In vitro and *In vivo* Anticancer Activity of Extracts, Fractions, and Eupomatenoid-5 Obtained from *Piper regnellii* Leaves

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Key words
- Piperaceae
- *Piper regnellii* (Miq.) C. DC. var. *regnellii*
- eupomatenoid-5
- neolignans
- *in vitro* antiproliferative activity
- Ehrlich solid tumor

Abstract

Despite numerous studies with the *Piper* genus, there are no previous results reporting *in vitro* or *in vivo Piper regnellii* (Miq.) C. DC. var. *regnellii* anticancer activity. The aim of this study was to investigate *P. regnellii in vitro* and *in vivo* anticancer activity and further identify its active compounds. *In vitro* antiproliferative activity was evaluated in 8 human cancer cell lines: melanoma (UACC-62), breast (MCF7), kidney (786–0), lung (NCI-H460), prostate (PC-3), ovary (OVCAR-3), colon (HT29), and leukemia (K-562). Total growth inhibition (TGI) values were chosen to measure antiproliferative activity. Among the cell lines evaluated, eupomatenoid-5 demonstrated better *in vitro* antiproliferative activity towards prostate, ovary, kidney, and breast cancer cell lines. *In vivo* studies were carried out with Ehrlich solid tumor on Balb/C mice treated with 100, 300, and 1000 mg/kg of *P. regnellii* leaves dichloromethane crude extract (DCE), with 30 and 100 mg/kg of the active fraction (FRB), and with 30 mg/kg of eupomatenoid-5. The i.p. administration of DCE, FRB, and eupomatenoid-5 significantly inhibited tumor progression in comparison to control mice (saline). Therefore, this study showed that neolignans of *Piper regnellii* have promising anticancer activity. Further studies will be undertaken to determine the mechanism of action and toxicity of these compounds.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

Cancer is a pool of many diseases diagnosed in thousands of people worldwide and represents the second major cause of death [1]. The loss of normal cell growth control is one of the main events in cancer development, followed by angiogenesis, metastasis, and apoptosis, considered hallmarks of cancer [2].

Medicinal herbs are a significant source of active compounds that also could be used as lead compounds for new anticancer agents [3]. The development of the antiproliferative screening by the U.S. National Cancer Institute (NCI) has contributed to the discovery of new natural anticancer agents. Nowadays over 60% of all anticancer drugs have their origin directly or indirectly from natural resources [4].

Piperaceae species are mostly pioneer shrubs with economic and medicinal importance and are widely spread in tropical regions [5]. The search for active constituents from *Piper* species has been intensive in recent years, revealing several *Piper* species as a source of a great number of active compounds responsible for biological activities such as antifungal, anti-PAF, antioxidant, antiplasmodial, and tripanocidal properties [6]. Many substances reported for the *Piper* genus have presented cytotoxicity in a variety of tumor cell lines [7,8], highlighting the anticancer potential of these plant species [9–11].

Piper regnellii (Miq.) C. DC. is popularly known as caapeba or pariparoba in Brazil [12]. The leaves and roots are used as extracts, infusions, or plasters for wound treatment, swelling reduction, skin irritations, and infections [13]. Phytochemical studies of *P. regnellii* leaves have shown the accumulation of several phenylpropanoids and four dihydrobenzofuran neolignans identified as eupomatenoid-6, eupomatenoid-5, eupomatenoid-3, and conocarpan [14].

Lignans and neolignans are a class of secondary plant metabolites produced by oxidative dimerization of two phenylpropanoid units [15,16].

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They are widely distributed in nature and display an impressive range of biological activities [17]. Because of the high structural diversity of this chemical class, there is obviously an extraordinary range of medicinal properties, and this area continues to be a fruitful research topic. There is a growing interest in lignans and their synthetic derivatives due to applications in cancer chemotherapy and various other pharmacological effects [16].

Despite several studies related to the *Piper* genus, no evidence of *in vitro* or *in vivo* anticancer activity of *Piper regnellii* had been reported previously.

