

Effect of *Porophyllum ruderale* (Jacq.) Cass. in the Liver of the B16-F10 Murine Melanoma Model and Antioxidant Potential

Efeito de *Porophyllum ruderale* (Jacq.) Cass. no Fígado do Modelo de Melanoma Murino B16-F10 e Potencial Antioxidante

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Abstract

The aim of this study was to evaluate the *Porophyllum ruderale* effects on the liver in murine melanoma model B16-F10 and to determine the antioxidant potential. The plant's aerial parts of the were collected in Campo Grande, MS, Brazil, dried, extracted with ethanol, ultrasonic bath and static maceration. The hydroalcoholic formulation at 100 and 200 mg/kg were submitted to antioxidant activity (DPPH) and hepatoprotective effect. Thirty-eight black mice with a mean weight of 30 g were distributed in 4 experimental groups: negative control, positive control (doxorubicin), *P. ruderale* at 100 mg/kg and *P. ruderale* at 200 mg/kg. All the animals received subcutaneous injection of 5×10^5 B16F10 cells on the 1st day of the experiment and were treated until the 14th day. Although a significant antioxidant activity was verified for the two concentrations analyzed, they were not sufficient to minimize the melanoma effects toxic in the mice liver as observed by histological changes and that leads us to conclude that the hydroalcoholic extract of aerial parts of *P. ruderale*, in the concentrations used, possesses antioxidant properties, good performance in inhibiting the proliferation of human melanoma cells, but it has no hepatoprotective effect in the melanoma cutaneous treatment.

Keywords: Cutaneous Melanoma. Asteraceae. Hepatoprotective Effect. Phenolic Compounds. DPPH.

Resumo

O objetivo deste estudo foi avaliar os efeitos de *Porophyllum ruderale* no fígado em melanoma murino modelo B16-F10 e determinar o potencial antioxidante. As folhas da planta foram coletadas em Campo Grande, MS, Brasil, secas, extraídas com etanol, banho ultrassônico e maceração estática. A formulação hidroalcoólica de 100 e 200 mg/kg foi submetida à atividade antioxidante (DPPH) e ao efeito hepatoprotetor. Trinta e oito camundongos pretos com peso médio de 30 g foram distribuídos em 4 grupos experimentais: controle negativo, controle positivo (doxorubicina), *P. ruderale* na concentração de 100 mg/kg e *P. ruderale* na concentração de 200 mg/kg. Todos os animais receberam injeção subcutânea de 5×10^5 células B16F10 no 1^o dia de experimento até o 14^o. Embora tenha sido verificada atividade antioxidante significativa para as duas concentrações analisadas, elas não foram suficientes para minimizar os efeitos tóxicos do melanoma no fígado dos camundongos, como observado nas alterações histológicas, o que nos leva a concluir que o extrato hidroalcoólico das folhas de *P. ruderale*, nas concentrações utilizadas, possui propriedades antioxidantes, bom desempenho na inibição da proliferação de células de melanoma humano, mas não apresenta efeito hepatoprotetor no tratamento do melanoma cutâneo.

Palavras-chave: Melanoma Cutâneo. Asteraceae. Efeito Hepatoprotetor. Compostos Fenólicos. DPPH.

1 Introduction

Among the several existing neoplasms, skin cancer is the most predominant, accounting for 25% of cancers (MORENO *et al.*, 2012). According to the National Cancer Institute (INCA, 2018), regarding skin neoplasms, 3% are cutaneous melanoma type, with alterations in the melanocytes cellular division. Such cells are melanin producing, responsible for the cellular nucleus pigmentation and protection.

However, despite great efforts to identify new therapies for the metastatic melanoma treatment, different treatment

methods for regression remain rare in patients with advanced disease and the organ often affected by metastatic dissemination of cutaneous melanoma is the lung (18-36%) (JUNQUEIRA; CARNEIRO, 2017) and the usual treatments also involve liver and kidneys (HOFMANN *et al.*, 2016).

In Brazil, as a complementary treatment, several plants are used in traditional medicine to fight cancer. This use was approved in 2006 by the Health Ministry (BRASIL, 2006), in basic health units and, then the Policy on Integrative and Complementary Practices (PICP) and National Policy on Medicinal Plants and Phytotherapies (BRASIL, 2008).

