

Daily intake of watercress causes inhibition of experimental Ehrlich tumor growth

O consumo diário de agrião causa inibição do crescimento do tumor experimental de Ehrlich

Débora Aparecida de Souza¹; Poliane M. Costa¹; Rosy Iara M. A. Ribeiro¹; Paula V. T. Vidigal²; Flávia C. H. Pinto¹

1. Universidade Federal de São João Del Rei (UFSJ), Minas Gerais, Brazil. 2. Universidade Federal de Minas Gerais (UFMG), Minas Gerais, Brazil.

ABSTRACT

Introduction: Increasing cruciferous vegetable intake has been associated with reduced risk of cancer. Experimental and epidemiological studies suggest that the watercress it is a cruciferous vegetable with high concentration of compounds with recognized antitumor activity. **Objective:** To investigate the effects of daily intake of an aqueous solution of watercress on the growth of the experimental Ehrlich tumor (EET). **Methods:** *Swiss* mice were divided into three groups A, B and C ($n = 6$). The animals from the control group (A) received, by gavage, 0.05 ml of saline throughout the experiment. The animals in group B, from the first day of the experiment, received daily, by gavage, 0.05 ml of watercress aqueous solution (0.5 g/ml). The animals from group C began, on day 21, daily intake of this solution. At day 21.2×10^6 EET cells were inoculated subcutaneously in the left footpad of all mice, and tumor growth was assessed by measuring the thickness of the paw. On day 42, the animals were sacrificed and their footpads removed for histopathological analysis. **Results:** The animals from groups B and C have showed a suppression of tumor growth and a small area of necrosis compared to the animals of group A. **Conclusion:** The present study demonstrated that the daily intake of aqueous solution of watercress was able to activate a suppression of the EET growth, probably due to the main compounds with antitumor properties present in this vegetable.

Key words: carcinoma Ehrlich tumor; *Brassicaceae*; pathology.

INTRODUCTION

Increased intake of cruciferous vegetables has been associated with the reduction of numerous human pathologies, in particular, cancer. *Nasturtium officinale*, commonly known as watercress, belongs to the *Brassicaceae* family, is a cruciferous vegetable that has a high concentration of compounds with recognized antitumor activity in several types of cancers^(1, 2). Experimental and epidemiological studies suggest that diets rich in cruciferous vegetables are associated with reduce in relative risk of colorectal carcinoma, lung cancer and possibly prostate cancer⁽³⁾.

Cruciferous vegetables are characterized by the high presence of glucosinolates, which are converted to isothiocyanates (ITCs) by myrosinase enzymes, present in plants or in the human intestinal microflora⁽³⁾. ITCs represent a class of chemopreventive agents that may play an important role in modulating the carcinogenesis process and are very effective in protecting against tumors in

experimental models^(4, 5). Administration of ITCs in the diet of animals prior to and during exposure to the tobacco-derived carcinogen resulted in inhibition of the development of lung tumors compared to the animals of the control group⁽⁶⁾. Some studies mention harmful effects of glucosinolates on health, such as goiter formation, cytotoxic effects and induction of chromosomal changes in humans⁽⁷⁾.

In addition to the ITCs, watercress also has high concentrations of carotenoids, such as lutein and beta carotene, which are phytochemicals also associated with antitumor activity^(1, 8). According to Stringheta *et al.* (2001)⁽⁹⁾, lutein is a potent antioxidant that prevents damage caused by free radicals in the tissues, and is associated with the prevention of colon cancer. Epidemiological studies indicate that beta-carotene may exert a protective effect against cancer in healthy populations, and in combination with antioxidant substances, if ingested in physiological amounts as found in a diet rich in fruits and vegetables^(10, 11).

There is ample evidence that nutrition plays an important role in the onset, promotion and spread of cancer stages, standing out among other risk factors. Among cancer deaths attributed to environmental factors, diet contributes about 35%. Studies suggest that from an adequate diet about three to four million new cases of cancer could be avoided per year^(12,13).

The experimental Ehrlich tumor (EET) model has been widely used as a tool in studies that seek to elucidate mechanisms of antitumor action of new substances. This model is easy to handle, easily transplanted and allows tumor to growth in the solid form when inoculated in the subcutaneous tissue, and in ascites when inoculated in the peritoneal cavity of the animals⁽¹⁴⁾.

Therefore, based on the probable antitumor potential of cruciferous vegetables, the objective of the present study was to evaluate the effect of the daily intake of watercress in the growth of EET.