The development of experimental models has contributed to the study of antineoplasic compounds and to the understanding of their mechanism of action. Solid tumors are structures resembling organs in their complexity and heterogeneity, with a microenvironment formed by tumor and stroma cells which are embedded in the extracellular matrix and in the presence of a vascular network. These parameters often contribute to tumor resistance to chemotherapy due to irregular distribution of drugs inside the tumor matrix. Therefore, the development of *in vivo* experimental models to complement *in vitro* drug screening is necessary due to the limitations inherent to cell cultures to predict the behavior of solid tumors to chemotherapy [18].

There are a number of experimental models based on laboratory animals including the Ehrlich solid tumor, derived from a mouse breast adenocarcinoma which is an aggressive and fast growing carcinoma able to develop both in the ascitic or solid form [19]. Transplanted tumors afford an advantage due to the previous knowledge of the amount and initial features of the tumor cells and to the fast development of the neoplasia, thus reducing the duration of the study [20].

Continuing previous studies conducted on the Piperaceae plant species [21], the aim of this study was to evaluate the *in vitro* and *in vivo* anticancer activities of *P. regnellii* crude extract, active fraction, and isolated neolignan eupomatenoid-5.

Materials and Methods

General experimental procedures

^{1}H, ^{13}C NMR, and 2D experiments: Varian Inova-500 spectrometer (11 tesla). Chemical shifts were recorded in $CDCl_3$ solutions and quoted relative to TMS (δ 0.0, ^{1}H NMR) and $CHCl_3$ (δ 77.0, ^{13}C NMR). Column chromatography (CC): 8 × 12 cm, silica gel 60 (0.063 × 0.200 mm, Merck®, 2 × 50 cm). TLC (thin-layer chromatography): precoated plates (775554 Merck®), UV detection, and anisaldehyde solution.

Plant material

Piper regnellii (Miq.) C. DC. var. *regnellii* leaves were collected and identified at the Chemistry, Biology, and Agriculture Research Center (CPQBA, State University of Campinas, São Paulo, Brazil). A voucher specimen was deposited at CPQBA Herbarium (n° 221) and identified by Dr. Elsie Franklin Guimarães.

Extraction and isolation of compound 1

Dried leaves (218 g) were ground prior to use in a Stephen mill (model UM 40) and extracted by maceration (1 : 5 plant : solvent, 3 × 1 h) successively with hexane (Merck®) and dichloromethane (Merck®) at room temperature, providing the crude hexanic (HCE, 4% yield) and dichloromethanic (DCE, 6% yield) extracts, after solvent evaporation. *In vitro* cytotoxic assay monitored the extracts activity, showing that DCE was active. An aliquot (5 g) of DCE was chromatographed over silica gel (150 g), eluted first with hexane. The eluent polarity was increased by gradients of dichloromethane and then methanol, providing eleven fractions (50 mL) that were grouped according to the thin-layer chromatography (TLC) profile, visualized with anisaldehyde reagent (50 mL acetic acid, 0.5 mL sulfuric acid, and 0.5 mL anisaldehyde) followed by heating at 110 °C. Further similar fractions were grouped according to their TLC profile in three fractions as fraction A (FRA – apolar fraction), fraction B (FRB – medium polar fraction), and fraction C (FRC – polar fraction). *In vitro* cytotoxic assay monitored the fractions activity, indicating that FRB (1 g) was the best. Compound **1** (210 mg) was obtained by FRB precipitation and recrystallization with a mixture of hexane/dichloromethane (1 : 1). Eupomatenoid-5 was evaluated by *in vitro* anticancer assay against 8 human tumor cell lines.

Chromatographic analysis

The GC/MS analysis of fraction FRB was carried out using an HP-6890/5975 system equipped with an HP-5 (30 m × 0.25 mm × 0.25 µm). Temperature program: 60 °C (3 °C · min^{-1}) – 240 °C (60 min), injector 220 °C, detector 250 °C. Helium was used as a carrier gas (0.7 bar, 1 mL · min^{-1}). The MS were taken at 70 eV. Scanning speed was 0.84 scans s^{-1}, from 40 to 550. Sample volume was 1 µL. Split: 1 : 40. The mass spectra were compared with previously reported data [14]. This analysis suggested the presence of compounds **1**–**4**. Spectral data of compounds **1**–**3** were in agreement with previous reported data [14]. Copies of the original spectra are obtainable from the author of correspondence.

