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# INHIBITION OF TUMORAL GROWTH BY HYDROETHANOLIC EXTRACT OF PEEL FROM *CAMPOMANESIA ADAMANTIUM* (CAMBESS) O. BERG. IN MELANOMA

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**ABSTRACT**

Cerrado, Brazilian biome, presents numerous species of plants with pharmacological potential, among these, there is Campomanesia adamantium (Cambess.) O. Berg. (guavira) whose fruits are associated with various biological activities such as antiproliferative and antimutagenic action. Purpose of study was evaluate anticancer activity of hydroethanolic extract of guavira fruit peel and determine its acute oral toxicity.For this were used 25 male BALB/c mice inoculated with murine melanoma B16-F10 cells (5 x 105 / 0,2 µL), treated after 10 days in a single intraperitoneal dose, at doses of 2.5, 5, and 10 mg / animal, from guavira peel extract. For acute toxicity, female and male Swiss mice, 15 of each, were treated with concentrations of 50, 300 and 2000 mg / kg in single oral dose. Results showed anticancer action at 5 mg / animal concentration with up to 82% inhibition of tumoral growth. Acute oral toxicity test demonstrated a satisfactory result, since concentration of 2000 mg / kg did not lead any animal death, without significant changes related to hippocratic screening. Therefore, results found for guavira extract has great importance for confirmation of antitumor activity and stimulate deepening of studies to identify and isolate active compounds present in plant.



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## INTRODUCTION

Cancer is a term associated with a set of more than 100 diseases, characterized by the disordered (malignant) growth of cells that invade organs and tissues, and can spread (metastasize) to other regions of the body (INCA, 2018). According to National Cancer Institute (2018), cancer is among leading causes of death in world and predicts that by 2030, number of new cases per year will rise to 23.6 million, estimated approximately 440 new cases per 100,000 people, according to data collected by 2015.

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According to data from 2018, most common types of cancers are breast, lung, prostate, colon and rectum, melanoma skin, bladder, lymphoma, kidney, endometrial, leukemia, pancreatic, thyroid and liver. Skin cancer can be classified as non-melanoma and melanoma. Melanoma-like cancer originates from melanocytes, which are skin cells that produce a pigment called melanin, pigment responsible for protection against damage caused by ultraviolet (UV) radiation. Development of melanoma is a consequence of loss of genetic mechanisms (mutations) of cellular control caused mainly by UVA and UVB radiation (Liu and Herlyn, 2005). Brazil has a very diverse flora, possessing a great wealth of medicinal plants whose active principles are often still unknown and yet used for treatment, cure and prevention of diseases (Varanda,2006; *et al*, 2008; Veiga Junior *et al*, 2005). Cerrado is second largest

Brazilian biome in extension with approximately 204 million hectares (Sano *et al*., 2010; Silveira *et al*., 2015) and features more than 12,000 plant species (Mendonça *et al*., 2008) of great commercial importance (food, landscape and logging) and high pharmacological potential (Klink and Machado, 2005). Thereby, several studies have been carried out in order to encourage use of cerrado fruits, since they are known to be natural sources of bioactive compounds (Bailão *et al*., 2015; Bortolotto *et al*., 2016; Rocha *et al*., 2011). *Campomanesia adamantium* (Cambess.) O. Berg. is a specie of plant belonging to Myrtaceae family, whose fruits are known as guavira or guabiroba and that is found in cerrado of central region of country (Sobral *et al*., 2014). From different parts, mainly leaves and fruits, were attributed different biological activities associated with presence of phenolic compounds, flavonoids and chalcones, present in this species such as: anti-inflammatory (Ferreira *et al*., 2013; Souza *et al*.,2017), antidiarreal (Lescano *et al*., 2016), antioxidant (Ramos *et al*., 2007; Coutinho *et al*., 2008; Pascoal *et al*., 2011), antimicrobial (Pavan *et al*., 2009), antiproliferative (Pascoal *et al*., 2014) and mainly of antimutagenic action (Martello *et al*., 2016). Studies carried out by Lima e Silva *et al*. (2018), demonstrate antiproliferative activity of pulp and peel dichloromethane extracts of guavira in tumoral lines B16-F10, MCF-7, PC-3 and HEP-G2, showing concentrations of GI50 (concentration that inhibits 50% of cell growth) between 23.67 and 27.90 µg / ml. In vitro assays contribute to screening of compounds for their anticancer activity. Neoplastic cells are used in evaluations of antiproliferative activity (Holbeck, 2004; Skehan *et al*., 1990). However, selective cytocidal activity on cancer cells observed *in vitro* models may not necessarily be reflected *in vivo,* since in this assay information on pharmacokinetics and pharmacodynamics of drug under study is considered, unlike *in vitro* assays. Therefore, combined study of these two models is indicated for a more complete evaluation regarding activity of a new substance with anticancer potential (Smith *et al*., 2005). Murine melanoma is one of most used experimental models in studies of antitumor activity, with lineage B16-F10, in mice of lineage BALB/c, allowing evaluation of substances regarding their anticancer potential (Junqueira, 1997; Nakamura *et al*., 2002). Considering potential antiproliferative activity of specie, reported by Lima e Silva *et al*. (2018), present study aims to evaluate anticancer activity of hydroethanolic extract of fruit peels of guavira in an experimental model of murine melanoma and its acute toxicity.

