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Effect of *Poikilacanthus glandulosus* (Nees) Ariza branches and gallic acid against viability of *Echinococcus ortleppi* protoscoleces

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ABSTRACT

The aim of this study was to assess the antioxidant capacity and to evaluate effect of *P. glandulosus* on the viability of protoscoleces of *Echinococcus* spp. as well as determine the possible substance in charge for this action. *Echinococcus ortleppi* protoscoleces were exposed front of the crude extract and butanol fraction and gallic acid at different times.Phytochemical study revealed the presence of triterpenoids, steroids, coumarins, catechins, phenols, anthocyanins, anthocyanidins and cardioactiveheterosides. The highest polyphenol and flavonoids content was found in the ethyl acetate(151.34±1.71mg/g) and dichlorometane (123.30±1.46mg/g), respectively. For assays with DPPH the ethyl acetate fraction was highlighted (IC_{50}95.48±0.35 µg/mL) and the dichlorometane fraction significantly reduced lipid peroxidation (IC_{50} 135.75±2.39 µg/mL) and also the oxidation of DCFH (15.6 µg/mL). The crude extract, butanol fraction and gallic acid showed scolicidal action of 100% at 15, 10 and 15 minutes, respectively.

INTRODUCTION

Poikilacanthus glandulosus (Nees) Ariza belong the family Acanthaceae, is a shrub and is distributed in South and Southeast of Brazil, Paraguay, Argentina and Uruguay, presenting as synonyms *Justicia umbrosa*, *Justicia flexuosa* and *Poikilacanthus flexuosus* (Wasshausen and Smith, 1969*;* Ariza, 1984*;* Mabberley, 1997*;* Wasshausen and Wood, 2004).

The external morphology of *Poikilacanthus* is very similar to the genus *Justicia* and many of their species have been described belonging to this. Only from the analysis of pollen morphology that was possible to maintain *Poikilacanthus* as a genus the part. Pollen characteristics seem to be the only form of distinction between the two genus (Daniel, 1991, 1998). The plant under study lacks chemical studies, pharmacological, biological, toxicological, phytochemical, etc.

There is only one study in the literature which addresses about the antimicrobial screening through the acetone extract of *P. glandulosus* that showed no inhibition for the tested microorganisms (Barneche *et al*., 2010). The phytochemical compounds of the plant extracts are the major basis of pharmacological activities of natural products (Oloyede, 2005) whereas flavonoids and polyphenols are antioxidants (Pietta, 1998*;* Mau *et al*., 2002) and play significant roles in many processes taking place in living systems.

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The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). Synthetic antioxidants are used in foods to prevent lipid oxidation. However, some studies suggest synthetic antioxidants are harmful to human health (Valentao *et al*., 2002). Consequently, there is increasing interest in finding naturally occurring alternatives from plants for use in food and medicines.

According to Turrens, (2004) all aerobic organisms are exposed to reactive oxygen species (ROS) generated by their metabolism and so, the redox system plays an important role in the survival of the parasite in the host. Parasitic protozoa not only have to eliminate their endogenous toxic metabolites but they should also cope with the oxidative (or respiratory) burst of the host immune system. In general, antiparasitic drugs, which have the ability to inhibit vital redox reactions or promote oxidative stress in parasites, are considered redox-active antiparasitic drugs (Seeber *et al*., 2005).

Considering the growing interest and the need for research on medicinal plants, especially those species not yet studied, and the need for the development of new agents in the treatment of several diseases,led to development of this work so there is a greater understanding about the chemical constituents, antioxidant capacity and scolicidal effect of P. glandulosus branches front Echinococcus spp. protoscoleces, as well as determine the possible substance in charge for this action.

MATERIAL AND METHODS

Animals

Male Wistar rats (3.0–3.5 months of age and weighing 270–320 g) were maintained in groups of 3–4 rats per cage. They had continuous access to food and water in a room with controlled temperature (22±3 °C) and on a 12-h light/dark cycle with lights on at 7:00 am. The animals were maintained in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA) (project number 030/2012).