Compound **4** (not identified compound): GC/MS: Rt 58.500 min, MS *m/z* (int. rel.%): $[M]^+$ 296 (100), 281 (21), 267 (4), 253 (10), 137 (29), 91 (25), 44 (46).

In vitro anticancer activity assay

Human tumor cell lines, UACC-62 (melanoma), MCF7 (breast), 786–0 (kidney), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovary), HT29 (colon), and K-562 (leukemia), were kindly provided by the NCI. Stock cultures were grown in medium containing 5 mL RPMI 1640 (GIBCO BRL) supplemented with 5% fetal bovine serum. Gentamicine (50 µg mL^{-1}) was added to experimental cultures. Cells in 96-well plates (100 µL cells well-1) were exposed to sample concentrations in DMSO/RPMI (0.25, 2.5, 25, and 250 µg mL^{-1}) at 37 °C, 5% of CO^2 in air for 48 h. Final DMSO concentration did not affect cell viability. Afterwards cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Doxorubicin chloridrate (0.1 mg/mg; Europharma) was adopted as a positive control. Using the concentration-response curve for each cell line, TGI (total growth inhibition) was determined through nonlinear regression analysis (**Table 1**) using software ORIGIN 7.5 (OriginLab Corporation) [22].

The assay results for the extracts screened were separated into four categories, according to Fouche et al., 2008 [23]: inactive (TGI > 50 µg/mL), weak activity (15 µg/mL < TGI < 50 µg/mL), moderate activity (6.25 µg/mL < TGI < 15 µg/mL), and potent activity (TGI < 6.25 µg/mL). Extracts from the latter two categories were selected for further *in vivo* testing for anticancer activity.

In vivo assay – acute toxicity

Balb/C mice were treated intraperitoneally (i.p.) with DCE at doses of 300, 1000, and 2000 mg/kg. Groups were observed during 4 h and then daily for 14 days. The following general toxicity

Table 1 Antiproliferative activity of doxorubicin, DCE, FRB, and eupomatenoid-5 against human cancer cell lines[a].

	TGI (µg/mL)[b]			
Cell Lines	**DCE**	**FRB**	**EUP-5[c]**	**DOX[d]**
Melanoma (UACC-62)	26.45	10.18	8.21	0.05
Breast (MCF7)	39.58	10.77	6.24	3.10
Kidney (786–0)	21.93	8.33	1.93	4.97
Lung (NCI-H460)	20.39	6.09	10.26	0.81
Prostate (PC-3)	10.97	5.44	6.17	3.19
Ovary (OVCAR-3)	12.05	17.61	5.50	0.07
Colon (HT29)	42.09	12.39	14.28	14.88
Leukemia (K-562)	158.44	19.21	99.63	1.29

[a] Assessed by the SRB assay. [b] TGI values represent the necessary concentration (µg/mL) for total inhibition of cancer cells proliferation. Values were determined through nonlinear regression analysis using the ORIGIN 7.5® (OriginLab Corporation). Dose range tested: 0.25 to 250 µg/mL. [c] Eupomatenoid-5; [d] doxorubicin (positive control)

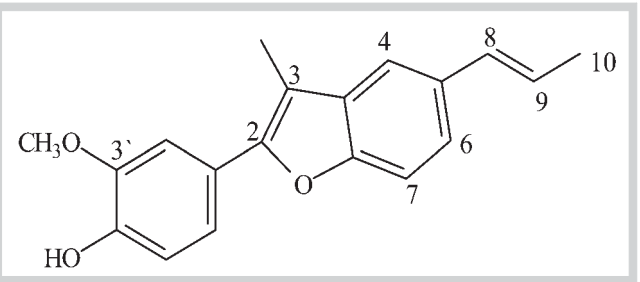

Fig. 1 Eupomatenoid-5 chemical structure [2-(4′-hydroxy-3′-methoxyphenyl)-3-methyl-5(E)-propenylbenzofuran].

parameters were evaluated: body weight loss, locomotion, behavior (agitation, lethargy), respiration, salivation, tearing eyes, cyanosis, and mortality [24, 25].

