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Genotoxicity, anti-melanoma and antioxidant activities of *Hymenaea courbaril* L. seed extract

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Abstract: *Hymenaea courbaril* has been used to treat different diseases, although its properties are yet to be scientifically validated. The objective of this study was to determine the cytotoxicity, genotoxicity, antigenotoxicity and antioxidant potentials of hydroethanolic extract from *H. courbaril* seeds. Therefore, for the cytotoxicity test an anti-melanoma assay was performed in B16F10 strain cells. The genotoxicity and antigenotoxicity was evaluated in bone marrow cells (Permit number: 002/2010) of mice, the antioxidant activity was determined by the DPPH test and the total flavonoid content was also determined. The hydroethanolic extract showed antigenotoxic effect and antioxidant activity. It was verified that total flavonoid content was 442.25±18.03 mg RE/g dry extract. HPLC-PAD chromatogram revealed presence of flavones as majority compound in evaluated extract. The results allowed us to also infer that the hydroethanolic extract from seeds shows cytotoxic activity against B16F10 melanoma cells line and it has dose-and-time-dependency.

Key words: B16F10 melanoma cells, micronucleus test, chromosomal aberration, DPPH radical scavenging, HPLC-DAD.

INTRODUCTION

Cancer is one of the major causes of death worldwide (Assaf et al. 2013). Development of cancer in humans is a multifactorial process (Choudhari et al. 2014). It can rise as a result of genetic alterations which are mediated by endogenous and exogenous factors. Inactivation of tumor suppressor genes, inactivation of genes responsible for apoptosis, activation of oncogenes, DNA damage due to lack of repair or incomplete repair are some of the factors related to carcinogenesis (Kryston et al. 2011).

The side effects presented by current chemotherapeutics and synthetic drug resistant cancer cells have increased the demand for new

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anticancer agent's development. In this context, the plants have been highlighted. They have been the basis of traditional medicine for thousands of years and now have been widely studied for the anticancer activity (Krishnaiah et al. 2011). Several compounds isolated from plants are still not used as therapeutical drugs, but they have potential for the development of anticancer agents. Phenolic compounds such as flavonoids have been highlighted by presenting a range of pharmacological activities such as antioxidant, antimutagenic, anti-inflammatory and anticancer activity.

Hymenaea courbaril (Fabacea, Caesalpinioideae) is commonly known in Brazil as"Jatobá"(Veggi et al. 2014). Apart from its ecological importance, Jatobá presents agronomic potential for its stem and fruit. This species has mealy pulp surrounding the seeds of the fruit, rich in nutrients and the resin exuded from the trunk is rich in terpenes making this an important species for commercial use (Dias et al. 2013). The Jatobá has medicinal properties and its different parts are used in local folk medicine as tonic, expectorant, hepatoprotective and dewormer (Bezerra et al. 2013). Previous studies have demonstrated the biological activities of *Hymenae courbaril* species, for example, anti-inflammatory activity (Jayaprakasam et al. 2007), while others studies have been identified and isolated compounds with antioxidant and myorelaxant activities of *H. courbaril* (Bezerra et al. 2013).

Nevertheless, there are no studies in the literature regarding the anti-melanoma and genotoxicity activity in this species. Therefore, this work brings a contribution to the knowledge of Brazilian medicinal plants, offering important information about its pharmacological effectiveness and its safe use. This study aimed to evaluate genotoxic, antigenotoxic, anti-melanoma and antioxidant activities of the hydroethanolic extract from *H. courbaril* seeds and also evaluate the presence of flavonoid compounds.

MATERIALS AND METHODS

REAGENTS

RPMI medium (Gibco Life Technologies), Cyclophosphamide (CAS n. 50-18-0; Endoxan, Baxter Oncology Gmb, German), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich Chemical Co., St Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade.