Plant Collection and Extractions

The branches of *P. glandulosus* were collected in Santiago, RS, Brazil in May of 2012. A voucher specimen is preserved in the herbarium of the Department of Biology at UFSM (SMBD 12.442 and 13.178). Plant material was dried at room temperature and powdered in a knife mill. The branches (2.061 Kg) were macerated at room temperature with 70% ethanol for a week with daily shaking*;* the solvent was renewed several times. After filtration, the hydroalcoholic extract (HE) was evaporated under reduced pressure at a temperature below 40°C, in order to obtain the aqueous extract (AE)*;* part of this AE was evaporated to dryness to furnish a crude extract (CE). The remaining AE was partitioned with solvents of increasing polarity: dichloromethane (CH_2Cl_2-F), ethyl acetate (AcOEt-F) and *n*-butanol (BuOH-F). Finally, the fractions obtained were concentrated to dryness on a rotary evaporator. The fractionation method is illustrated in the Figure 1.

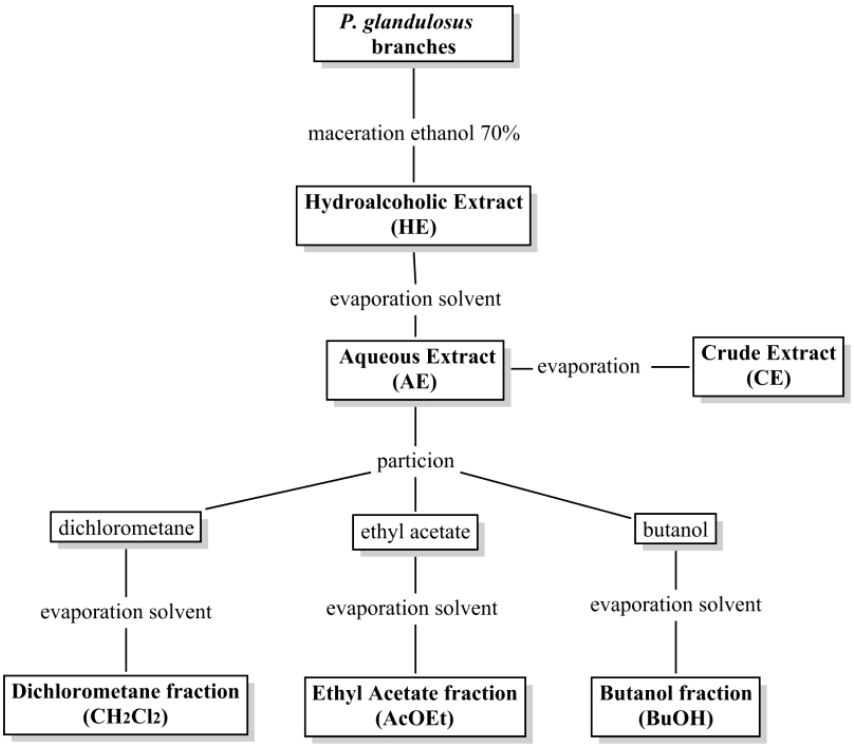

Fig. 1: Method of fractionation of *P. glandulosus* branches extracts.

Phytochemical analysis

Qualitative Analysis of Compounds

The phytochemical analysis was realized in according with literature specialized (Moreira, 1979; Matos, 2009), observing the individual methodology for each reaction and verify the possible presence of the chemical groups of secondary metabolism in the plant drug.

Determination of Total Phenolics

Total phenolic contents were measured using Folin–Ciocalteu method, slightly modified as described by Chandra *et al*. (2004). Total phenolic content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction, using GA as standard.

Determination of Total Flavonoids

Total flavonoids were determined according to the colorimetric method described by Woisky and Salatino (1998), using a 2% aluminum chloride solution. The flavonoid content was established in milligrams equivalents of rutina (RE) per gram of each fraction, using R as standard.