In vivo assay – Ehrlich solid tumor

DCE, fraction FRB, and eupomatenoid-5 were evaluated *in vivo* on an Ehrlich solid tumor assay. Ehrlich tumor cells were maintained in the ascites form by peritoneal passages in mice by weekly transplantation of 5×10^5 tumor cells. For testing, cells were prepared at a density of 2.5×10^6 cells/60 µL/animal [26].

Male Balb/C mice (10/group) aged 8–10 weeks and weighing 25–30 g were used for Ehrlich solid tumor experiments. The animals were obtained from CEMIB-Unicamp and maintained under controlled conditions of temperature (22–24 °C), light (12-h light/12-h dark), and humidity (45–65%), with food and water ad libitum. All procedures were in accordance with the principles and guidelines adopted by the institutional Committee for Ethics in Animal Research at the State University of Campinas (CEEA, UNICAMP, protocol 1964-1, September 14th, 2009).

Ehrlich tumor cells (2.5×10^6) were implanted on the right footpad and the animals were treated with the samples, i. p., every 72 h. The animals were divided into groups: negative control group (saline), positive control group (5-fluorouracil, 25 mg/mL; Europharma) at dose of 20 mg/kg, and sample treated groups: DCE, FRB, and eupomatenoid-5. DCE was administered at doses of 100, 300, and 1000 mg/kg; FRB at doses of 30 and 100 mg/kg, and eupomatenoid-5 at a dose of 30 mg/kg. To determine the solid tumor growth, footpad volume was measured every 3 days, using a plethysmometer (Panlab), until the fifteenth day, approximately, when the animals were sacrificed. Anticancer activity was assessed by comparing the tumor volumes using the equation: (mean tumor volume of negative control group – mean tumor volume of treatment groups)/mean tumor volume of the negative control group × 100.

Statistical analyses

Results are expressed as mean ± standard deviation (SD) from 10 animals per group. Statistical evaluation was done by the ANOVA test followed by Duncan's test using StatSoft® software. Graphics were designed using the Origin® software. Differences were considered significant at $p \leq 0.05$ and are represented by an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Supporting information

Chromatographic profiling of compounds **1**–**4** identified in DCE and FRB and tables indicating tumor volume (µL) and tumor reduction percentage of samples are available as Supporting Information.

Results

The GC/MS analyses of FRB (**Fig. 1S**, Supporting Information) suggested the presence of compounds eupomatenoid-5 (**1**), eupomatenoid-3 (**2**), and conocarpan (**3**) in the active fraction with comparison to previous reported data [14]. Another neolignan with *m/z* 296 (M^+) (**4**) was detected, and the mass spectra suggested that the compound could be a methoxyl conocarpan derivative. The relative rates of compounds **1**–**4** were determined by GC/MS as 4.05, 8.99, 17.51, and 2.77%, respectively. Eupomatenoid-5 (**Fig. 1**) was isolated from FRB, and spectral data (^1H and ^{13}C NMR data) were in accordance with those previously reported [14]. Furthermore, melting point ranged between 113.9–115.2 °C, consistent with the literature [27]. According to GC/MS analysis (**Fig. 2S**, Supporting Information) and melting point data, the compound purity is above 98%, detected within the detection limit of equipments.