## MATERIALS AND METHODS

**Botanical Material - Collection and identification of specie:** fruits and leaves of specie *Campomanesia adamantium* (Cambess.) O. Berg were collected in months of November and December in municipality of Santa Rita do Pardo in state of Mato Grosso do Sul. Samples were identified and exsicata of specie was deposited in Herbarium Campo Grande Mato Grosso do Sul (CGMS), n° 53328.

**Botanical Material - Preparation of fruit peel extract:** For preparation of hydroethanolic extract (30:70) fruit peels were separated from pulp and added to solvent in their in natura form, where they underwent exhaustion maceration for 15 days at room temperature, with solvent exchange every 5 days. At the end, extract was filtered, concentrated in a rotary evaporator to completely remove the solvent and lyophilized. Sample was stored in a freezer at a temperature of -20 °C until moment of use.

**Cell culture:** Cells from B16-F10 strain (ATCC-CRL-6322, murine melanoma), cryopreserved in liquid nitrogen, were thawed and cultured in sterile vials in RPMI 1640 culture medium and *Dulbecco*'s modified minimal essential medium (DMEM) supplemented with 10 % fetal bovine serum and 50 µg gentamycin / ml (Europharma) (complete medium). They were placed at 37 °C in a humid atmosphere containing 5% $CO_2$(Freshney, 2005). Once cells are adherent, it was removed with trypsin solution (0.25% + 1mM EDTA) in PBS buffer, pH 7.4. They were transferred to conical tubes containing complete culture medium. After centrifugation at 1000 rpm for 4 minutes, the trypsin medium was discarded and cells was resuspended in small volume of complete medium. Viable cell counts were made with *Trypan Blue*® in Neubauer's Chamber in order to obtain ($5 \times 10^5$ / 0.2 mL PBS) for subcutaneous inoculation in the interscapular region of mices.

**Evaluation of anticancer activity of *Campomanesia adamantium* extract:** For the accomplishment of these tests, project was submitted toEthical Committee on Use of Animals of Federal University of Mato Grosso do Sul (CEUA - UFMS), protocol n °684/2015, according to determination of the National Council of Control of Animal Experimentation (CONCEA, 2013). The place to carry out experiments was Biotherium of experimentation attached to Laboratory of Biophysiopharmacology of UFMS. 25 male BALB / c mice weighing between 25 and 35 g were used. All from Central Biotherium of Federal University of Mato Grosso do Sul (UFMS). Animals were kept in collective cages (size 40 x 35 x 17 cm), in amount of 5 animals / cage, at temperature of approximately 25 ° C, with light / dark cycle of 12 hours, receiving standard feed (NUVITAL® CR1) and water at will. They were acclimatized to laboratory conditions for 3 days before being used experimentally. On the 10 th day after inoculation of cells the animals were divided into groups (n = 5) and treated as described below:

- Group Negative Control: Olive Oil (0,2 mL, intraperitoneal - i.p.);
- Group Positive Control: Doxorrubicina (5 mg / Kg / 0,2 mL, i.p.);
- Group *C. adamantium*fruit peel extract (2,5 mg / animal / 0,2 mL, i.p);
- Group *C. adamantium*fruit peel extract (5 mg / animal / 0,2 mL, i.p);
- Group *C. adamantium*fruit peel extract (10 mg / animal / 0,2 mL, i.p).