PLANT MATERIAL

The fruits of *Hymenae courbaril* were collected in Assis located in Southeast Brazil between November 2012 and February 2013 (*H. courbaril*-22º39'00.66''S and 50º26'15.86''W, at an altitude of about 845m. *H. courbaril* have been taxonomically identified and a voucher specimen has been deposited in the Herbarium Assisense (HASSI) under number of 186. The seeds were separated from the pulp, cleaned and dried at 40ºC in an oven with air circulation (Cienlab, Brazil) and posteriorly grounded in a Wiley type mil (Cienlab, CE-430, Brazil).

PREPARATION OF EXTRACTS

The ground material (50 g) was macerated with hydroethanolic solution (70% ethanol-water) (500 mL) as solvent for 24h, at room temperature, in the dark with constant stirring. The process was repeated twice with the same powder. The resulting extracts were combined, filtered and concentrated under vacuum (Marconi, MA-120, Brazil). After this process, the hydroethanolic extracts were frozen and subsequently lyophilized (Liotop, L101, Brazil). The dried crude extract (EHC) was kept in small opaque bottle at room temperature until use.

In vitro CYTOTOXICITY ASSAY IN B16F10 MELANOMA CELLS

The cytotoxicity assay was performed using B16F10-Nex2 murine melanoma cell line. The B16F10-Nex2 cells ($2x10^3$ cells) were seeded into

the wells of a 96 well plate (Corning Costar) and treated with 100µL of different concentrations of *H. courbaril* extract previously dissolved at different concentrations (12.5, 25, 50, 100 and 200mg/mL) in DMSO (Dimethylsulfoxide) and RPMI such that the concentration of DMSO did not exceed 2%, or DMSO 2% or Negative control (RPMI medium and B16F10-Nex2 cells), resulting in a final volume of 200µL. *T*hen, they were incubated for 24 and 48 h at 37ºC in a 5% CO_2 atmosphere. Cell viability was quantified using the Trypan Blue exclusion method. All experiments were performed in triplicate. The results were expressed as percentage of cell viability.

MOUSE BONE MARROW MICRONUCLEUS AND CHROMOSOMAL ABERRATION TEST

Antigenotoxic effects of *H. courbaril* extract on cyclophosphamide-induced micronucleus in mice was tested using seven-week to twelve-week old male Swiss albino mice (*Mus musculus* Muridae, Rodentia) weighing 25-35 g purchased from an animal breeding center (Vallé, Uberlândia, MG, Brazil) and acclimatized in cages at 24±1°C under 12h light period for one week. During the acclimatization and throughout the experiments, the mice had free access to standard granulated chow and drinking water. Each cage contained five mice, which were randomly assigned to one of the 8 following groups: negative control group, given distilled water by oral gavage; positive control group, given a single intraperitoneal injection containing the equivalent of 0.2 mg per 100 g of body weight (bw) of cyclophosphamide (CAS n. 50-18-0; Endoxan, Baxter Oncology Gmb, Germany), dissolved in distilled water; the extract groups, given the equivalent of 50 mg, 100 mg or 200 mg per 1000g (bw) of extract each day for 7 days by oral gavage; and the experimental groups, given the same treatment as for the extract groups except that on the seventh day the mice also received the same treatment as the positive control group. The experimental protocol followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee for animals use (Permit number: 002/2010). All the mice were sacrificed by cervical dislocation on day eight. This study conforms to the relevant Brazilian guidelines regarding the ethical use of living animals. Genotoxic effects were evaluated in the mouse bone marrow by the micronucleus test (Schmid 1975). Immediately after sacrificing the mice, both femurs were removed from each mouse and the bone marrow flushed out into centrifuge tubes containing 2 mL of fetal calf serum and 1000 revs.min^{-1} for 10 min, after which the supernatant was discarded and the pellet resuspended in a drop of serum and a smear made on a clean slide. The smear was air-dried, fixed with absolute methanol for 5 min then air-dried and either stored at room temperature or directly stained for 5 min with a freshly prepared working solution of Giemsa stain diluted 1:1 v/v in 0.06 M sodium phosphate buffer and 0.06 M potassium phosphate buffer (both at pH 6.8). After staining the slides were rinsed in distilled water, dried at room temperature and scored for micronucleus according to the criteria of Krishna and Hayashi (2000) using objective lens of 100 x magnification and a Carl Zeiss optical microscope. 2000 polychromatic erythrocytes (PCE) per mouse were scanned and the number of micronucleated PCE (MNPCE) was recorded. To compare the frequencies of MNPCE and normal PCE between treated and control groups, the results were expressed as mean ± standard deviation based on differences among the mice within same group. For the chromosomal aberration test it was scanned 250 metaphases and gaps, chromosome breakage, as well as rings were recorded.