HPLC/DAD Analysis of GA

The CE and fractions of the *P. glandulosus* were analyzed by HPLC performed with a Prominence Auto-Sampler (SIL-20A) equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU-20A5 degasser and CBM-20A integrator, UV–VIS detector DAD SPD-M20A and LC Solution 1.22 SP1 software. Reversed phase chromatographic analyses were carried out under gradient conditions using a C-18 column (250 mm×4.6 mm). The GA analysis was carried out using a gradient system using Solvent A (water containing 2% acetic acid) and Solvent B (acetonitrile), according to Boligon *et al*., (2012). The flow rate was 0.6 mL/min and the injection volume was 40 µL, with 60 minutes of time duration of analysis. Identification of compounds was performed by comparing retention times and the Diode-Array-UV spectra with those of standards.

Antioxidant Capacity Methods

DPPH Assay

The antioxidant capacity of the fractions and the CE was evaluated by monitoring its ability in quenching the stable free radical DPPH, according to Choi *et al*., (2002). The DPPH quenching ability was expressed as IC_{50} and a solution of DPPH (1 mL; 0.3 mM) in ethanol (2.5 mL) was used as a negative control and AA in the same concentrations used for the samples provided the positive control.

TBARS Assay

TBARS production was determined as described by Ohkawa *et al*., (1979). Rats were killed and the encephalic tissue was rapidly dissected and placed on ice. Tissues were immediately homogenized in cold 10 mMTris-HCl, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4,000 × *g* to yield a pellet that was discarded and a low-speed supernatant (S1). An aliquot of 100 µL of S1 was incubated for 1 h at 37 °C with freshly prepared $FeSO_4$ (10 µM), in the presence of *P. glandulosus* fractions.

DCFH-DA Method

The substrate DCFH-DA was utilized to measured intracellular formation of reactive oxygen species, according to Myrhe *et al*. (2003). The supernatant of rat brain homogenate was incubating at 37°C with different concentrations of *P. glandulosus* fractions. The fluorescence was measured using 488 nm for excitation and 520 nm for emission. ROS levels (expressed as µmol of oxidized 2',7'-dichlorofluorescin (DCF) per mg protein) were calculated by interpolation in a standard curve of oxidized DCF (constructed in parallel), corrected by the content of protein (Lowry *et al*., 1951). Ethanol was used as negative control.

Effect of CE, BuOH-F and GA on the protoscoleces viability of *E. ortleppi*

Biological samples and molecular analysis

Hydatid cysts in lungs and lives of naturally infected bovine were collected in a slaughterhouse in the central region of RS. The protoscoleces viability was assessed with 0.1% eosin (Moazeni *et al*., 2012). The percentage considered suitable for the development of our experimental test was 100% of viability.

DNA extraction was performed using an aliquot of the liquid containing hydatidprotoscoleces, using a commercial kit (QIAamp tissue) (QIAGEN Inc. Chatsworth, CA) according to the manufacturer's instructions. After extraction it was performed the polymerase chain reaction using a pair of primers (5' TTTTTTGGGCATCCTGAGGTTTAT 3'
5' TAAAGAAAGAACATAATGAAAATG 3')
to amplify a fragment of COX-I gene (Bowles *et al*., 1992). To detect the pattern of bands, the electrophoresis in 1% agarose gel was performed, stained with ethidium bromide and visualized under UV light. The samples were submitted to DNA sequencing using a pair of primers (cox I) gene. The similarity of DNA sequencing samples was carried out with using BLAST program (Basic Local Alignment Search Tool).

Scolicidal and viability assay

In this study, it was used CE and BuOH-F in concentrations of 350 mg/mL of *P. glandulosus* and GA in 7 mg/mL. Distilled water was used as dilution vehicle of the agents. In each experiment, 500 µL of the solution was placed in a test tube, to which 7 µL of protoscolex rich sediment (about 1000 protoscoleces) was added. The contents of the tube was gently mixed, this procedure was repeated in times of 5, 10, 15 and 30 minutes for each concentration tested. At the end of each incubation the upper phase was removed and 500 µL of 0,1% eosin stain was then added to the protoscoleces and mixed gently. After 15 min of incubation the pellet of protoscoleces was

examined in microscope optic. The percentages of viability were determined by counting minimum of 700 protoscoleces (Moazeni *et al*., 2012). The viability was evaluated by the same optical microscope, at different times described, observing motility and eosin staining, performing a count of 500 protoscoleces (7 µL hydatid cyst fluid) each evaluation. The protoscoleces were considered viable, when they presented motility and unstained, and not viable with no motility and stained (Yones *et al*., 2011; Moazeni *et al*., 2012).