In vitro antiproliferative evaluation (**Table 1**) of DCE demonstrated high potency for most human cancer cell lines, with selectivity for prostate (PC-3) and ovary (OVCAR-3) cell lines and TGI values of 10.97 and 12.05 µg/mL, respectively. The active fraction FRB showed *in vitro* antiproliferative effects against kidney (786–0), melanoma (UACC-62), breast (MCF7), and colon (HT29) cells with TGI values of 8.33, 10.18, 10.77, and 12.39 µg/mL, respectively, showing better potency for prostate (PC-3) and lung (NCI-H460) cell lines, with TGI = 5.44 and 6.09 µg/mL, respectively. As well as the fraction, eupomatenoid-5 presented *in vitro* antiproliferative effects for kidney (786–0), prostate (PC-3), and breast (MCF7) cell lines, with TGI values of 1.93, 6.17, and 6.24, respectively; being especially potent for kidney, with a TGI value lower than the positive control. Additionally, the compound was also selective for the ovary (OVCAR-3) cell line (TGI = 5.5 µg/mL), as well as DCE. Furthermore, this compound presented moderate activity against melanoma (UACC-62), lung (NCI-H460), and colon (HT29) cell lines, with TGI values of 8.21, 10.26, and 14.28, respectively. This strong *in vitro* antiproliferative activity prompted the study of antitumor activity in murine models.

Treatment with *P. regnellii* every three days proved to be effective to control tumor progression, since this plant was able to inhibit

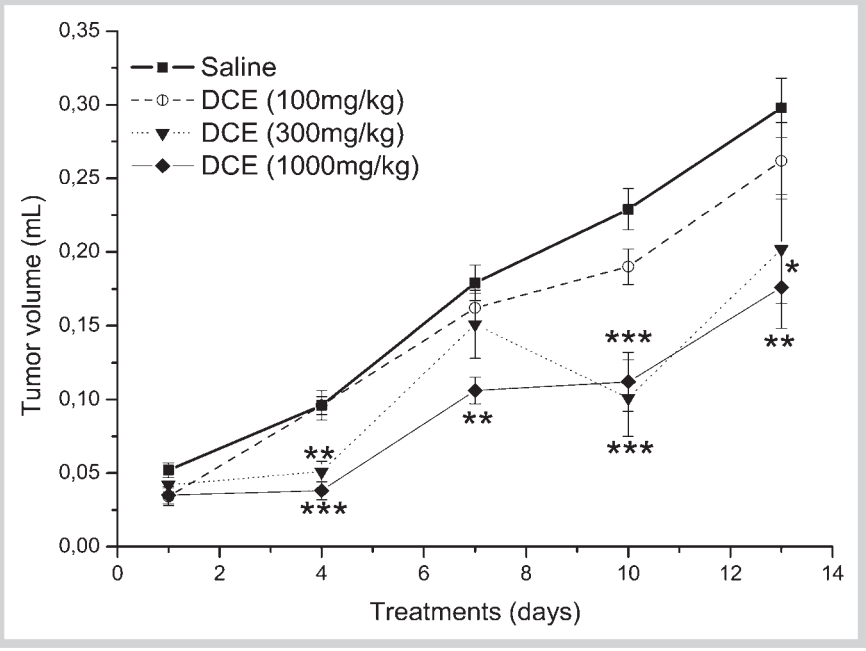

Fig. 2 Tumor volume variation induced by Ehrlich cells in mouse hind footpad during the treatment with *P. regnellii* DCE; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Duncan's test, $p < 0.001$ (ANOVA).