METHODS

Animals

Eighteen female Swiss mice weighing around 25-30 g and aged 2-3 months provided by the Central Vivarium of the Universidade Federal de Minas Gerais (CEBIO/UFMG), were used. The animals were divided into three groups (A, B and C), with six animals each, housed in polycarbonate cages (40 × 45 × 25 cm) and submitted to artificial control of light cycles (12 hours light/12 hours darkness). Throughout the experimental period, all the animals received water and balanced commercial feed at will; they were kept at the Experimental Vivarium of the Campus Centro-Oeste Dona Lindu (CCO), at the Universidade Federal de São João Del Rei (UFSJ), in Divinópolis, Minas Gerais. The animals were submitted to a 10-day adaptation period prior to the start of the experiment. The Committee on Animal Research and Ethics of the UFSJ (CEPEA) (n° 53/2010) evaluated all the procedures performed in this study.

Preparation of the aqueous solution of watercress

The aqueous solution of watercress was prepared daily, one hour prior to administration to the animal. Fifty grams of the leaves of the fresh watercress from a local grocery store and 10 ml of distilled water were placed in a mechanical mixer for 1 minute at high speed, according to Dyba *et al.* (2010)⁽¹²⁾. The resulting suspension was filtered through two layers of gauze, and the liquid obtained was completed to the final volume of 100 ml.

Treatment of animals with aqueous solution of watercress

The animals of the control group (A), group B – from day 1 – and group C – from day 21 – received daily, through gavage, throughout the experiment, respectively, 0.05 ml of saline solution, 0.05 ml of *Nasturtium officinale* aqueous solution (0.5 g/ml), and 0.05 ml of *Nasturtium officinale* solution (0.5 g/ml), until the end of the experiment. The body weight of each animal was evaluated at the beginning and at the end of the experiment. Our study lasted 42 days.

Preparation and inoculation of EET

The EET cells were maintained in the ascetic form by weekly inoculations of 10⁶ cells through *i.p.* in Swiss mice. For the experimental procedures, from pre-inoculated mice a 3 ml of the ascitic fluid were removed, centrifuged (3 min, 200 ×g) and the supernatant removed. The EET cells were washed three times in saline and counted in the Neubauer's chamber, and the viability was determined by the trypan blue dye exclusion staining. On day 21, all mice were inoculated subcutaneously with 2 × 10⁶ (0.05 ml) EET cells in the left footpad for the development of the solid tumor^(15,16).

Tumor growth evaluation

For the evaluation of the tumor growth curve, measurements in duplicate of the dorsal-plantar thickness of the left hind limb of each animal were carried out. This measurement was performed with a digital caliper immediately before and after inoculation of the tumor, three times a week for 21 days. The mean values obtained were considered as the value of the tumor growth of the day. After the end of the experiment, on the day 42^o, the animals were euthanized with anesthesia overdose and necropsy for the collection of the left footpad and the left popliteal lymph nodes for histopathological analysis. All material was fixed in buffered formaldehyde, dehydrated in successive solutions of alcohols and embedded in paraffin. The paraffin blocks were sectioned in a microtome, obtaining 4 mm sections, which later were stained with hematoxylin and eosin (HE) for qualitative assessment of tumor mass and necrosis areas⁽¹⁶⁾. From the histological slides stained by HE, semiquantitative analysis of the areas of necrosis were performed using a digital image analyzer (KS300 Program; version 2.0, Kontron Elektronik), the images were generated by a Zeiss Axiolab microscope connected to a camera interconnected to a board computer scanner. A total of five fields per case/slide were analyzed at a magnification of 100×, these fields were selected at random.

Statistical analysis

Statistical procedures were performed using the GraphPad Prism software, by analysis of variance (Anova), followed by Tukey-Kramer test, with significance level of 5%. Data were expressed as the mean ± standard deviation.

RESULTS

The effects of consuming aqueous solution of watercress were evaluated in the growth of EET in mice. The study showed that, from the second week after tumor inoculation, a reduction in tumor growth was observed in the animals receiving daily dose of aqueous watercress solution (groups B and C) in relation to the animals of the control group (Anova, $p < 0.05$). The reduction in footpad thickness was significant from the day 10, and was maintained until the end of the experiment, as shown in **Figure 1**.

