recife, Brazil during the period of September to March, during the harvest time when the plants produce fruits. The plant was identified by Marlene

Carvalho de Alencar, Curator of the Geraldo Mariz Herbarium-UFPE. The collected species was deposited in the Herbarium under the register 50.541. The leaves were washed and dried in the laboratory at room temperature, then dried in an oven for fifteen days at ± 45 °C, after that they were triturated manually. Ethyl alcohol and water was added at a proportion of 70:30 (v/v). The leaves, water and alcohol mixture was then rested for 48 h, followed by mechanical agitation also for 48 h, then submitted to rote-evaporation. The extract was dissolved in a physiologic serum solution (Almeida, 2002).

Animals used in the pharmacological tests

For the tests, Swiss albino female mice (*Mus musculus*) were used, weighing between 30-35 g, with an average age of two months. The animals were monitored according to the norms of the National Institute of Health Guide for Care and Use of Laboratory Animals. The experiments were conducted according to the National Cancer Institute protocol (Geran et al., 1972) and approved by the UFPE-Animal Experimentation Ethic Committee (Protocol nº 018301/2007-96).

Neoplastic cells used in the tests

The cytotoxicity tests were conducted using three lines of cells: NCI-H292 cells (mucoepidermoid lung carcinomacells), HEp-2 cells (larynx carcinoma epidermoid) and KB cells (mouth Carcinoma epidermoid).

Cytotoxic activity

The cells (HEp-2, KB and NCI-H292) were maintained in DMEM-Minimum Essential Medium Eagle modified Dulbecco's (Sigma), (Eagle, 1971) supplement with 10% fetal bovine serum (Gibco), 1% solution of antibiotic (penicillin 1000 UI/mL + streptomycin 250 mg/mL) and 1% de L-glutamin 200 mM. For cytotoxicity determination a cellular suspension of 10^5 cells/mL, was prepared in DMEM. The suspension was distributed in culture plaques with 96 wells (225 μ L in each well). The plaques were incubated at 37 °C in a CO₂ (COLE PARMER) incubator. After 24 h (25 μ L/well) was added to the tested substances and the plaques were incubated at 37 °C (Costa & Nascimento, 2003). Evaluation of cytotoxic activity was carried out by MTT colorimetric assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] in a PBS concentration of 5 mg/mL. The plaques were placed in an oven at 37 °C for 2 h. At the end of this time, the culture medium along with the excess MTT material were aspirated, mixed with 100 μ L of DMSO and added to each well to dissolve the Formazan crystals (Alley et al., 1988; Machon et al., 1981). An optic reading was carried out in an automatic plaque reader BIOPLUS (BIO 2000) at 540 nm.

Antitumor Activity

The animals received 0.2 mL of a suspension (5×10^6 cells/animal) prepared from a cellular suspension of 25×10^6 cellules/mL (animal donator). The treatment of 0.2 mL leaf suspension was initiated 24 h later in the right axillary region of the healthy animals. The donator animals were sacrificed with xilasin. These animals were divided into groups, the first being the control group, receiving a saline solution (NaCl 0.9%). The second group was submitted to a treatment with *C. sicyoides* hidroalcoholic extract to evaluate the antitumor activity. The extract dosages were based on DL50 as referred by (Almeida et al., 2006b). The animals received a dose of 300 mg/kg and 600 mg/kg of body weight via peritoneal injection. At the end of the treatment, the animals were sacrificed with a fatal dose of xilasin. The tumors were removed and weighed to evaluate the tumor inhibition. The same procedure was adopted for the control group of animals. The tumor inhibition was calculated according to (Machon et al., 1981). The average corporal weight of the animals in the control and treated groups was also observed.

Migration of cells verification (peripheral blood)

The animals were anesthetized with a solution of 100 μ L (2:8) containing xylazine/ketamine to be able to count total leukocytes and lymphocytes from the blood. Samples were collected by cardiac puncture to the amount of 0.5 mL and preserved in 0.001 mL of EDTA 10% and subsequently subjected to automated counting (3200 BC) of the blood slides which were prepared, in duplicate, immediately after collection. Slides were stained with hematoxylin and the eosin analysis cell was counted under optic microscope (1000 x). The analysis was performed 2, 6 and 24 h after treatment.