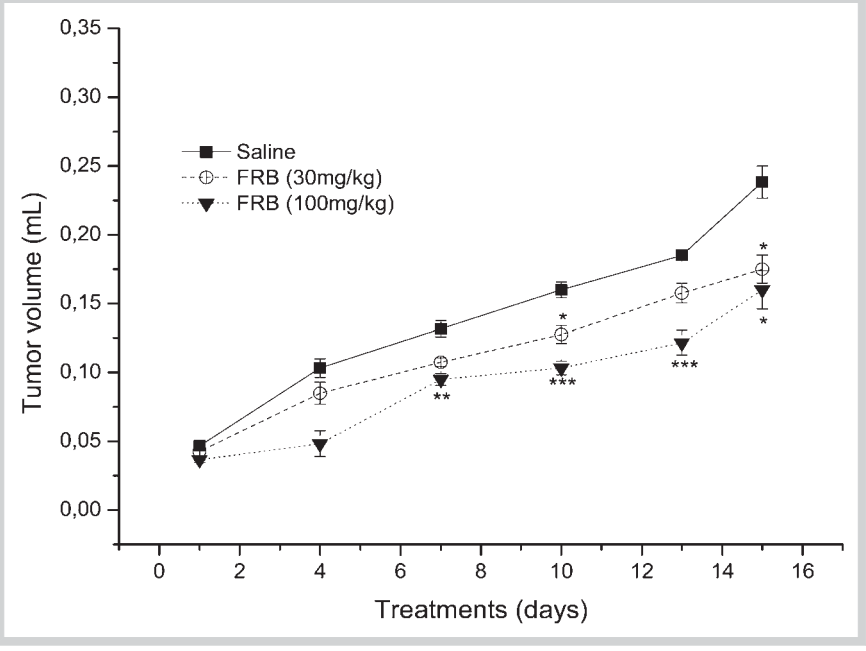

Fig. 3 Tumor volume variation induced by Ehrlich cells in mouse hind footpad during the treatment with *P. regnellii* FRB; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Duncan's test, $p < 0.001$ (ANOVA).

tumor growth in lower doses than the negative control group (vehicle) with all tested samples (DCE, FRB, and eupomatenoid-5).

At doses of 300 and 1000 mg/kg, DCE reduced the tumor volume by 33.3 and 41.9%, respectively (**Table 1S**, Supporting Information). These treatments also resulted in toxic effects, such as piloerection, evacuation increase, weight loss, and even death after 7 days of experiment [28]. No evidence of acute toxicity was observed 4 h after administration of 300, 1000, and 2000 mg/kg doses of DCE as well as during the following 15 days of observation. However, when DCE 300 and 1000 mg/kg were administered in repeated doses, some of the most important signs of toxicity appeared. On the other hand, although the group treated with 100 mg/kg did not show a statistical difference from the vehicle (**Fig. 2**), no toxic effects were observed throughout the experiment.

The purification process conferred a more potent fraction (FRB) that promoted a reduction of 30.9% in tumor volume at a dose of 100 mg/kg and 26.6% at a dose of 30 mg/kg (**Table 2S**, Supporting Information; **Fig. 3**). During these treatments, the animals did not show signs of toxic effects [28].

Compound eupomatenoid-5 (**1**) at a dose of 30 mg/kg reduced the tumor volume by 30.4% (**Table 3S**, Supporting Information). In this experiment, the positive control 5-fluorouracil was effective, as well as compound **1**, from the 7th day on and reduced the tumor volume by 58.6% (**Table 3S**, Supporting Information, **Fig. 4**).