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical scavenging activity of the extracts was determined according to the technique described by Blois (1958). One mL of the 100 mM acetate buffer solution (pH 5.5) were mixed with 1.25 mL of absolute ethanol, then, 250µL of

500 μM DPPH solution (in ethanol) and 50μL of tested samples (250μg/mL in ethanol) or standard (gallic acid- 80μg/mL in ethanol) was added. After incubation at room temperature for 30 min, the absorbance of each sample or standard was determined at 517 nm in spectrophotometer UV-VIS (Femto, 800XI, Brazil). All determinations were performed in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation: Antioxidant activity (%) =[(Acontrol-Asample)/Acontrol]x 100, where A control is the absorbance of the control and A sample is the absorbance of the sample.

TOTAL FLAVONOID CONTENT

The total flavonoid content were measured by the colorimetric-based method assay (Christ and Mueller 1960). Briefly, 1.0 mL of the plant extracts was mixed with 4 mL 70% aqueous ethanol, and 0.5 mL $NaNO_2$ (5% w/v) was added. After 6 min, 0.5mL $AlCl_3$ (10% w/v) and 3 mL NaOH (1M) were added, followed by the addition of distilled water to reach 10 mL. The solution was mixed and incubated for 15min at room temperature. The absorbance of the extracts was measured using a UV- spectrophotometer (Femto, 800XI, Brazil) at 510 nm against the control. The control contained all the reaction reagents except the sample. The flavonoid content was calculated from the calibration curve and expressed as mg of rutin equivalents per gram of dried extract (mg RE/g dry extract). Each sample was tested in triplicate.

ANALYSIS OF FLAVONOIDS BY HPLC

Aiming to identify classes of compounds that may be linked to the activities evaluated in this study, the hydroethanolic extract from seeds of *H. courbaril* was analyzed by analytical HPLC. A sample (10 mg) of the hydroethanolic extract of *H. courbaril* seeds were dissolved in 1 mL of methanol /water (7:3 v/v) solution and filtered through a syringe filter with PTFE membrane (0.45 μm). The filtered solution was applied to a solid-phase extraction cartridge (Strata-X, Phenomenex®) containing 500 mg of C18 silica to remove non-polar compounds and tannins.

Chromatographic separation was performed in HPLC, quaternary gradient pump PU-2089S plus Jasco®, coupled photodiode array detector (PAD) with scan range 200-900 nm, MD-2015 Plus (Jasco®), AS-2055 autosampler (Jasco®) and CO-2060 plus column oven. The HPLC analyses were performed using a reverse phase column (Phenomenex® Luna C_{18}, 250 × 4.6 mm internal diameter; 5 μm) and Phenomenex® (4 × 3 mm internal diameter). The mobile phase was composed of solvent A (0.1% formic acid in methanol), and solvent B (0.1% formic acid in water). The data acquisition and treatment were performed using the Jasco Chrom Pass chromatography data system (Version 1.8.1.6). The samples were monitored in PDA detector into the visible region of the spectrum (UV-Vis) in the range 200-600 nm.

STATISTICAL ANALYSIS

The data were expressed as the mean ± SD by measuring three independent replicates. The chromosomal aberrations were analyzed statistically using the nonparametric Mann-Whitney U-test. Antioxidant activity was realized an analysis of variance using one-way ANOVA followed by Tukey's test the significance of differences between means obtained among the treatments at the level $\alpha \leq 0.05$ of significance using the BioEstat software version 5.0 (Ayres et al. 2007).