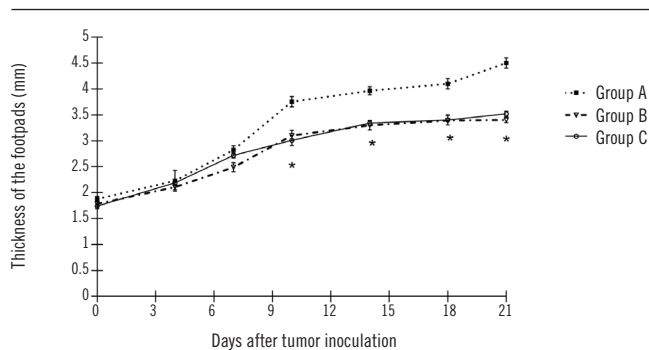


FIGURE 1 – Tumor growth curve

EET growth curve in the footpads of mice for 21 days. Group A: control; group B: beginning of intake prior to tumor inoculation; group C: beginning of intake after tumor inoculation. * $p < 0.05$ compared to the control group, results expressed as mean and standard deviation with $n = 6$ /group.

EET: experimental Ehrlich tumor.

The body weight of each animal was evaluated at the beginning and at the end of the experiment. There was no significant difference between the weights of the animals that ingested aqueous solution of watercress and the animals of the control group, as shown in **Figure 2**.

The subcutaneous implantation of EET cells in the footpad resulted in the development of a solid tumor, represented by compact masses. The histopathological analyzes of the footpad of the animals of group A showed extensive eosinophilic and amorphous areas of necrosis, preferably located in the deep dermis, with granular appearance and imprecise limits, indicative of cellular damage, as shown in **Figure 3A**. In relation to the

animals of groups B and C, which ingested aqueous solution of watercress, the histopathological analyzes of the footpad showed reduced necrotic areas, also reaching the deep dermis, as shown in **Figures 3B** and **3C**. The semiquantitative analyzes of the necrotic areas confirmed significant differences between the animals of the control group and those who ingested aqueous solution of watercress (**Figure 3D**).

Figure 4 shows that in all groups the tumor cells showed, by optical microscopy, invasion of the underlying connective tissue, large pleomorphism, high degree of anaplasia, with polygonal morphology, scarce cytoplasm, prominent central nucleus and numerous and evident nucleoli.

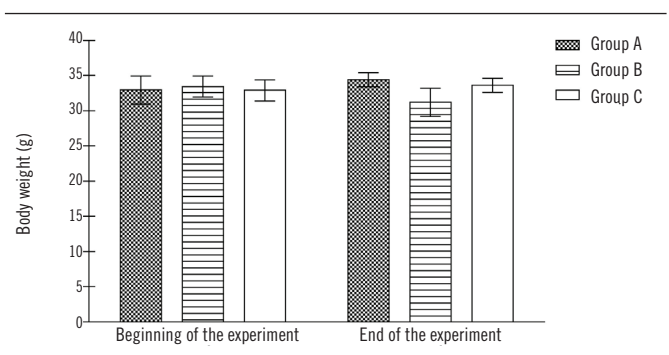


FIGURE 2 – Body weight

Absolute values of the body weight of the mice at the beginning and at the end of the experiment. Group A: control; group B: beginning of intake prior to tumor inoculation; group C: beginning of intake after tumor inoculation. Results expressed in mean and standard deviation with $n = 6$ /group. There was no significant difference between the groups.

DISCUSSION

Epidemiological studies suggest that a diet rich in cruciferous vegetables, such as broccoli, cabbage and watercress, is associated with reduced risk of several types of cancers⁽¹⁷⁾. The effect of daily consumption of 0.5 g/ml of aqueous watercress solution administered by gavage on the growth of EET in mice was investigated. The study demonstrated suppression of tumor growth in both animals that started this intake prior to inoculation of tumor cells (group B) and in animals that started the ingestion after tumor inoculation (group C). This suppression probably occurred due to the concentration of phytochemicals with antitumor activities present in the watercress.