Among the species used by traditional communities, to many different therapies, including cancer, *Porophyllum ruderale* (Jacq.) Cass., Family Asteraceae stands out, which is an herb habit plant ruderal, of medium size, native in Brazil, pointed out as an invasive species of open agrosystems with its high dispersal capacity, and fast growth in the environment. It is an aromatic plant with strong fragrance, known as Brazilian arnica or “arnica” (KISSMAN; GROTH, 1999; ATHAYDE *et al.*, 2019).

Recently, our research group carried out studies with the ethanolic extract and fractions of *P. ruderale*, performing a toxicological bioassay using *Artemia salina* (Linnaeus, 1758) (MENDONÇA *et al.*, 2020) and antiproliferative *in vitro* activity with four tumoral strains, being potentially active to PC-3, HT-29 and MCF-7 (MENDONÇA *et al.*, 2019) (KISSMAN; GROTH, 1999). Recently, our research group carried out studies with the ethanolic extract and fractions of *P. ruderale*, performing a toxicological bioassay using *Artemia salina* (Linnaeus, 1758) (MENDONÇA *et al.*, 2020) and antiproliferative *in vitro* activity with four tumoral strains, being potentially active to PC-3, HT-29 and MCF-7 (MENDONÇA *et al.*, 2019). In both studies, phytochemical analysis was performed and phenolic compounds, flavonoids and the presence of thiophenoids were evidenced. In order to continue the plant validation for the cancer treatment and at the same time to seek complementary therapies that may help patients with melanoma, the present study aims to evaluate *in vivo* anticancer activity of hydroalcoholic solutions aerial parts (100 and 200 µg/kg) of *P. ruderale* in liver of mice and their antioxidant potential.

2 Material and Methods

2.1 Collection of botanical material and extraction

The aerial parts of *P. ruderale* were collected in native vegetation areas in Campo Grande, MS, Brazil (S20°26'20.64" O54°32'26.78"). After identification, a voucher specimen was cataloged and incorporated into the Herbarium of Federal University of Grande Dourados – DDMS (Dourados, Mato Grosso do Sul, Brazil) (number 6363). To collect and research purposes authorization was obtained for access to genetic resources of the “The Genetic Patrimony Board of Management (GPBM)” under the registration number 010579/2013-3.

After the plant material was drying in an stove with air circulation at 40 °C (MARCONI®, MA35), it was triturated in electrical mill (MARCONI®, MA048). The crude ethanolic extract was obtained following the methodology of Andrade *et al.* (2018), with some modifications, using 800 g of the plant powder extracted with ethanol (99.5%) in ultrasound bath (ultrasonic Cleaner®) for 60 minutes, followed by extraction by maceration during 7 days. The resultant solution was filtered, and the solvent was evaporated, obtaining the ethanol extract, which was subjected to chemical analysis.

2.2 Phenolic compounds and flavonoids determination

Crude ethanolic extract and hydroalcohol solutions (1:10, C₂H₅OH:H₂O) at doses of 100 and 200 mg/kg were used to quantify total phenols by the Folin-Ciocalteu method with gallic acid (Vetec®) (10 to 350 mg mL⁻¹) as standard ($Y = 0.7182x + 0.0927$, $R^2 = 0.982$), and the flavonoids were evaluated by the aluminum chloride method and, as standard, quercetin (Sigma®) ($Y = 0.1114x + 0.0030$, $R^2 = 0.999$) (DO *et al.*, 2014) in spectrophotometer (Femto®, model 800XI).

2.3 Antioxidant Activity

The antioxidant activity of crude ethanolic extract and hydroalcohol solutions (1:10, C₂H₅OH:H₂O) at doses of 100 and 200 mg/kg was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) (BLOIS, 1958), using as standard BHT at concentrations of 0.5 to 100 µg/mL and 0.1 mL of solution 1 mM DPPH free radical. After 30 minutes incubation at ambient temperature, the free DPPH free radical reduction was measured by reading the absorbance at 517 nm against a specific blank at each evaluation, on UV-visível (Femto®, 800XI) spectrophotometer (BLOIS, 1958). All analyzes were performed in triplicate. To detect differences between means and evaluate these analysis of variance (ANOVA) and Tukey test with 95% confidence level. %Inhibition= [(Control Absorbance – Absorbance of the sample)/control absorbance] X 100. The EC₅₀ values were calculated and BHT was used as standards with a concentration of 100 mg/L (MENSOR *et al.*, 2001).

