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Anthelmintic and antioxidant potential of *Fagopyrum esculentum* Moench *in vitro*

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One of the limiting factors of sheep breeding is helminth infection, mainly resulting in poor weight gain. The combination of parasite control strategies, including the use of medicinal herbs can reduce the use of chemical anthelmintics. Tanniferous plants, by having phenolic compounds, mainly condensed tannins were associated with anthelmintic action. *Fagopyrum esculentum* Moench (buckwheat) has flavonoids, phenolic acids, tannins and high content of lysine, and the highest levels of these compounds are found in the seeds. Tannins exert direct anthelmintic action in reducing the the fertility of female nematodes, and indirect by increasing the immune response to protect the ingested protein of rumen degradation. The objective of this study was to evaluate the anthelmintic potential of the hydroalcoholic extract of *F. esculentum* Moench seeds (ETM) in the control of gastrointestinal nematodes of sheep *in vitro* and the antioxidant activity of ETM. Faeces from sheep presenting at least 2,000 eggs per gram of faeces were used. Hatchability and larvae migration tests were performed to evaluate the treatments ETM at concentrations of 0.625; 1.25; 2.5 and 5 mg mL^{-1}, negative and positive controls and DMSO control (0.75% + distilled water). The treatment means were compared by Tukey test at 5% of probability. Subsequently, the total content of polyphenols, flavonoids, tannins and antioxidant activity of ETM were determined. For the conditions evaluated in this study, it was possible to verify anthelmintic activity of ETM in both tests *in vitro*. The ETM inhibited 19.66% of hatching in concentration 1.25 mg mL^{-1} and 17.66% of larvae migration in the concentration of 5 mg mL^{-1}. The anthelmintic activity may be due to condensed tannin content found (288.89 mg equivalent tannic acid per gram of extract). Antioxidant activity was observed at all concentrations, reaching 38.71% at 3 mg mL^{-1} with $IC_{50\%}$ = 3.83 mg mL^{-1} and 468.12 µM equivalent Trolox per gram of extract. At the same concentration to flavonoids and total polyphenols was observed respectively 31 mg equivalent rutin and 54.33 mg equivalent gallic acid per gram of extract. In addition to the direct effect of ETM on trichostrongylids of sheep, future research is also justified by the possibility of an indirect effect due to immune stimuli that protein diet provides on fostering in combating worms. It was concluded that it was possible to verify anthelmintic and antioxidant activity, demonstrating the potential of ETM in parasitological control of sheep.

Key words: Agroecology, buckwheat, condensed tannins, gastrointestinal nematodes, organic production, sheep breeding.

INTRODUCTION

Sheep breeding is an expanding activity (Güetter, 2011; Gianlorenço, 2013). Brazil is the 18th largest producer with herd estimated at 25.43 million of sheep and goats (De Zen et al., 2014). However, gastrointestinal nematodes are a major limiting factor to sheep production, especially in tropical regions (Vieira, 2008).

Damages caused by helminth infection include less weight gain, poor quality of wool, higher mortality, lower yield carcass, lower milk production and low fertility. These losses are due to clinical signs such as diarrhea, anemia, hemorrhage, prostration and weakness, adversely affecting profitability and animal welfare (Szpatowski, 2010). In addition, there are increased costs for the acquisition of antiparasitic drugs and labor use.

Among the parasites that infect sheep, are the trichostrongylid from Trichostrongylidae family, which includes species of genera *Trichostrongylus, Haemonchus, Ostertargia, Nematodirus* and *Cooperia*.

The integration of management systems is the main concept in the search for sustainable control of helminths (Hoste and Torres-Acosta, 2011). The search for strategies such as integrated grazing to other species and the use of natural therapies has been frequently observed (Batatinha et al, 2011; Joshi et al, 2011).