Statistical Analysis

The results obtained for DPPH, TBARS, scavenging of ROS, total phenolics and flavonoids assays were analyzed statistically by one-way analysis of variance (ANOVA), followed by Tukey's test using the statistical package SAS (2001). For viability protoscoleces analysis, survival curves were plotted using Kaplan-Meier and differences were analyzed using the chi-square test. The experiments were performed in triplicate and when appropriate $p < 0.05$, $p < 0.01$ or $p < 0.001$ were considered significant. Data were expressed as mean ± SD.

RESULTS AND DISCUSSION

In the present work, was possible verify the presence of the main secondary metabolites (Table 1). Among the secondary metabolites found this phytochemical analysis, the coumarins possess several pharmacological activities, such as antithrombotic, anti-inflammatory, antiviral, antitumor and antioxidant properties (Martín-Aragón *et al*., 1996).

Table 1: Phytochemical analysis of AE and HE extracts of *P. glandulosus* branches.

Secondary metabolites	AE	HE
Heterosides anthocyanic	+	+
Heterosides cyanogenic	-	-
Amino-groups	+	+
Volatile acids	-	-
Tannins	-	-
Anthocyanins, anthocyanidins and flavonoids	+	+
Leucoanthocyanidins and flavones	-	-
Flavonols, flavanones, flavanonóis and xanthones	-	-
Triterpenes	+	+
Catechins	+	+
Resins	-	-
Heterosides cardiotonics	+	+
Phenols with ortho and meta free	+	+
Phenols with the position para free	+	+
Coumarins	+	+
Organic acids	+	+
Phenols	+	+

+ Presence, - Absence.

The presence of phenols was verified, being reported among many other activities, the antioxidant and anti-inflammatory, cardioactive heterosides as the hypotensive (Simões *et al*., 2010), triterpenoids and steroids have a variety of medicinal properties, highlighting anti-inflammatories, analgesics, cardiovascular and antitumor effects (Niero and Malheiros, 2009). Note that negative results do not necessary imply in his absence, it is possible that the quantity of these are small to be detected. Total polyphenols and flavonoids values are given in Table 2.The highest concentration of polyphenols in the AcOEt-F found in this study was expected, because according to the scheme proposed by CechinelFilho and Yunes (1998) and studies qualitative composition of plant extracts second Canadovic *et al*. (2008)show that phenolic compounds in general, tend to concentrate in this fraction when working with fractions of different polarities.The findings of this work are higher than studies with species of the genus Justicia, as *J. gangetica* (16.513 mg/g) (Stewart *et al*., 2013), *J. gendarussa* (12.68 mg/g) (Amid *et al*., 2011) and *J. pectoralis* (1.18 mg/g) (Lizcano *et al*., 2010).

Table 2: Total polyphenols and flavonoids for crude extract and fractions of *P. glandulosus.*

Extract/fraction	TP ± SD (mg GAE/g)	TF ± SD (mg RE/g)
Crude extract	90.37 ± 0.49^{d}	27.68 ± 0.85^{c}
Dichloromethane	118.65 ± 2.34^{c}	123.30 ± 1.46^{a}
Ethyl acetate	151.34 ± 1.71^{a}	51.02 ± 1.53^{b}
Butanolic	136.98 ± 1.30^{b}	27.28 ± 0.76^{c}

Values are expressed as mean ± standard deviation. $^{a–d}$ Means with the different letters in each column are significantly different ($p < 0.001$) according to the Tukey test (n = 3).