RESULTS AND DISCUSSION

In vitro CYTOTOXICITY ASSAY IN B16F10 MELANOMA CELLS

The Figure 1 shows the comparison between the cytotoxic effects of different concentrations of hydroethanolic extract of *H. courbaril* seeds

under cell line B16F10-Nex2 after 24 and 48 h of incubation.

One of the most important characteristics of cancer is the lack of control of the cell proliferation, differentiation and death (Loizzo et al. 2014). Therefore, compounds with antiproliferative activity becomes an important tool in combating this disease. The *in vitro* assay for the 24 and 48 h showed a marked decrease in the number of cells demonstrating that hydroethanolic extract from *Hymenaea courbaril* seeds has concentration and time-dependent cytotoxic effect against B16F10 melanoma cells line.

For the incubation period of 24 and 48 hour, the extract at a concentration of 50µg/mL reduced the number of cells by 58 and 91%, respectively, when compared to the negative control. This demonstrates that the time variation may interfere in the effect of a given concentration of the substance.

Figure 2 shows the cytotoxic activity of the extract at concentrations of 12.5, 50 and 100 µg/mL compared to the negative control after 48 h exposure period, demonstrating a significant cell growth reduction.

The cytotoxicity assay is a rapid method for screening natural and synthetic products with potential antitumor (Oliveira et al. 2013), this allows to determine the concentration and the time required for the occurrence of cell death in response to treatments. Several authors have reported cytotoxic activity of plants on melanoma cells by *in vitro* as well as *in vivo* methods. Krifa et al. (2014) demonstrated *in vitro* and *in vivo* significant anticancer effect of aqueous gall extract from *Limoniastrum guyonianum*. Rajasekar et al. (2012) showed cytotoxic activity of *Lithospermum erythrorhizon* on B16F10 cells, same activity was demonstrated by Oliveira et al. (2013) evaluating the latex of *Synadenium grantii*.

MICE BONE MARROW MICRONUCLEUS TEST AND CHROMOSOMAL ABERRATION TEST

An important feature to be considered in studies of compounds with pharmacological applicability is

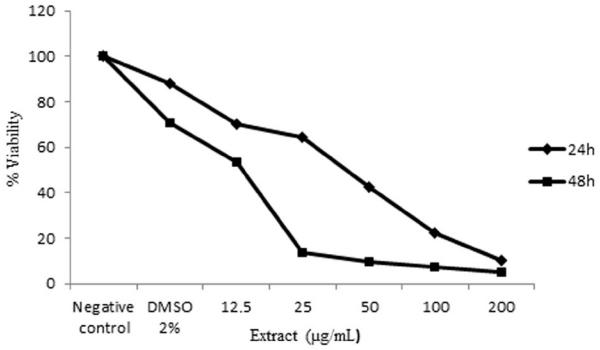

Figure 1 - Effect of *H. courbaril* extract on B16F10- Nex2 melanoma cell line using Trypan blue viability assay for 24 and 48 h exposure periods. The values represent the means ± S.D.

the balance between toxic and therapeutic effects (Cariddi et al. 2012). The increased frequency of micronucleated cells acts as a biomarker of genotoxic effects (Felzenszwalb et al. 2013). Table I shows the frequency of micronuclei in polychromatic erythrocytes (MNPCE) of bone marrow in mice after administration of *H. courbaril* seed extract, alone and in combination with Cyclophosphamide. In the groups treated with extract only, there was no significant difference in the number of MNPCEs between the evaluated concentrations, but they presented a reduction in the number of MNPCEs in comparison to the negative control. For the groups treated with the extract association and Cyclophosphamide, the number of MNPCEs observed did not differ between the concentrations evaluated and also in comparison with the negative control. These results reveals that the extract of *H. courbaril* does not present genotoxic properties, besides inhibiting the genotoxicity of cyclophosphamide in bone marrow cells of mice at the evaluated concentrations.