Migration of cells verification (peritoneal cavity)

The peritoneal cavity received, 4 mL of sterile solution PBS 10 mM EDTA. Around 3 mL of peritonitis were aspirated and submitted to the refrigerated centrifuge for 7 min at 1500 rpm. The supernatant was collected and the pellet diluted in 100 μ L and 500 μ L of PBS+0.1% of BSA. The slides were analyzed as described above.

Statistical analyses

Cytotoxic and antitumor data were analyzed by statistical analysis software GraphPad Instat 3.0. The results were submitted to the paired T test and analysis of variance (ANOVA) followed by the test of multiple comparisons of Tukey-Kramer. The migration activity was analyzed by (Wilcoxon) and Mann-Whitney tests.

RESULTS

Cytotoxic activity

The *Cissus sicyoides* L., Vitaceae, hidroalcoholic leave extract did not demonstrate cytotoxic activity at tested concentrations, (CI50 > 50 μ g/mL).

Antitumor activity

The hidroalcoholic extract from the leaves of *C. sicyoides* showed an inhibition of tumor activity in sarcoma-180 of 48.7 and 62% to the doses of 300 and 600 mg/kg respectively, when compared to tumors with the control group. (Table 1) When Ehrlich Carcinoma was considered, the inhibition was 69 and 84.4% to the doses of 300 and 600 mg/kg weight respectively in comparison with the control group (Table 2). Considering the change in weight of animals, significant differences were seen in the weight of animals belonging to the Ehrlich carcinoma group in the dose of 300 mg/kg. The results were considered significant when they showed tumor inhibition growth above 40% according to the protocol of Geran e colaboradores (1972).

Leukocytes migration evaluation

Regarding the number of leukocytes in peripheral blood, the hidroalcoholic extract of *C. sicyoides* did not lead to change in normal values of these cells in any of the times studied, as compared to the control group animals. In the case of leukocytes in the peritoneal cavity, the extract had a strong chemotactic effect on the 24 h period, since there was a greater quantity of these cells in the group that received the extract in relation to that which received saline (Table 3). Considering the number of lymphocytes, it was observed that within 24 h the extract led to lymphocytopenia in the peripheral blood (Table 3). Otherwise, the analysis of the peritoneal cavity showed that there was a significant amount of lymphocytes due to the presence of the extract within 24 h, compared to the control group. These results suggest that the lymphocytopenia observed reflected the migration of these cells to peripheral blood to the peritoneal cavity (Table 3).

Table 1. Antitumor activity of *Cissus sicyoides* L., Vitaceae hidroalcoholic extract (Sarcoma 180).

Groups	Animal weight g \pm SD	Tumor weight g \pm SD	Inhibition (%)
Control (NaCl 0.9%)	33.1 \pm 0.44	1.97 \pm 0.21	0
Treated (300 mg/kg)	33.8 \pm 0.32	1.01 \pm 0.08	48.7**
Treated (600 mg/kg)	35.9 \pm 0.21	0.75 \pm 0.05	62.0**

Used doses 300 mg/kg and 600 mg/kg by peritoneal injection
SD = standard deviations

** p <0.01

DISCUSSION

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, from which several studies are being made to find active substances against cancer (Costa & Nascimento, 2003). There are various indications of *Cissus sicyoides* L., Vitaceae, being used as popular medicine; many confirmed

Table 2. Antitumor activity of *Cissus sicyoides* L., Vitaceae hidroalcoholic extract (Ehrlich Carcinoma).

Groups	Animal weight g±SD	Tumor weight g±SD	Inhibition (%)
Control (NaCl 0.9%)	36.5+0.96	2.09+0.24	0
Treated (300 mg/kg)	32.6+0.71*	0.65+0.07**	69.0**
Treated (600 mg/kg)	35.0+0.79	0.32+0.05**	84.4**

Dose used = 300 mg/kg and 600 mg/kg peritoneal injection.

SD = standard deviations

* $p < 0.05$ ** $p < 0.01$

Table 3. Leukocyte migration to peritoneal cavity and peripheral blood.