The CH2Cl2-F showed the highest concentration of flavonoids in a very significantly front the other fractions, following the AcOEt-F. In the extractor solvent, dichloromethane, can be found the methoxylated flavonoids, which can explain the largest content of flavonoids in this fraction (Cechinel Filho and Yunes, 1998), The BuOH-F and CE obtained the lowest values, there being no significant difference between them. In a study conducted by John *et al*., (2013) with six species of *Justicia*, they verified the flavonoid content in different parts of the plant, whereas the maximum value was found in the leaves of *J. betonica* (2.86 mg/g). Lizcano and collaborators (2010), reported the flavonoid content of the leaves and stem *J. pectoralis* (0.75 and 0.12 mg/g), respectively. These results were lower than those found for all extracts of *P. glandulosus* used in this study for polyphenols and flavonoids. Through HPLC/DAD analysis was verify the presence of GA in all extracts of *P. glandulosus* branches, showing 5.09±0.12 mg/g, 0.68±0.02 mg/g, 2.17±0.15 and 6.72±1.06 mg/g for CE, CH_2Cl_2-F, AcOEt-F and BuOH-F, respectively. According Ferguro and Harris (1999), the antioxidant activity of the phenolic acids is generally determined by the number of hydroxyl groups present in the molecule. Besides the proven antioxidant activity of this compound, preclinical studies have shown that the GA has a variety of pharmacological actions, such as anti-inflammatory, antimicrobial, antiviral and antitumor activities (Kim, 2007; Özçelik *et al*., 2011). The GA was found by the first time in *P. glandulosus* and others compounds can be investigated, once through this study was observed the significant presence of others peaks in the chromatograms that were not identified.

The DPPH assay is the most common methods to determine antioxidant capacity in a practical, rapid and sensitive manner (Arnao *et al*., 2000). The best sequestrant capabilities of

DPPH in this study were observed in the AcOEt-F (IC_{50} 95.48 µg/mL), followed by BuOH-F (IC_{50} 118.92 µg/mL). The CH_2Cl_2-F and CE showed weaker antioxidant capacity (IC_{50}144.05±0.23 and409.5±0.76 µg/mL), respectively (Table 3).

Table 3: Antioxidant capacity for crude extract and fractions of *P. glandulosus*.

Extract/fraction	IC_{50} ± SD (µg/mL)	
	DPPH	TBARS
Crude extract	409.5^{a} ± 0.76	#
Dichloromethane	144.05^{b} ± 0.23	135,75 ± 2,39
Ethyl acetate	95.48^{d} ± 0.35	#
Butanolic	118.92^{c} ± 0.28	#
Standarts		
Ascorbic acid	$15,98±0,42^{e}$	-
BHT	-	*

Values are expressed as mean ± standard deviation. $^{a-e}$ Means with the different letters in each column are significantly different ($p < 0.05$) according to the Tukey's test (n = 3). The IC_{50} value found by linear regression corresponding to a value smaller than the concentrations tested. # The value of IC_{50} found by linear regression corresponding to a value higher than the concentrations tested.

The results are in agreement with the content of polyphenols found in the fractions, so is possible to attribute the antioxidant capacity seen in the DPPH assay to the presence of these compounds. This positive correlation can be explained on the basis of the similarity between compounds with high antioxidant activity extracted by these organic solvents (Silva *et al*., 2006*;* Schubert *et al*., 2007).

TheCH_2Cl_2-F showed highest antioxidant capacity, it may be suggested by the high flavonoid content found for this fraction. Our results show that *P. glandulosus* was more potent in sequestering the DPPH radical when compared with *J. gendarussa* (IC_{50}482.5 µg/mL) (Krishma *et al*., 2010) and *J. spicigera* (IC_{50} of 944.67 µg/mL) (Sepúlveda-Jiménez *et al*., 2009). For the TBARS analysis, theCH_2Cl_2-Fwas the one that presented significant reduction in lipid peroxidation compared to baseline, from the concentration of 31.2 µg/mL and $IC_{50}$of 135.75 µg/mL (Table 3 and Figure 2). However, this correlation was not possible to check with other fractions that showed values polyphenols and flavonoids relatively high. By linear regression analysis, was possible to perceive that the other fractions were not capable of reducing the lipid peroxidation, requiring largest concentrations, which may produce a toxic effect.