The groups treated with different concentrations of *H. courbaril* seed extract did not showed significant differences in mitotic index when compared with each other and the negative control group, indicating that these treatments were not cytotoxic (Table II). The groups treated with extract and CP exhibited significant mitotic index reduction in comparison with the groups treated only with extract, but less

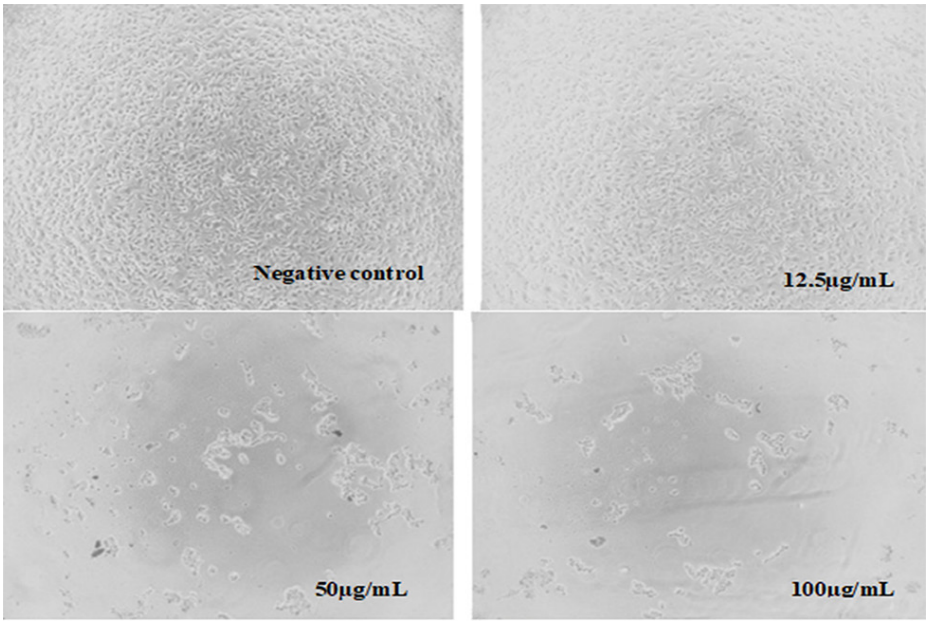

Figure 2 - Effect of *H. courbaril* extract on B16F10- Nex2 melanoma cell line for 48 h exposure period.

than the CP positive control. Maximum decrease in chromosomal aberrations were noticed in animals treated with E200 (4.8%), however, chromosomal aberrations decreased correspondingly with the increasing concentration of the extract in both treatments with and without CP. Among the different types of chromosomal aberrations a larger number of breaks were observed, followed by gaps and rings. When animals were treated with E50, E100 or E200, the percentages of breaks, were dramatically reduced compared to CP-induced aberrations.

Cyclophosphamide is a mutagenic substance that has been widely used as a positive control for genotoxicity testing (Leite-legatti et al. 2012). CP positive control in this study showed a significant induction of chromosomal damage in comparison to the negative control (distilled water), but *H. courbaril* extract was able to reduce these chromosomal damage thus demonstrating effect chemopreventive against cyclophosphamide induced damage. Several studies reported antigenotoxic activity in plant and its extracts, such as *Mandevilla velutina* (Silva et

TABLE I
Frequency of erythrocytes polychromatics micronucleuds (MNPCEs) of bone marrow cells of Swiss mice in experimental groups treated with hidroethanolic extract of *H. courbaril* seed.

Treatments	Nº of animals	Nº MNPCE	MNPCE Mean± SD (%)
NC	5	35	0.35± 0.09a
E50	5	09	0.09± 0.01b
E100	5	06	0.06± 0.01b
E200	5	03	0.03± 0.03b
E50 + CP	5	50	0.50 ±0.17a
E100 + CP	5	34	0.34 ±0.11a
E200 + CP	5	22	0.22 ±0.03a
CP	5	286	2.86± 1.78c

NC= Negative Control was distilled water; Animals were treated with the following doses of extract: E50: 50mg/kg bw, E100: 100mg/kg bw, E200: 200mg/kg bw, E50+CP: 50mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), E100+CP: 100mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), E200+CP: 200mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), CP: 2mg/kg bw of cyclophosphamide. Same letters within the same column indicate no significant diferences among samples by Tukey test ($\alpha \leq 0.05$).*They were analyzed 2000 erythrocytes polychromatics (EPC) for each experimental and control group.