Treatment scheme used was a single dose / animal. After administration of extract diluted in olive oil at different concentrations, animals were under observation for evaluation of physical signs (grip strength, tremors, piloerection, convulsions, breathing, appearance, changes in abdomen) and behavioral (irritability, response to touch, spontaneous and provoked behavior) at times of 30, 60, 120 and 240 minutes (Brito, 1994). On 21st day after inoculation of cells (11 days after treatment), animals were euthanized. Afterwards, nodules formed in dorsal region of mice were removed and evaluated weight (g) and volume ($mm^3$) of tumors. In order to determine tumor mass, nodules were weighed in RADWAG® analytical balance. Diameters were measured using the WORKER® digital caliper for volume determination, according to Steel's

formula (Dxd2 / 2), where D and d give the largest and smallest diameters respectively (Neto *et al.,* 2002). From this, calculation was made for defining inhibition rate of tumor growth.

**Statistical analysis:** Results obtained *in vivo* test of anticancer activity against solid tumors were expressed as mean ± standard error of mean (SEM) and compared with the controls by analysis of variance (ANOVA) followed by the Benferroni test.

**Evaluation of acute toxicity of extract of *Campomanesia adamantium*:** Acute oral toxicity was performed according to Acute Toxic Class Method (OECD-423/2001). For acute toxicity test, 15 female mice and 15 male mice weighing between 25 and 35 g, both Swiss (*Mus musculus*) were used. All from Central Biotherium of Federal University of Mato Grosso do Sul (UFMS). Animals were kept in collective cages (size 40 x 35 x 17 cm), in amount of 3 animals / cage, at temperature of approximately 25 ° C, with light / dark cycle of 12 hours, receiving standard feed (NUVITAL® CR1) and water at will. Animals were divided into groups of three animals for both sexes, GFC and GMC being male and female control groups, respectively, that received vehicle (olive oil) and treated groups, GFT (female) and GMT (male), receiving doses of 2000 mg / kg, 300 mg / kg, 50 mg / kg and 5 mg / kg body weight, of *Campomanesia adamantium* extract, diluted in olive oil, by single dose gavage. For acute toxicity test, animals were fasted for 6 hours prior to administration, with free access only to water. Initial dose tested was 50mg / kg, due to lack of toxicity data in literature. If there were no deaths of animals in first 24 hours of exposure, the other doses were tested. After each treatment, animals were observed in first 30 minutes, 1, 2, 3, 4, 6, 12, 24 hours, and periodically for 14 days (OECD, 2001) regarding behavioral, motor and sensorial functions to access the potential effects neurotoxic from hippocratic screening (systematic behavioral analysis). Animal's body weight was checked on day of administration of compounds and on fourteenth day after administration. On fourteenth day animals were euthanized. Organs (heart and lung) were observed macroscopically and liver and kidneys of each animal were removed and weighed.

**Evaluated Parameters:** During experiment, body mass was evaluated, in addition to relative weight of organs. Hippocratic screening was applied in an open location in which observed parameters were (anesthesia, general activity, grasping strength, touch response, tremors and death). Signs of toxicity, timing of onset, intensity, duration, and progression were recorded by tabulating them on a scale of 0 to 4 (absent, rare, mild, moderate, severe) for further analysis.

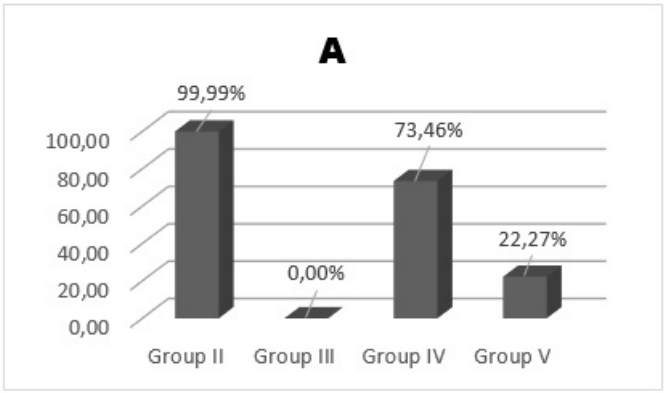
**Euthanasia method:** After fourteen days of analysis, all groups were weighed and euthanized in a $CO_2$ chamber.