2.4 Animals and Procedures

Thirty-eight mice (*Mus musculus* L.) of the Black C57BL/6 line, family Muridae, with a mean weight of 30 g, were obtained from the Federal University of Mato Grosso do Sul (UFMS). The animals were kept in polypropylene boxes and fed with commercial feed and filtered water *ad libitum*. The temperature and luminosity were controlled with a 12-hour photoperiod (12 hours clear: 12 hours dark), with temperature control (22 ± 2 °C) and relative humidity (55 ± 10%). All the procedures were performed in accordance with the standards established by the National Council for Animal Experimentation Control (CONCEA, 2018) and the experimental protocols were approved by the Ethics Committee on Animal Use of Uniderp University under opinion no. 3051 de 19/12/2017.

On the first day of experiment all the animals received a suspension of B16F10 cell line (murine melanoma) at the cell density of 500,000 cells/animal. The treatment was performed on the 14th day of the experiment, with administration of hydroalcoholic solution of aerial parts of *P. ruderale*, intraperitoneal (i.p.), at concentrations of 100 and 200 mg/kg of animal weight.

The animals were divided into 4 experimental groups: Group 1 - Negative Control (n = 10): i.p. (200 µL) of PBS. Group

2 - Positive Control (n = 10): i.p. (0.2 mL) of the dose of 5 mg/kg doxorubicin (DOXO). Group 3 – *P. ruderalis* formulation (100 mg/kg) (n = 9): i.p. of 100 mg/kg extract. Group 4 - *P. ruderalis* formulation (200 mg/kg) (n = 9): i.p. of 200 mg/kg extract. For the hypodermic nodule development, suspensions of B16-F10 cells (500.000 cells/0.2 mL PBS) were inoculated subcutaneously into the mice's interscapular region on the 1st day of the experiment. Treatment was carried out on day 14th only and the animals received the administration of 200 µL PBS (phosphate buffered saline solution), i.p. (G1); of 5 mg/Kg/0.2mL Doxorubicin, i.p. (G2); *P. ruderalis* formulation 100 mg/kg concentration, i.p. (G3) and *P. ruderalis* formulation 200 mg/kg concentration, i.p. (G4). On the 24th experimental day the animals were submitted to euthanasia under the effect of ketamine (30 mg/kg) and xylazine (10 mg/kg) for liver collection and histopathology evaluation (CONCEA, 2018).

2.5 Histopathological analysis

The liver of each animal was sectioned and fixed in 10% neutral buffered formalin solution. Then, dehydrated in ethyl alcohol and xylol baths. The impregnation with paraffin was then carried out, which was performed in histological

cassettes. They were dipped in paraffin and xylene solution (1: 1) for 30 minutes. Then, they went through a paraffin solution I for 2 hours, followed by paraffin II for another two hours and ending with inclusion in the final paraffin. Once the blocks were obtained, they were sectioned in microtome rotative (mod. MRP2015, LUPETEC®) (5 µm) and placed on a slide for subsequent staining with Hematoxylin Eosin (HE). To make the permanent slide, they were placed on the slide, covered with cover slip with Entellan® (LUNA, 1992). The material was analyzed by light field microscopy and morphometric and histological analyzes were performed.

2.6 Statistical analysis

Statistical analyses were carried out using Graph Pad Prism software version 4. Student *t* and Wilcoxon test were used for the *in vivo* test, in intragroup comparisons.

3 Results and Discussion

The crude ethanolic extract and hydroalcoholic solutions (1:10, C₂H₅OH:H₂O) at concentration of 100 and 200 mg/kg indicated the presence of phenols, flavonoids and antioxidant activity shown in Table 1.