TABLE II
Number and mean percentages of chromosomal aberrations and mean number of mitotic index in mice bone-marrow cells after treatment of the hidroethanolic extract of *H.courbaril* seed with or without CP. They were analyzed 2000 erythrocytes polychromatics (EPC) for each experimental and control group.

Treatments	Mitotic index (mean±SD)	Different types of chromosomal aberrations			Number of cells with aberrations	
		Breaks	Gap	Rings	Total	Mean± SD(%)
NC	1.38±0.52a	24	6	0	30	12.0±3.74a
E50	1.58±0.61a	22	4	0	26	10.4±2.53a
E100	1.42±0.45a	18	2	0	20	8.0±1.09a
E200	1.32±0.56a	11	1	0	12	4.8±0.98b
E50 + CP	0.93±0.34b	34	4	1	39	15.6±1.17c
E100 + CP	1.08±0.27b	21	5	2	28	11.2±0.97a
E200 + CP	1.12±0.32b	17	3	1	21	8.40±1.02a
CP	0.47±0.21c	121	26	8	155	62.0±11.22d

NC= Negative Control was distilled water. Animals were treated with the following doses of extract: E50: 50mg/kg bw, E100: 100mg/kg bw, E200: 200mg/kg bw, E50+CP: 50mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), E100+CP: 100mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), E200+CP: 200mg/kg bw + 2mg/kg bw of cyclophosphamide (CP), CP: 2mg/kg bw of cyclophosphamide. Same letters within the same column indicate no significant diferences among samples by Tukey test ($\alpha \leq 0.05$).

al. 2008), *Azadirachta indica* (Vinod et al. 2011) and *Liquidambar orientalis* Mill. var. *orientalis* (Saraç and Sen 2014). The results of the present study suggest that extract from *H. courbaril* seeds does not produces bone marrow micronucleus or chromosomal aberration, and thus, might be considered a source of antigenotoxic compounds.

DPPH RADICAL SCAVENGING ACTIVITY

Free radical-scavenging capacity of the hydroethanolic extract from *H. courbaril* seeds, measured by DPPH assay are shown in Table III.

Reactive oxygen species contribute to cancer development and progression by increasing DNA mutations, DNA damage and cell proliferation (Reuter et al. 2010). The antioxidants act as protective mechanisms which are able to donate electron and neutralize the reactive oxygen species (Saeidnia and Abdollahi 2013). DPPH assay is an easy, rapid and inexpensive colorimetric method for the evaluation of antioxidant properties; it is commonly used to measure the antioxidant content of several vegetables in different solvent systems (Loizzo et al. 2014).

The antioxidant capacity of the seed extract from *H. courbaril* (78.94%) did not significantly differ from gallic acid standard (79.98%). This data support previous studies such as Dias et al. (2013) who evaluated the seed oil of *H. courbaril* and found antioxidant capacity of 83.49% and Bezerra et al. (2013) who reported antioxidant activity in extracts and fractions from stembark of *Hymenaea courbaril* L.

TOTAL FLAVONOID CONTENT

The result of flavonoids observed to this extract (442.25 mg RE/g of dry extract) suggests that this class of compounds is responsible for significant antioxidant activity observed in the extract, this is due to its structure formed by the phenolic aromatic rings attached to one or more hydroxyl groups, which makes them capable of binding to free radicals (Saraç and Ben 2014).

Flavonoids possess a wide spectrum of pharmacological activities. Among these, the anticancer activity is highlighted. Therefore, different types of flavonoids and their mechanisms of action have been investigated in the search

TABLE III
DPPH radical scavenging activity and total flavonoids content (TFc) of the hidroethanolic extract of *Hymenaea courbaril* seed (HEHCS).