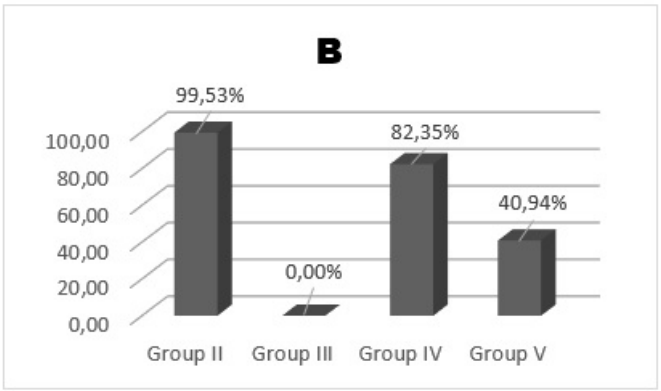
**Relative mass of organs:** After euthanasia of animals, liver and kidneys were removed and weighed. Relative mass of organs of each animal was calculated by dividing weight of each organ by body weight of each animal on day of collection and multiplying result by 100. Result was expressed in g / 100 g of live weight (g / 100g l.w.).

**Statistical analysis:** For obtained data the analysis of variance (ANOVA one-way) followed by Benferroni test was applied.

## RESULTS

As regards evaluation of *in vivo* anticancer activity of *Campomanesia adamantium* against B16-F10 (murine melanoma cell) in mice, it was observed that animals treated with doxorubicin presented tremors, wheezing and piloerection in first 60 minutes, remaining thus until 120 minutes. Animals of negative control group presented normal behavior, as well as treated animals. There was no death, up to 24 hours after administration. Results showed a decrease in weights and volume of nodules of animals of Groups II positive control, IV (5 mg / animal) and V (10 mg / animal) when compared to Group I negative control ($p < 0.05$). Group III (2.5 mg / animal) had no effect on reduction of tumor growth, with a significant result when compared to Group I negative control (Table 1). Once percentage of inhibition of tumor growth has been compared, promising effect of extract compared to chemotherapy of choice (doxorubicin) is notorious. Graph 1 shows percentage inhibition of tumor growth in relation to weight (A) and volume (B) in relation to Group I negative control.




Group II: positive control (doxorubicin 5 mg / kg); Group III: 2.5 mg / animal; Group IV: 5 mg / animal; Group V: 10 mg / animal of *Campomanesia adamantium* fruit peel extract.

**Graph 1. Percentage inhibition of tumor growth in relation to weight (g) (A) and volume (m3) (B)**

Acute toxicity test evaluations conducted in this study demonstrated that, during the 14 days of experimentation, no abnormal or major behavioral changes were found. Normal values were found in hippocratic screening of both females and males control (GFC and GMC), as well as their treated 50 mg/kg equivalents (data not shown). However, at concentration of 300 mg / kg, animals showed a slight change in relation to general activity and grabbing force (Tables 2 and 3). These parameters evaluated and considered more relevant were observed only in first 24 hours, later, they remained normal until end of experiment.

**Table 1. Nodules weight and volume obtained from BALB / c mice after treatment with *Campomanesia adamantium* fruit peel extract**

| Treated Groups | Weight (g) | Volume (mm³) |
|---|---|---|
| Negative Control (olive oil) Group I | 2.11 ± 0.14 | 4.25± 1.22 |
| Positive Control (doxorrubicina) Group II | 0.02 ± 0.0* | 0.02 ± 0.0* |
| 2,5 mg / animal Group III | 3.5 ± 0.74* | 6.28± 1.75 |
| 5 mg / animal Group IV | 0.56 ± 0.30* | 0.75±0.4* |
| 10 mg / animal Group V | 1.64 ± 0.49 | 2.51±0.96 |

*= significant difference between negative control group (Group I) and the other groups (ANOVA, followed by Bonferroni test, p <0.05). Results expressed as mean ± standard error of mean (SEM).

**Table 2. Parameters evaluated in hippocratic screening of female animals treated with 300 mg / kg of *Campomanesia adamantium* fruit peel extract in comparison to female control group**

| Evaluated | Group | Time(hours) | | | | | | | Time(days) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| parametersa | (n=3) | 0,5 | 1 | 2 | 4 | 6 | 12 | 24 | 3 | 7 | 9 | 10 | 14 |
| Anesthesia | GFC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GFT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| General | GFC | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Activity | GFT | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 3 |
| Grabbing | GFC | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Force | GFT | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Touch | GFC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Response | GFT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tremors | GFC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GFT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Score: 4: severe; 3: moderate; 2: mild; 1: rare and 0: absent / GFC: female control group (treated with olive oil); GFT: treated female group.