According to Chiao *et al.* (2004)⁽¹⁷⁾ and Thomson *et al.* (2006)⁽¹⁸⁾, the isothiocyanates are one of the major compounds present in large quantities in watercress. ITCs are active in the post-initiation phase of carcinogenesis and, therefore, have a chemotherapeutic potential, impeding tumor progression through several mechanisms, such as

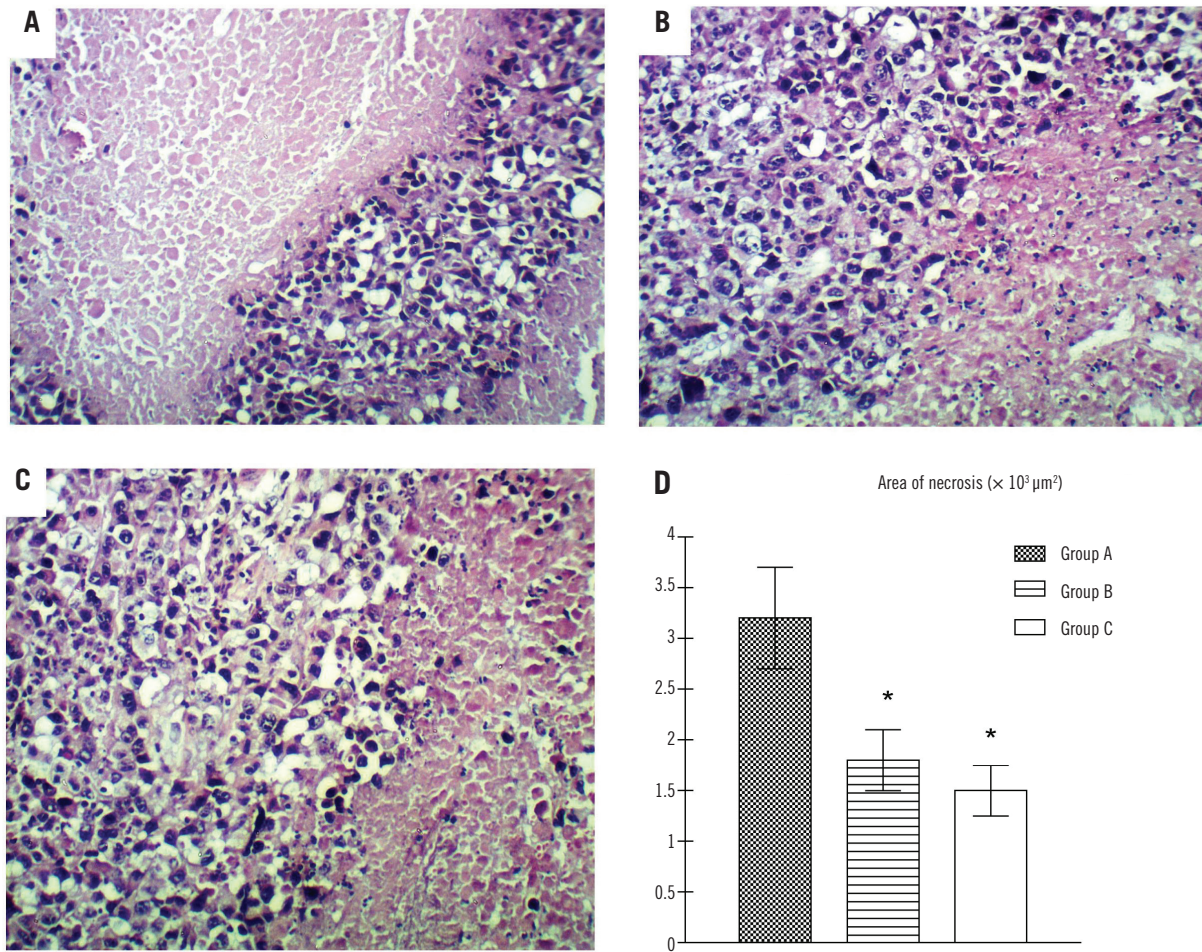


FIGURE 3 – Analysis of necrotic areas

Histopathological analyzes of the footpads of mice inoculated with EET after 21 days, HE 100×. A) control group: presence of extensive eosinophilic area of necrosis; B) group B beginning of watercress intake prior to tumor inoculation. Presence of necrosis area to a lesser extent; C) group C: beginning of watercress intake after tumor inoculation. Presence of necrosis area to a lesser extent; D) semi quantitative analysis of the areas of necrosis. *p < 0.05 compared to the control group, results expressed as mean and standard deviation with n = 6/group.

EET: experimental Ehrlich tumor; HE: hematoxylin and eosin.