Sample	% Antioxidant activity	TFc (mg *RE/g of dry extract)
HEHCS (250µg/mL)	78.94±0.46a	442.25±18.03
Gallic acid (80µg/mL)	79.98±1.60a	

*RE=Routine Equivalent. Same letters within the same column indicate no significant diferences among samples by Tukey test (α≤0.05).

for natural source substances with potential application in different therapies for various diseases (Ravishankar et al. 2013).The results of this study demonstrate the potential of this species as a source of flavonoid. Furthermore, there are few scientific studies investigating or demonstrating the flavonoid content of extracts and preparations from species of the genus *Hymenaea*.

ANALYSIS OF FLAVONOIDS BY HPLC

Chromatogram of the hydroethanolic extract from *H. courbaril* seeds obtained by HPLC-PAD showed four major peaks (Figure 3). Flavonoid compounds have a characteristic absorption spectrum in the UV region with two bands of higher absorption, which the band I absorbs between 300-400 nm and refers to ring B and the band II absorbs between 240-285 nm and refers to ring A.

HPLC-PAD chromatogram obtained from the hydroethanolic extract of *H. courbaril* seeds in analytical scale revealed the presence of flavonoid compounds. Peaks were eluted between 19.36 and 30.15 minutes, the peak 4 eluted at around 30.15 minutes is highlighted due to the higher intensity (at 350 nm) compared to the other peaks (Figure 4).

The UV spectra of all numbered peaks showed absorption between 284-350nm relating to band I and therefore, suggests that the peaks of the chromatogram refer to flavones. The flavone band I occur at 304-350 nm (Almeida et al. 2012).

The data obtained from the HPLC-PAD analysis support previous studies with other species of this genus. c. Maranhão et al. (2013) identified

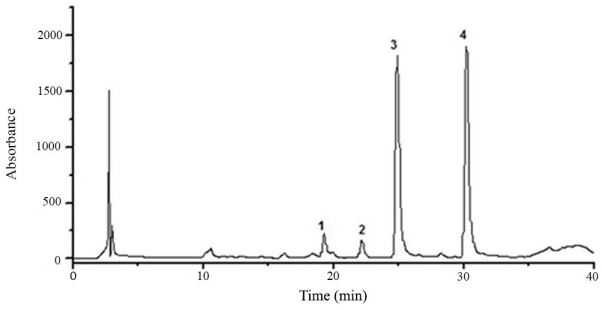

Figure 3 - HPLC-PAD flavonoids profile of the hydroethanolic extract from seeds of *H. courbaril*. Phenomenex® Luna Column (C18 250 x 4.6 mm d. i., 5 µm). Eluent A (0.1% formicacid in methanol); Eluent B (0.1% formic acid in water). Ranging from 20-50% of A in 30 min and 30-35% of A in 5 min at a flow rate of 1mL.min^{-1}. Injection volume: 40µL. Column Oven: 40˚C. λ = 350 nm.

a flavonone (hultenin) and other three flavonoids (taxifolin, quercetin and 7-methoxycatechin) in extract of heartwood of *H. stigonocarpa*, Dimech et al. (2013) reported presence of flavonoids in extracts of this same species.

CONCLUSION

The *in vitro* cytotoxic activity and *in vivo* genotoxic and antigenotoxic activities of the extract of *H. courbaril* seeds on murine melanoma cells was first time reported in this paper. The results obtained in this study exposed that *Hymenaea courbaril* has cytotoxic effect against B16F10 melanoma cells line, antigenotoxic effect on mice bone marrow cells and present antioxidant activity. Furthermore, the results obtained in this study exihibited that *Hymenaea courbaril* has flavonoids which possibly belong to the class of flavones. This can explain the biological activities observed in this study.