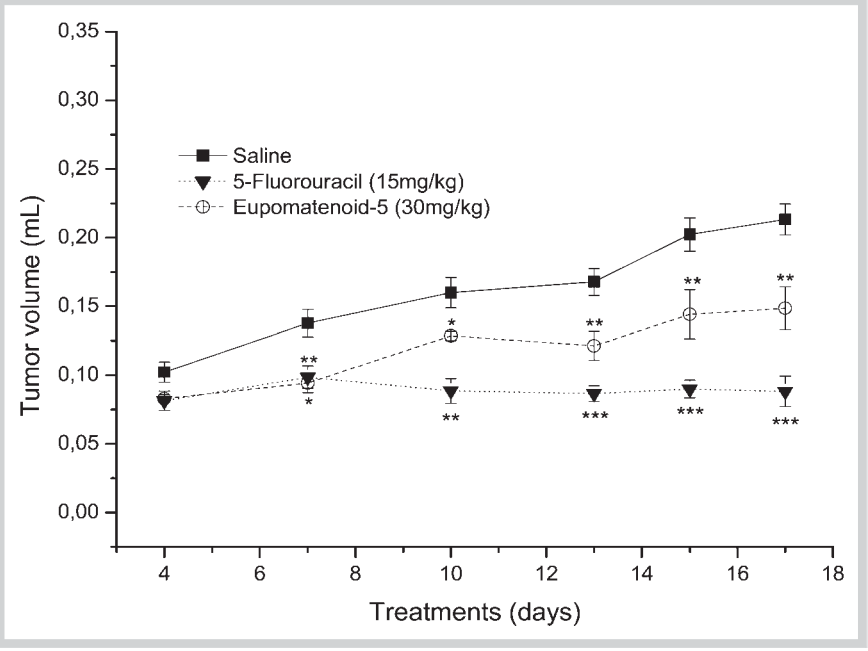

Fig. 4 Tumor volume variation induced by Ehrlich cells in mouse hind footpad during the treatment with eupomatenoid-5 and 5-fluorouracil; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Duncan's test, $p < 0.001$ (ANOVA).

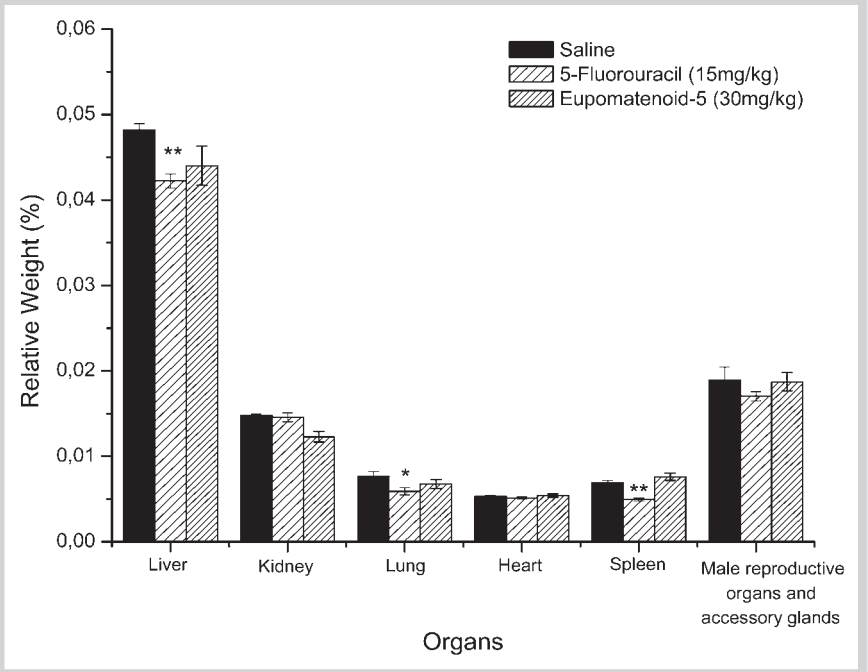

Fig. 5 Relative organ weights of experimental groups treated with eupomatenoid-5 and 5-fluorouracil; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Duncan's test, $p < 0.001$ (ANOVA).

Besides the efficiency, 5-fluorouracil caused drastic weight loss [28], presenting significant toxic signs, especially in the liver, lung, and spleen, as observed in the relative weight of these organs (**Fig. 5**). In contrast, eupomatenoid-5 (30 mg/kg) was effective in controlling tumor progression, with no toxic effects.

Discussion

Over the past few decades, cancer has turned out to be the second most frequent cause of death around the world and a major concern for pharmaceutical industries. The discovery of new drugs derived from medicinal plants still plays an important role in cancer chemotherapy, despite the development of synthetic drugs [4].

Although numerous studies have elucidated the anticancer activity of the Piperaceae species [29–31], no reports are found for *P. regnellii* specifically.

In vitro studies revealed that *P. regnellii* dichloromethanic extract was effective against almost all human tumor cell lines evaluated. The purification process of this extract provided a lignan enriched fraction (FRB) that enhanced the specific anticancer activity. From FRB, enough of eupomatenoid-5 (**1**) was isolated to perform *in vitro* and *in vivo* studies. The isolated compound presented better *in vitro* antiproliferative activity than FRB, which emphasizes that this compound might be involved with the *in vitro*

anticancer activity of fraction FRB, with potent activity against kidney (786–0), ovary (OVCAR-3), prostate (PC-3), and breast (MCF7) tumor cell lines. Among these cell lines, OVCAR-3 and MCF7 are hormone-dependent, suggesting further studies to evaluate the possible interaction of this compound with hormonal receptors.