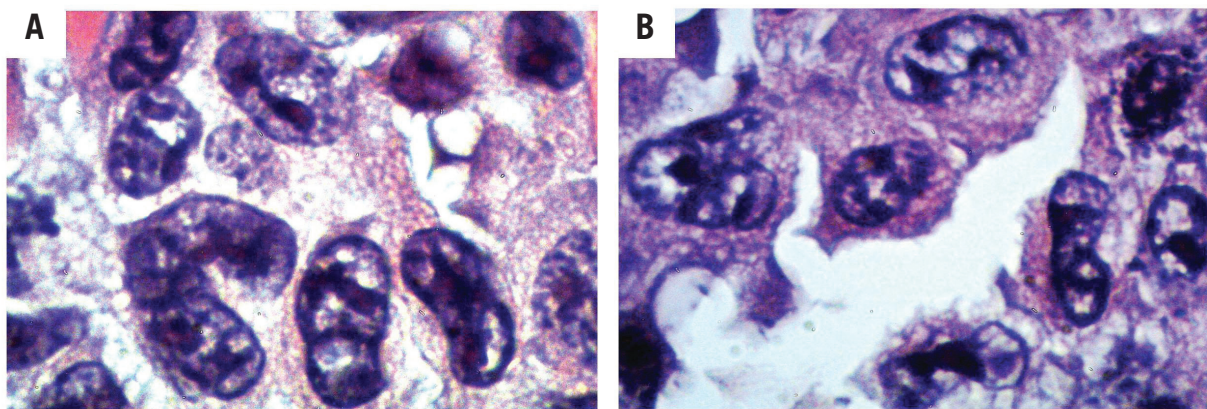


FIGURE 4 – Histopathological analyzes of tumor cells

Histopathological analyzes of the footpads of mice inoculated with EET after 21 days, HE 1000×. A) control group; B) group B beginning of watercress ingestion prior to tumor inoculation, HE 1000×. Tumor cells showed marked pleomorphism, evident nucleoli and enlarged nuclei.

EET: experimental Ehrlich tumor; HE: hematoxylin and eosin.

modulation of cell growth, inhibition of angiogenesis, suppression of metastasis and induction of apoptosis.

Experimental studies show that the ITCs inhibit carcinogenesis in several tumor cell lines. Hahm *et al.* (1989)⁽⁶⁾ demonstrated that the administration of ITCs delays the growth of human tumor cells implanted subcutaneously in rats. According to Jiao *et al.* (1994)⁽¹⁹⁾, the administration of a single dose of 0.2 μmol of ITCs in mice, 2 hours prior to the inoculation of tobacco-derived carcinogen, was able to inhibit lung tumorigenesis. According to Powolny *et al.* (2011)⁽²⁰⁾, the administration of 3 $\mu\text{mol/g}$ of ITCs in the diet for 19 weeks inhibited the progression of experimental prostate adenocarcinoma in mice associated with reduced cell proliferation and increased apoptotic index. Khor *et al.* (2008)⁽²¹⁾ demonstrated that *APC^{Min/+}* mice (genetically modified to form multiple intestinal adenomas), after feeding on a diet supplemented with 0.05% de ITCs for three weeks, showed a reduction in the development of these adenomas compared to mice fed with standard diet. The mechanism by which the isothiocyanates cause this type of cell death is not fully understood, but it is likely that this chemopreventive agent causes cell cycle arrest in the G2 phase of mitosis^(6, 22).

Cellular proliferation and apoptosis are important events in carcinogenesis, since tumor growth is related to the balance resulting from the sum of these two events⁽²³⁾. Increased rates of apoptosis have been associated with inhibition of growth of potentially oncogenic cells⁽²⁴⁾. Apoptosis plays an important role in tumor progression, and is reported as the cause of death of tumor cells during chemotherapy, radiation therapy and immunotherapy⁽²⁵⁾.

Another compound present in the watercress with the ability to exhibit antitumor effects is the carotenoid. Epidemiological studies show a negative correlation between the intake of carotenoids from vegetables and the risk of developing cancer⁽⁷⁾. According to Gill *et al.* (2007)⁽¹⁾, the daily consumption of watercress reduces the risk of cancer by reducing the damage to the deoxyribonucleic acid (DNA) caused by free radicals and by the antioxidant power of the carotenoids present in this vegetable. Carotenoids protect against carcinogenesis by neutralizing reactive oxygen species, reducing oxidative stress and DNA mutations, they have an important antioxidant potential, and are considered chemopreventive agents⁽²⁶⁾. Lutein is a carotenoid found in green leafy vegetables, especially dark leafy vegetables, such as watercress, with considerable antioxidant action, offering protection against different pathologies, such as atherosclerosis, cataracts and cancer^(8, 27). Beta carotene is also considered an important carotenoid found in watercress. According to Ziegler (1991)⁽²⁸⁾, observational epidemiological studies have shown that low serum levels of beta-carotene are associated with the risk of developing lung cancer. However, high-dose supplementation

of this substance in smokers and subjects exposed to asbestos increased the incidence of this type of cancer⁽⁷⁾. Through these studies, it becomes difficult to determine to what extent the intake of a single nutrient can interfere with cancer chemoprevention. The protective effect may be the result of the action or interaction of other components of foods of plant origin.