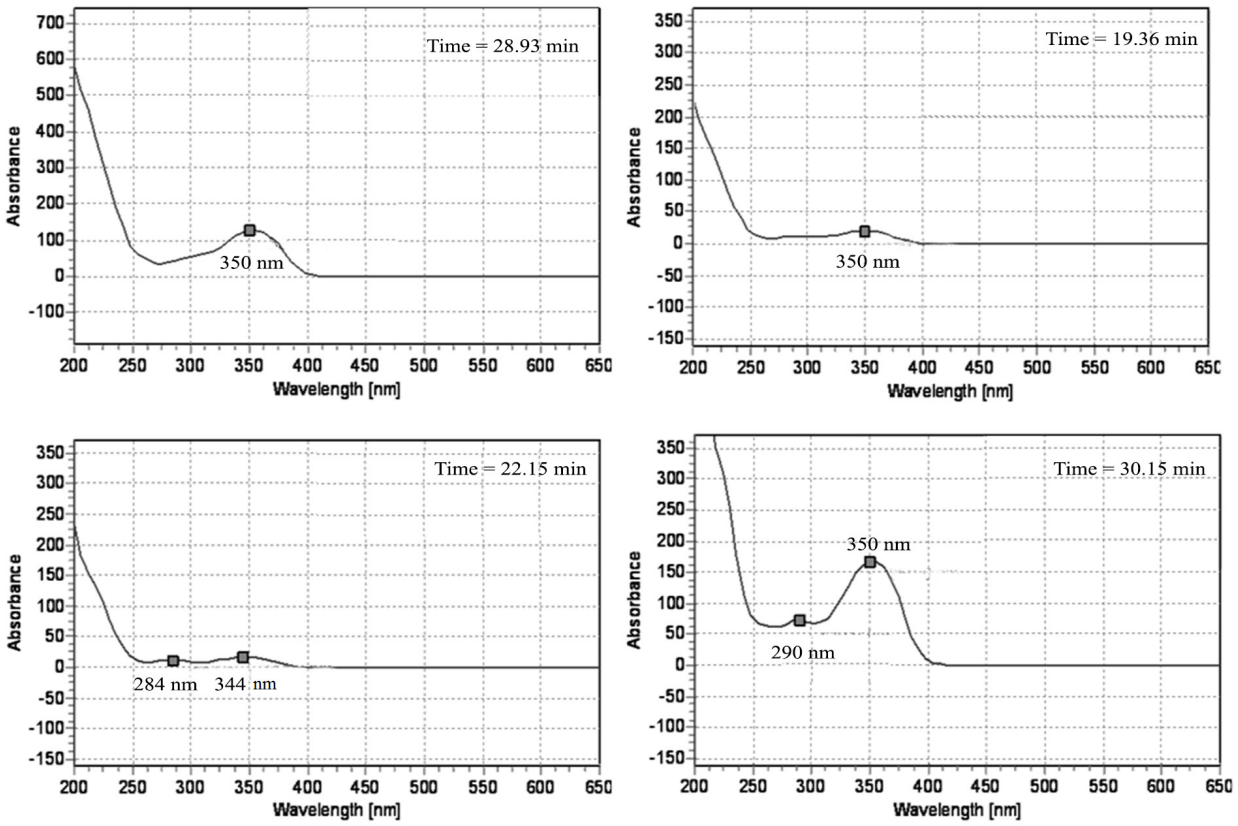

Figure 4 - Spectra in the UV-Vis region obtained by HPLC-PAD of the hydroethanolic extract from seeds of *H. courbaril*.

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AUTHOR CONTRIBUTIONS

The authors Kamille Daleck Spera and Pamela Cristina e Santos contributed to concept, design and experimental studies of antioxidant activity, Patrícia Aparecida Figueiredo contributed to concept, design and experimental studies of genotoxicity activity. Fernando Cesar Barbosa, Caio Pismel Alves and Carlos Rogério Figueiredo contributed to experimental studies of anti-melanoma activity and statistical analysis. Anne Lígia Dokkedal and Luiz Leonardo Saldanha contributed to phytochemical analysis, Paulo Cesar Ferreira, Luciana Pereira Silva and Regildo Marcio Gonçalves da Silva contributed to concept, design, experimental studies data acquisition, statistical analysis and manuscript review.

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