Table 1 - Total phenols, total flavonoids and antioxidant activity of the ethanolic extracts and hydroalcoholic solutions of the aerial parts of *Porophyllum ruderalis*

Test	Extract	Hydroalcoholic solutions (1:10, C ₂ H ₅ OH:H ₂ O)	
		100 mg/kg	200 mg/kg
Phenols (mg GAE/g) ¹	162.29 ± 1.09a	72.76 ± 4.28c	94.33 ± 2.50b
Flavonoids (mg QE/g) ²	140.40 ± 3.66 a	67.44 ± 3.06c	109.25 ± 1.78b
DPPH ³	11.3 ± 4.4a	32.6 ± 1.18c	28.7 ± 2.6b

1..Gallic acid (mg GAE/g). 2. Quercetin (mg QE/g). 3Antioxidant activity: expressed as IC₅₀ (µg/mL). Positive controls: BHT: IC₅₀ = 9.4 ± 0.25 µg/mL; DPPH Integral Intensity = 676.24 ± 0.34. Note: Values are the mean ± SD (n = 3).

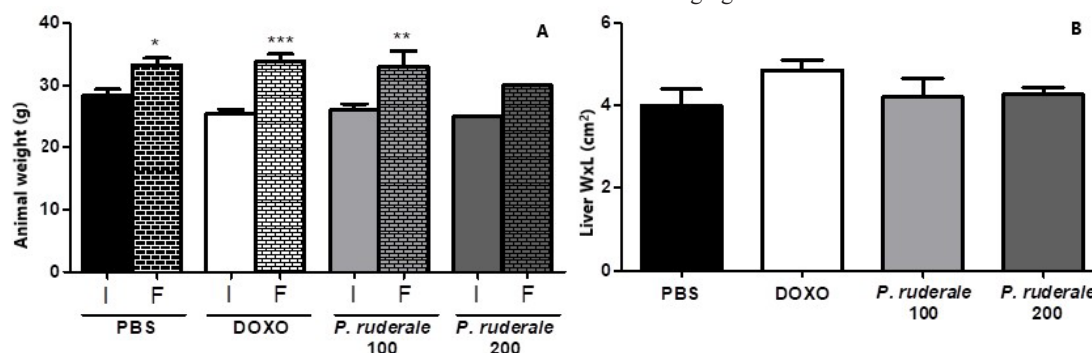
Source: Data research.

3.1 Macro and histopathological analyzes

Intergroup comparisons did not show differences in animal weights. When comparing intragroup, a difference was

observed in the comparison of initial and final weights in the PBS groups (p <0.05), the DOXO treated group (p <0.001) and the *P. ruderalis* treated group 100 mg/kg (p < 0.01) (Figure 1A).

Figure 1 - A = Inter - and intra-group comparison of animals treated with *Porophyllum ruderalis* formulations 100 and 200 mg/kg. DOXO: Doxorubicin. ANOVA, followed by Bonferroni. * p <0.05, ** p <0.01, *** p <0.001 compared to the starting weight of the respective group. B = Width x Length (WxL) liver intergroup comparison of the animals with melanoma and treated with *P. ruderalis* 100 and 200 mg/kg formulations



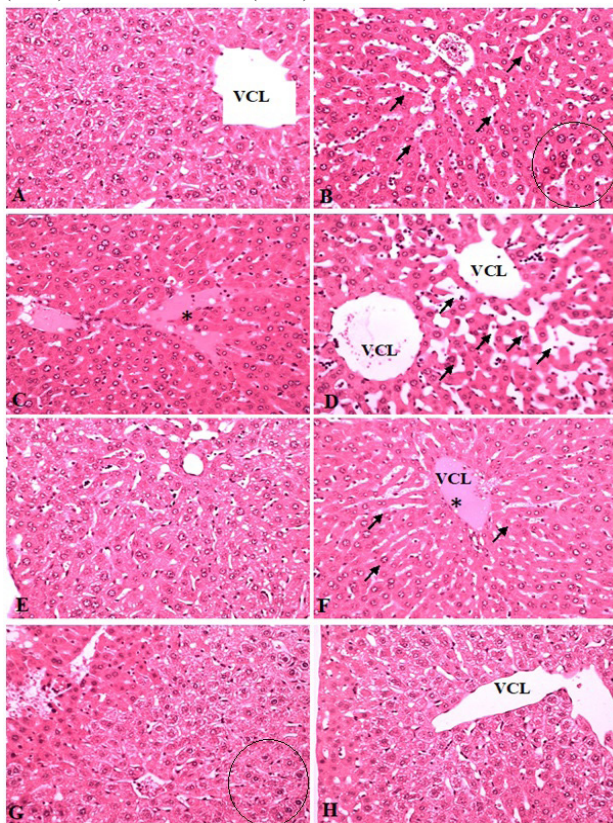
(ANOVA with post hoc Bonferroni test, ** p <0.01 compared to the PBS group, *** p <0.001 compared to the PBS group (Saline phosphate buffer), p <0.05 compared to the DOXO group, p = 0.01 compared to the DOXO group).