There are several methods to analyze the antioxidant capacity, mainly because there are different mechanisms that plant extracts can exert this action. The fluorogenic compound DCFH-DA has been utilized extensively as a marker for oxidative stress, and is suggested to reflect the overall oxidative status of the cell (Wang and Joseph, 1999).

In this work, the CH_2Cl_2-F significantly decreased the oxidation of DCFH at a concentration of 15.6µg/mL (Figure 3). The BuOH-F was able to significantly reduce levels of oxidation of DCFH from the concentration of 125µg/mL and the AcOEt-F, only in the most concentration tested (250 µg/mL). The CE was not able to reduce significantly the oxidation of DCFH. The results of this study suggest that *P. glandulosus* was able to reduce the levels of malondialdehyde and also the basal ROS generation in rat brains, especially CH_2Cl_2-F.

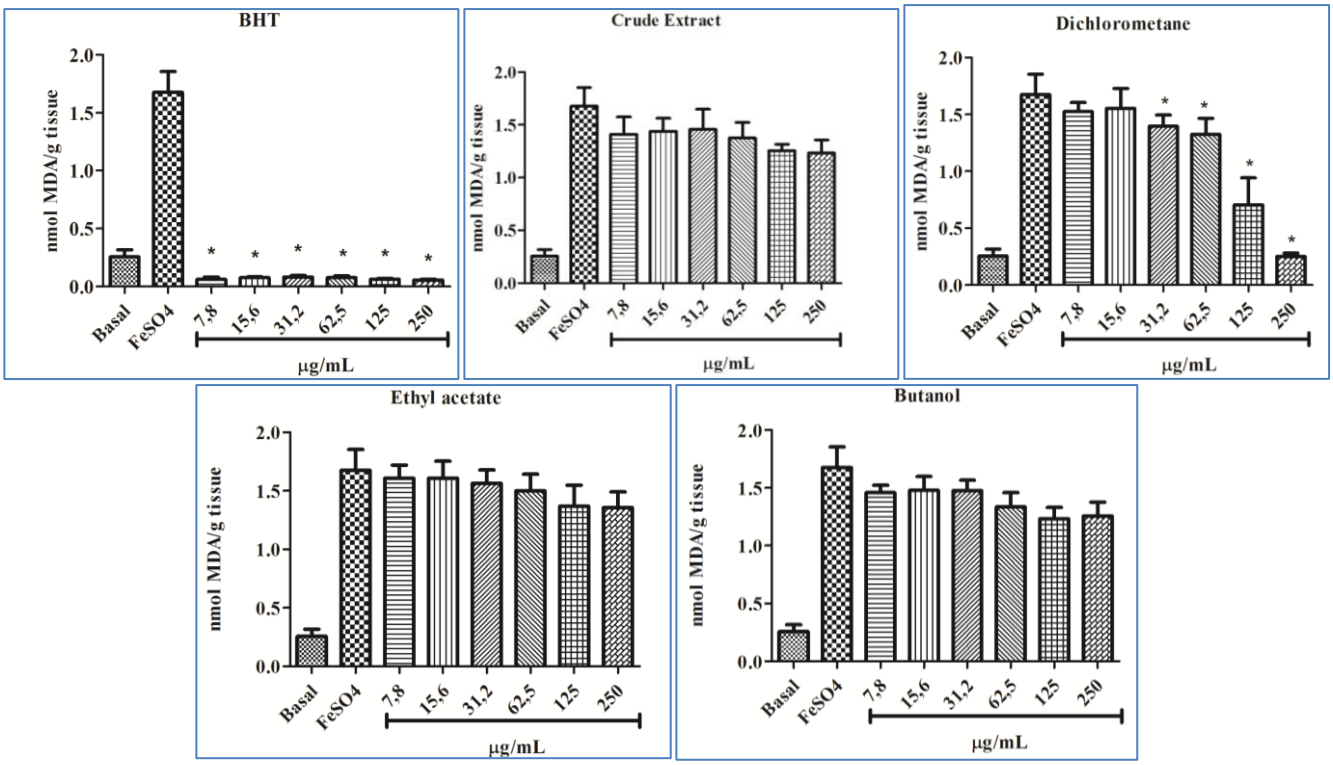

Fig. 2: Effect of different concentrations fractions from the branches of *P. glandulosus* on Fe(II) (10 µM)-induced TBARS production in brain homogenates. Dates are expressed as means ± S.D., (n=3). Significant differences are indicated by * $p \leq 0.05$ when compared with $FeSO_4$ group.

Echinococcosis is a zoonotic infection caused by cestode species of the genus *Echinococcus*. This neglected disease is distributed worldwide and causes illnesses in humans and animals (Cardona and Carmena, 2013). *Echinococcus ortleppi* is found in southern Brazil (Balbinotti *et al*., 2012) and in determined parts of Europe, Africa, South Asia and the Americas (Dinkel *et al*., 2004). There are also reports of *E. ortleppi* causing cystic echinococcosis in humans (Maravilla *et al*., 2004*;* Sharma *et al*., 2013). To date, many scolicidal agents have been used for inactivation of the hydatid cyst content, but there is no ideal agent that is both effective and safe. An ideal scolicidal agent is define as being