The *in vivo* assay aimed to confirm the *P. regnelli* extract, fraction, and compound **1** potential use in cancer treatment as demonstrated by the *in vitro* antiproliferative evaluation.

The Ehrlich tumor is a fast-growing and aggressive adenocarcinoma [20], being, therefore, a relevant model for screening of compounds with anticancer properties. DCE (300 and 1000 mg/kg), fraction FRB (100 mg/kg), and eupomatenoid-5 (30 mg/kg) were effective against Ehrlich solid tumor progression.

Although no evidence of acute toxicity has been observed when 300 and 1000 mg/kg of DCE were administered in repeated doses, some of the most important signs of toxicity appeared. The same was observed for chemotherapeutic 5-fluorouracil. Liver, lung, and spleen were the most affected organs by 5-fluorouracil. These data are consistent with the clinical data that reported toxicity in patients treated with this chemotherapeutic [32].

Data of sample toxicity are important and relevant once neoplasms are responsible for high mortality and many treatments used, such as chemotherapies and radiotherapies, have undesirable side effects. Therefore, there is an incessant search for efficient drugs that have low toxicity and, consequently, cause minimal collateral effects [33]. Eupomatenoid-5 showed significant antitumor activity, without any toxic signs in animals. This apparent lack of toxicity displayed by eupomatenoid-5 predestined this compound to further potential preclinical studies.

The *in vitro* and *in vivo* studies conducted with *P. regnellii* samples corroborate data in the literature regarding anticancer activity of lignans and neolignans [34,35]. Lignans are described as hormone-like diphenolic phytoestrogens and have been shown to influence not only sex hormone metabolism and biological activity, but also intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, and angiogenesis, which makes them strong candidates for natural cancer-protective compounds [36,37].

As confirmed by our data, the neolignan eupomatenoid-5 presented relevant *in vitro* antiproliferative activity for kidney, breast, ovary, and prostate cancer. Previous studies described in the literature showed that dietary lignans inhibit breast carcinoma [38] and suppress the growth of prostate cancer cells, and may do so via hormonally dependent and independent mechanisms [39]. The lignans may contribute towards the prevention of breast cancer as a result of their antiestrogenic properties, whereby they interact with the estrogen receptor and modulate the action of estrogen. Alternatively, they may act as antioxidants and prevent the production of carcinogens from estrogen, or they may inhibit aromatase enzyme activity and, thereby, contribute to the prevention of estrogen dependent cancers [40]. These could also be possible mechanisms of action for eupomatenoid-5. Also important to note is that other neolignans are currently in use for cancer treatment. Among those, podophyllotoxin was isolated as the active antitumor agent from the roots of various species of *Podophyllum* genus [17]. Extensive research led to the development of etoposide, an important chemotherapeutic agent used to treat a wide spectrum of human cancers, acting as mitotic spindle poisons, by inhibition of topoisomerase II, an important enzyme involved in the replication pathway of DNA during cell cycle progression [4,41]. Etoposide has been in clinical use for more than two decades and remains one of the most highly prescribed anticancer drugs in the world [42]. Taking into account that eupomatenoid-5 is part of this same class of compounds, further studies are suggested to evaluate possible similar mechanisms of cell cycle inhibition.

This is the first report of *Piper regnellii* antiproliferative potential, showing that this species could be considered a potential source of compounds against cancer. Moreover, eupomatenoid-5 is one of the active principles responsible for the antiproliferative activity of *P. regnellii* dichloromethane extract, showing efficacy at a low dose (30 mg/kg) with no toxic effects. These significant *in vitro* and *in vivo* activities encourage further studies to elucidate possible mechanisms of action. As reported for other lignans, eupomatenoid-5 may be acting in cell cycle progression, affecting cancer cell proliferation, or even by hormone-dependent mechanisms.

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