Source: Data research

Regarding the animal's weight, it was observed that in the group *P. ruderale* 200 mg/kg there was a reduction in weight when compared to the other groups ($p < 0.05$). No significant differences were observed in livers weight among the groups evaluated. Therefore, the livers were similar in all the groups (Figure 1B).

Morphological changes occurring in the liver inoculated with B16F10 interscapular region C57BL mice subcutaneously and treated with PBS, DOXO and *P. ruderale* formulations 100 and 200 mg/kg (Figure 2).

Figure 2 - Histological sections of Black mouse liver which received subcutaneous tumor cell inoculation PBS (A, B); DOXO (C, D); *Porophyllum ruderale* 100 mg (E, F); *P. ruderale* 200 mg (G, H). Central lobe vein (CLV). HE, 200x



Mild cell necrosis. B) Increased Disse space and metastatic cells infiltrate (arrows and circle, respectively). DOXO: C) Proteinaceous deposition (asterisk) D) Disse space with sharp increase (arrows). *Porophyllum ruderale* 100 mg: E) Marked cell necrosis; F) Disse space normalized and proteinaceous deposition (asterisk). *P. ruderale* 200 mg: G) Marked cell necrosis and metastatic cells infiltrate (circle); H) Marked cell necrosis.

Source: Data research.

No inflammatory foci and steatosis were observed. Figure 2 shows the liver in the four treatments performed. The PBS group (Figures 2A and 2B) presented degenerating cells, disorganized liver structure and foci of cells with melanin spots (Figure 2B), cell necrosis and increased Disse space, which was expected since this was the negative control group. The Doxorubicin (DOXO) group (Figure C and D), positive control, which could minimize the liver changes, showed changes such as proteinaceous deposition, hepatocyte eosinophilia and increased Disse space. In the animals treated

with the extracts at both concentrations (100 and 200 mg/kg) there was a reduction of protein deposition, necrosis was evident and in the Figure G, a focus of metastatic cells could be observed.

Macroscopic analysis analysis performed on the liver did not reveal the presence of metastases however microscopically there were some infiltrated cells indicating that the metastatic process was initiating (circle in Figure B and G). It was observed that the organ architecture was partially preserved with few metastatic cells, for example, in the organization of hepatocyte strings, interspersed by sinusoidal capillaries draining towards the central lobular vein (CLV).

The species of the genus *Porophyllum* Guett. are known to be rich in phenolic compounds and flavonoids and specifically to *P. ruderale* (Brazilian arnica) which has a diversity of applications in Brazilian popular medicine, as tea (infusions) in the treatment of stomach problems, liver, kidney, as a soothing agent, combating hypertension (Tea) and also to snakebite, leishmaniasis (BIESKI *et al.*, 2012; JORGE *et al.*, 1998) and joint problems, considering its healing and anti-inflammatory properties (ROSA *et al.*, 2008; YUI *et al.*, 1998). Anti-inflammatory action has been related to the species, being such effect related to the diversity of chemical constituents, just as the phenolic compounds and their derivatives (HASLAM, 1996), which were also evidenced in our findings (Table 1).