potent in low concentrations, acting in a short period time, being stable in cyst fluid, not affected by dilution with the cyst fluid, being able to kill the protoscoleces in the cyst, being non-toxic, having low viscosity, and being readily available and easily prepared, as well as being inexpensive (Moazeni and Nazer, 2010). The mainstay of treatment for hydatid disease is surgery. Surgical approaches depend on the parasitized organ and cyst size. In order to prevent recurrence of secondary hydatid cysts, inactivation of protoscoleces with scolicidal agents before the cyst removal is recommended. As it is also indicated the use of powerful scolicidal the alternative method of treatment puncture, aspiration, injection and recirculation(Park *et al*., 2009). For this study, the protoscoleces were identified molecularly as the specie *E. ortleppi*. GA was found in all fractions of *P. glandulosus*, but in larger quantities in the CE and BuOH-F, which ledus toconduct this studywith the standardand theseextracts in an attempt tojustifythis activity. The effect of *P. glandulosus* front *E. ortleppi* protoscoleces revealed that CE and BuOH-F at concentration of 350 mg/mL can kill all protoscoleces acting in a short period time (10 at 15 minutes of application, respectively). The GA was able to kill all protoscoleces in 15 minutes of exposure at concentration 7 mg/mL (Figure 4).

This significant effect of *P. glandulosus* extracts can be explained by good activity of GA standard that was identified by HPLC-DAD analysis in this specie. The GA has a variety of pharmacological actions, but as far as we know, this is the first report on the scolicidal activity this compound. The relation between antiparasitic and antioxidant activities of natural compounds have long been under investigation and discussed, especially on the generation of ROS, but remains unclear (Ribeiro *et al*., 1997*;* Moazeni *et al*., 2012).