**Tabela 3 – Parameters evaluated in hippocratic screening of male animals treated with 300 mg / kg of *Campomanesia adamantium* fruit peel extract in comparison to male control group**

| Evaluated | Gorup | Time(hours) | | | | | | | Time(days) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| parameters | (n=3) | 0,5 | 1 | 2 | 4 | 6 | 12 | 24 | 3 | 7 | 9 | 10 | 14 |
| Anesthesia | GMC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GMT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| General | GMC | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Activity | GMT | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Grabbing | GMC | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Force | GMT | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Touch | GMC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Response | GMT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tremors | GMC | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | GMT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 4. Values referring to body weight of animals and organ weight (kidneys and liver) after oral administration in a single dose of *Campomanesia adamantium* fruit peel extract**

| GFC | Initial weight | Final weight | Kidney (R) | Kidney (L) | Liver |
|---|---|---|---|---|---|
| | 37 | 43 | 0.273 | 0.235 | 1.974 |
| | 35 | 45 | 0.333 | 0.293 | 2.212 |
| | 30 | 37 | 0.245 | 0.272 | 1.798 |
| Mean ± MSE | 34±2.00 | 41.7 ±2.40 | 0.280 ±0.00 | 0.260 ±0.12 | 1.990 ±0.12 |
| GFT | Initial weight | Final weight | Kidney (R) | Kidney (L) | Liver |
| | 37 | 36 | 0.283 | 0.263g | 1.657g |
| | 43 | 42 | 0.234 | 0.273g | 1.926g |
| | 30 | 38 | 0.293 | 0.295g | 1.717g |
| Mean± MSE | 39.6 ±1.76 | 38.6 ±1.76 | 0.270 ±0.00 | 0.410 ±0.00 | 1.760 ±0.00 |

Results expressed in grams, mean and mean standard error (MSE). Initial Weight: first day of treatment; Final Weight: after 14 days of treatment; GFC: female control group (treated with olive oil); GFT: female group treated at the dose of 300 mg / kg; R: right; L: left.

Among concentrations tested, none of them took animals to death. Knowing that hippocratic screening is part of a previous screening useful in evaluation and drug-toxicological functioning of substances evaluated (Cunha *et al*., 2009), it can be considered that such changes did not result in relevant acuity signs. Results of toxicity obtained did not show a significant statistical difference between weights of control animals and animals that were submitted to administration of extract of *Campomanesia adamantium* in concentration of 300 mg / kg ($p = 0.37$). No significant difference was found in weight of kidneys right ($p > 0.05$) and left ($p = 0.6$) and liver ($p = 0.18$) of animals compared to control group and treated group (Table 4). Macroscopic evaluation of hearts and lungs did not indicate morphological changes when compared to the organs of untreated control group.

## DISCUSSION

Several studies have demonstrated the efficacy of *Campomanesia adamantium* with antioxidant, hepatoprotective (Fernandes *et al*., 2015), antimicrobial (Sá *et al*., 2018), anti-inflammatory, antinociceptive action (Ferreira *et al*., 2013; Viscardi *et al*. (2016), antiproliferative action against prostate cancer cells (Pascoal *et al*., 2014) and with potential antiproliferative action against breast cancer cells (Hermawan and Putri, 2018). According to Lima e Silva *et al.* (2018) compounds of flavone and chalcone class may be responsible for antiproliferative activity of *Campomanesia adamantium.* Based on results presented, present study demonstrated that fruit peel extract of *C. adamantium* had antiproliferative action against murine melanoma cells, at a concentration of 5 mg / animal. Regarding weight of tumors, a reduction of 73.46% was observed in relation to positive control, considering that doxorubicin in this assay presented a reduction of 99%. Regarding tumor volume, reduction at this same concentration was 82.36%, close to reduction caused by doxorubicin (99%). Maximum concentration of 10 mg / animal had a reduction of inhibitory action, which may be attributed to other compounds present in crude extract used. In acute toxicity test, in concentration of 300 mg / kg (approximately 12 mg / animal), none of animals died, as were not observed any significant changes related to behavior of treated animals, nor changes in weights of evaluated organs and own animals. Thus, a satisfactory result was obtained, since the concentration used for acute toxicity was twice as high as the concentration that presented highest antiproliferative activity (5 mg / animal) in *in vivo* assay (murine melanoma).

### Conclusion

Extract did not cause death of animals up to dose of 2000 mg / kg observed in *in vivo* acute toxicity experiment. In experimental model of murine melanoma, it was possible to observe that best dose-response for inhibition of tumor growth was 5 mg / animal, presenting aproximately 73 % and 82 % inhibition of growth in weight and volume of tumors, respectively. Hydroethanolic extract of guavira peel can be considered as a potential anticancer agent with promising effects in treatment of melanoma. Identification and isolation of active compounds, as well as, new dose and concentration studies, both *in vitro* and *in vivo*, is required.

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