The histopathological analysis of the footpad showed that the animals of the groups that daily ingested aqueous solution of watercress presented reduced areas of necrosis, these results are consistent with the reduction of the thickness of the tumor mass observed in these groups. The animals in the control group had extensive areas of necrosis and these results are in accordance with the literature data. According to Graham *et al.* (2013)⁽²⁹⁾, most solid tumors are characterized by regions of hypoxia that often are correlated with worse clinical prognosis. Hypoxia regions result from a high oxygen demand for tumor cells that rapidly multiples in the periphery of the tumor combined with disorganized vascularization, often resulting in necrosis and ulceration. These extensive areas of necrosis are typical in hyperproliferative tumor tissues, because of the difference between the high tumor proliferation and the formation of new blood vessels, which present slower development. According to Multhoff *et al.* (2014)⁽³⁰⁾ and Vaupel and Mayer (2014)⁽³¹⁾, necrosis is directly associated with tumor progression, increased aggressiveness and metastatic potential. The daily intake of aqueous watercress solution has been shown to be effective in inhibiting tumor growth, as observed in the reduction of tumor size and in the reduction of necrosis areas. Tumor cell morphology was similar in all groups, with a pronounced pleomorphism, slightly basophilic cytoplasm and oval nucleus, with several evident nucleoli, and is in agreement with the results of the literature^(32, 33).

CONCLUSION

The present study demonstrated that the daily intake of aqueous solution of watercress, both before and after the inoculation of EET cells, was able to cause suppression of tumor growth. This solution is likely to have chemopreventive and chemoprotective effects, and may be related to the main compounds present in watercress with antitumor properties. Further studies are being carried out to determine the rate of cell proliferation and the apoptotic index of these tumor cells through immunohistochemical analysis.

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RESUMO

Introdução: O aumento da ingestão de vegetais crucíferos tem sido associado à redução de risco de câncer. Estudos experimentais e epidemiológicos sugerem que o agrião é um vegetal crucífero que apresenta alta concentração de compostos com reconhecida atividade antitumoral. **Objetivo:** Investigar o efeito do consumo diário de solução aquosa de agrião no crescimento do tumor experimental de Ehrlich (TEE). **Métodos:** Camundongos Swiss foram separados em três grupos, A, B e C (n = 6). Os animais do grupo-controle (A) receberam, por gavagem, 0,05 ml de solução salina durante todo o experimento. Os animais do grupo B, a partir do 1º dia do experimento, receberam diariamente, por gavagem, 0,05 ml de solução aquosa de agrião (0,5 g/ml). Os animais do grupo C, no 21º dia, iniciaram a ingestão diária dessa solução. No 21º dia, todos os camundongos foram inoculados subcutaneamente no coxim plantar esquerdo com 2×10^6 células do TEE (0,05 ml), e o desenvolvimento tumoral foi avaliado pela mensuração da espessura das patas. No 42º dia, os animais foram sacrificados e suas patas, removidas para análise histopatológica. **Resultados:** Os animais dos grupos B e C apresentaram supressão do crescimento tumoral e menor área de necrose em relação aos animais do grupo A. **Conclusão:** O presente estudo demonstrou que a ingestão diária de solução aquosa de agrião foi capaz de causar supressão do crescimento do TEE provavelmente devido aos principais compostos presentes neste vegetal com propriedades antitumorais.

Unitermos: carcinoma de Ehrlich; Brassicaceae; patologia.

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CORRESPONDING AUTHOR

Flávia Carmo Horta Pinto

Universidade Federal de São João Del Rei (UFSJ); campus Dom Bosco; Departamento de Ciências Naturais; Praça Dom Helvécio, 74; Fábricas; CEP: 36301-160; São João Del Rei-MG, Brasil; Phone: +55 (32) 3379-2483; e-mail: fcarmo3@hotmail.com.