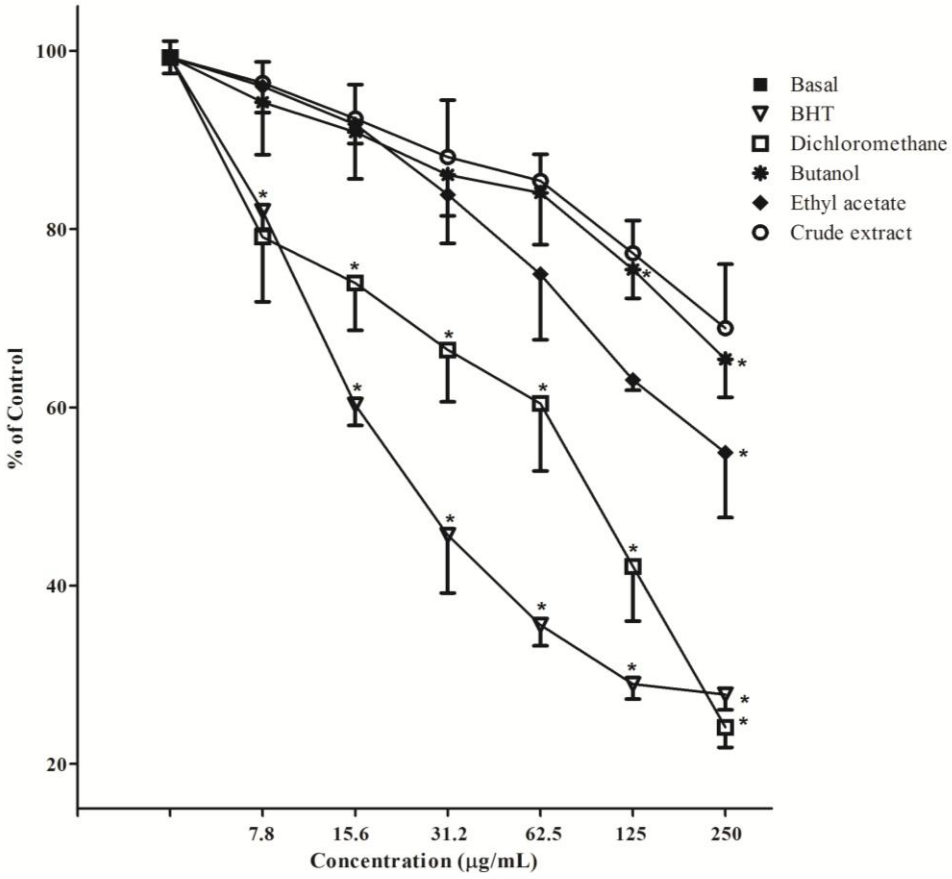

Fig. 3: Effect of the crude extract and fractions of the branches from *P. glandulosus* on scavenging of ROS in rat brain cells, by DCFH-DA method.
*Data are expressed as means ± S.D., (n = 3). *****Significant difference from the basal group ($p < 0.05$).

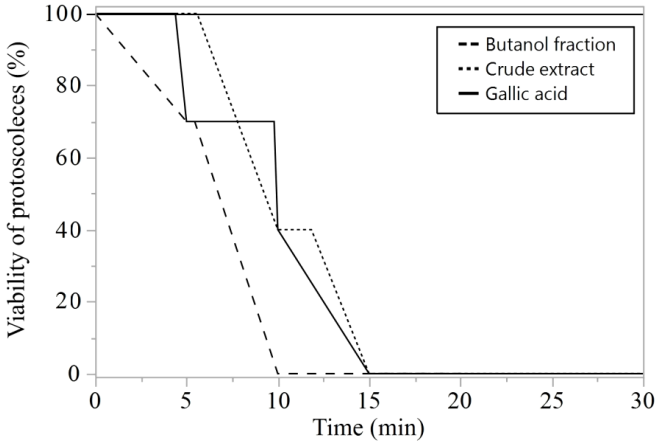

Fig. 4: *In vitro* effects on the viability of protoscoleces when submitted to CE 350 mg/mL, BuOH fraction 350 mg/mL of *P. glandulosus* and GA 7 mg/mL in different exposure times.

CONCLUSION

This study showed the antioxidant activity of P. glandulosus branches by different methods, the scolicidal effect and contributed to reveal some phytochemical compounds of this species for the first time. Taking into account that natural substances can be responsible for the protective effect against the risk of many disease processes, our results suggest that *P. glandulosus* has antioxidant potential and can be a promising source of natural antioxidants. Furthermore, was possible described the significant potential *in vitro* scolicidal activity of CE and BuOH-F of *P. glandulosus* front the protoscoleces of *E. ortleppi*. The results of this study allowed us to suggest that GA can be the responsible in part by scolicidal effect of *P. glandulosus*, for the reasons cited above. The present study open the possibility of more investigations of *in vivo* scolicidal effect of this traditional medicine.

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