Groups	Mean of cells+SD (cavity)		Mean of cells+SD (blood)	
	Leucocytes	Lymphocytes	Leucocytes	Lymphocytes
Control (2 h)	7.5+0.82	7.4+0.81	4.5+0.28	4.0+0.36
Treated (2 h)	1.4+0.43*	6.6+0.64	3.8+0.40	6.93+0.54
Control (6 h)	14.1+1.38	7.2+0.59	4.9+0.05	4.9+0.17
Treated (6 h)	15.4+1.5	4.0+0.84	4.9+0.63	4.5+0.66
Control (24 h)	4.9+0.03	2.5+0.4	7.2+0.75	6.7+0.49
Treated (24 h)	**25.3+1.45	**19.4+0.5	4.3+0.87	4.3+0.87

Dose used = 300 mg/kg of CS/peritoneal injection.

Control group = NaCl 0.9%

SD = Standard deviation

Referential values leucocytes (4-12).

Referential values lymphocytes (3-9).

* $p < 0.05$

** $p < 0.01$

by well known methodologies recognized in international literature, this plant is used as an anticarcinogenic in the treatment of gall bladder cancer (Quílez, 1996) and it was verified in this study that the hidroalcoholic extract produced from *C. sicyoides* leaves did not demonstrate cytotoxic activity at the concentrations tested, (CI50 > 50 µg/mL) and for the plant extract to be considered active the CI50 should be less than, or equal to, 30 µg/mL for plant extracts (Geran et al., 1972). Meanwhile, when the antitumor activity was carried out *in vivo* it was observed that the hidroalcoholic extract of the *C. sicyoides* leaves presented an inhibition to Sarcoma-180 of 48.7% to the 300 mg/kg and 62% to the 600 mg/kg treatment. In relation to the Ehrlich Carcinoma, the inhibition was 69% in the 300 mg/kg treatment and 84.4% in the 600 mg/kg treatment suggesting that the antitumor activity could be related to the metabolism of active compounds in secondary metabolites. This interaction is impossible to be visualized *in vitro*, seeing that the culture *in vitro* metabolic reactions, as in live organisms, does not exist. Tested substances and a gambit of reactions with hormones, enzymes and liver metabolism are capable of influencing the functions of tested substances in organism. This antitumor activity can be related to the presence of sitosterol which is pointed out as inducing apoptosis together with TNF- α and antitumor activity (Park et al., 2007) and hydroxystilbene resveratrol, another compound responsible for antitumor activity.

Resveratrol has pleiotropic effects, altering many different signalling pathways *i.e.*, nuclear factor- κ B, Rb-E2F, p53, phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinase pathways, leading to suppression of tumor cell proliferation, adhesion, invasion and metastasis, reduced signs of inflammation and angiogenesis, and induction of apoptosis and differentiation (Bharat et al., 2004). In relation to cells migration, no changes in the number of total leukocytes between the treated and control groups were verified, but the results demonstrated that the extract had a strong chemotactic effect on the 24 h study. In relation to the lymphocytes number (important against tumor development) the results showed that the extract led to a lymphocytopenia on the peripheral blood. Otherwise, the analysis of peritoneal cavity showed that there was a significant amount of lymphocytes due to the presence of the extract within 24 h, compared to the control group. These results suggest that lymphocytopenia observed reflects the migration of these cells to peripheral blood until the peritoneal cavity. Moreover, the stimulation of leukocyte influx becomes important in protecting against tumors. In this study, we found that the hidroalcoholic extract from the leaves of *C. sicyoides* was able to attract lymphocytes to the site of its application. Knowing that in the line lymphoid cells are cells that are specialized in the death of tumors, such as CD8+ lymphocytes cytotoxic cells and release of cytokines by T helper lymphocytes (CD4+) (Baumann & Gaudie, 1994; Ferreira et al., 2001). These results prompt the potential of hidroalcoholic extract from the leaves of *C. sicyoides* by lymphoid cell stimulation/attraction pathway. In conclusion, the hidroalcoholic extract of *C. sicyoides* presented antitumor activity which was prompt by T lymphocytes recruitment to the injured

tissue and suggests a new pathway to antitumor activity by activation of lymphoid lineage.

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