Among the pharmacological activities studied for the species of the genus by *Porophyllum*, there is the anti-inflammatory potential (SOUZA *et al.*, 2003) (LIMA-NETO, 1996), antifungal and antibacterial (RONDÓN *et al.*, 2008), photoprotective properties (ROSA *et al.*, 2008) stand out and are attributed to essential oils and phenolic compounds and their derivatives, potent antioxidants (MENDONÇA *et al.*, 2020; MENDONÇA *et al.*, 2019), potential also observed for the ethanolic extract and the hydroalcoholic solutions in the two concentrations (Table 1).

On the other hand, the use in the leishmaniasis lesions treatment caused by *Leishmania (Leishmania) amazonensis*, was attributed to thiophene and its derivatives strong activity against amastigotes and axenic promastigotes of *Leishmania*; it is inferred that this group has the ability to selectively bind to the DNA sequence, inducing apoptosis in tumor cells (TAKAHASHI *et al.*, 2011), which can lead to liver damage as observed in Figures E, F, G and H.

Regarding the weight of experimental animals treated at 200 mg/kg of the hydroalcoholic solution of *P. ruderale* with the histological findings, it is possible to infer that weight loss of this group is related to liver damage (ANASTÁCIO *et al.*, 2012). Possible explanations can be explored, it is known that circulating tumor cells spread from the primary tumor, thereby inducing tumor cells to move through the blood vessels and enter the bloodstream. This whole process occurs with the production of several molecules, mainly tumor cells adhesion to the endothelial cells lining the blood vessels intimate layer

and also to the extracellular matrix cells of the middle vessel layer (TAKAHASHI *et al.*, 2011) and consequently they are prone to metastasize the liver (XUE *et al.*, 2017), in a way that the liver lesions could be a consequence of melanoma, since the lesions occurred in all the groups.

Additionally, the treatment with *P. ruderale* at both concentrations (100 and 200 mg/kg) may have favored hepatic damage, which was evident in the experimental group with weight reduction of 200 mg/kg animals. Systemic toxic side effects, apathy and weight loss were related to liver damage in a study conducted by Strüh *et al.* (2013) and Kobayashi; Kodaera, (2017) in a murine melanoma *in vivo* model B16.F10 when treated with *Malus domestica* Borkh extracts, Family Rosaceae, and enriched with triterpenes, even after tumor regression.

The hydroalcoholic solutions of *P. ruderale* at concentrations of 100 and 200 mg/kg, we observed damage in liver tissue. In a study with treatment *Solanum americanum* Miller (Solanaceae), with the same model of melanoma, there was reduction of hepatic damage regardless of the concentrations tested (RASHID, 2017; WANG *et al.*, 2010) In this study, the animals were treated with two concentrations of *P. ruderale*, similar to the study carried out by Rashid (2017), but unlike the result obtained by the group of this research, there was significant anticancer activity in the C57BL/6 cells inoculated subcutaneously with B16F10 cells and treated with *P. ruderale* proving that the proposed therapy did perform well in inhibiting the human melanoma cells proliferation, but with liver damage.

Based on this information and considering the histological findings in this study, it was evident that the proposed therapy for melanoma in the two concentrations up to 14 days brought damage to the animal's liver compared to the positive control, and thus it is possible to infer that the treatment proposed longer than 14 days may lead to further damage to the assessed organ.

Although antitumoral *in vitro* studies with *P. ruderale* have already been described (MENDONÇA *et al.*, 2020; MENDONÇA *et al.*, 2019), in addition to widespread use in Brazilian popular medicine, it is a plant that still deserves attention of the Brazilian authorities regarding its use mainly due to liver damage observed in this study.

Although the antioxidant action of the crude extract and formulations (100 and 200 mg/kg) of *P. ruderale* was verified, this was not sufficient to minimize the effects that melanoma caused in the liver of mice inoculated with B16F10 cell line (murine melanoma). It was verified that in the evaluated concentrations, the plant has no protective effect in the melanoma treatment.

4 Conclusion

The hydroalcoholic extract of aerial parts of *P. ruderale* possesses antioxidant properties, but it has no protective

effect in the melanoma treatment. The population should be aware of the hepatotoxic risks of the indiscriminate use in the cutaneous melanomas treatment.

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