Another important aspect relates to the pharmacological resistance to chemical anthelmintics (Szpatowski, 2010). Natural measures for parasite control can minimize resistance to chemical anthelmintics (Houdijk et al., 2012). There are records of resistance to many drugs commonly used (Kaplan, 2004). Resistance has been observed in various chemical groups. The indiscriminate use of these drugs select resistant isolates, and therefore, these products do not end up performing the control of nematodes infections satisfactorily. In Brazil, the problem occurs in various regions (Vieira et al., 1992; Soccol and Pohl-de-Souza, 1997; Rosalinski-Moraes et al., 2007; Sczesny-Moraes et al., 2010, Vila Nova et al., 2014; Madruga et al., 2015). The resistance is gradually advancing on the latest available chemical groups. It is necessary to change the concept that the chemical anthelmintics are an inexhaustible source and only alternative for the control of parasites (FAO, 2003).

Today, the consumer market are showing growing demand for food products free from chemical residues (Resende, 2013). Organic farming, agroecology, biodynamic and organic have expanded rapidly in the world. These production systems contribute to the socio-economic sustainability of the producer (Neves et al., 2016). These systems are grounded in agroecological principles, not allowing the use of chemical pesticides.

Buckwheat (*Fagopyrum esculentum* Moench) is a dicotyledonous plant belonging to the Polygonaceae family, which has a high protein content, with high content of essential amino acid lysine (Zhou et al., 2012). The seeds have condensed tannins, flavonoids and phenolic acids (Steadman et al., 2001). Rutin, is a useful bioflavonoid in the treatment of various medical conditions, mainly due to its antioxidant action (Karamac, 2010). The highest concentration of rutin in buckwheat is found in the leaves and flowers (Vojtíšková et al., 2012).

Tannins exert direct anthelmintic action in reducing of the the fertility of female nematodes (Otero and Hidalgo, 2004), and indirect by increasing the immune response to protect the ingested protein of rumen degradation, increasing their availability in the lower gastrointestinal tract of animals (Ketzis et al., 2006). The anthelmintic activity *in vitro* of tannins was characterized by reduction of hatching, development and motility of larvae and adults (Brunet et al, 2008; Joshi et al 2011.). *In vivo* cause reduction of eggs per gram of faeces (OPG) and the parasite load (Minho et al, 2008; Max et al, 2009; Mupeyo et al, 2011; Oliveira et al, 2011).

Therefore, the objective of this study was to evaluate the anthelmintic potential of the hydroalcoholic extract of *Fagopyrum esculentum* Moench seeds (ETM) in control of gastrointestinal nematodes of sheep *in vitro*, as well as to evaluate the antioxidant activity, total content of polyphenols, flavonoids and condensed tannins.

MATERIALS AND METHODS

Plant material and extract preparation

Buckwheat seeds were collected in commercial farming in São João do Ivaí, Paraná, Brazil. The botanical material was selected by the absence of macroscopic changes in its surface constitution. Drying was carried out in a forced ventilation air oven at a temperature of 40°C and then maceration in a Wiley mill.

To obtain the hydroalcoholic extract of buckwheat seeds to 10%, 90 g of seed, 240 mL of distilled water and 570 mL of absolute ethanol were used. After this, the solution was kept under constant mechanical stirring at environment temperature for 24 h. Subsequent vacuum filtration was performed three times, after each filtration adding new hydroalcoholic solution in the same above ratios. The extract was concentrated in rotaevaporator and lyophilized.

Obtainment of eggs and larvae of nematodes

Faeces were collected directly from the rectum of naturally infected sheep and free from previous chemical treatments at least 60 days. Eggs per gram of faeces (EPG) was performed for selection of

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animals with EPG value above 2,000 eggs.

For eggs isolation for hatchability test, the methodology described by Coles et al. (1992) adapted by Bizimenyera et al. (2006) was used. Faeces were macerated in water at 40°C, sieved through sieves of 250, 150, 75 and 25 µm. After the material was centrifuged at 3000 revolutions for 5 min, the supernatant was discarded. This material was then transferred to Falcon tubes, filled with saturated NaCl solution suspending the eggs. After further centrifugation, the supernatant was again filtered on sieve of 25 µm and washed under the conditions described above. To obtain larvae coproculture was performed (Ueno and Gonçalves, 1998).

Anthelmintic evaluation

The hatchability test was carried out in quadruplicate (Von Samson-Himmelstjerna et al., 2009), evaluating the following treatments: ETM at concentrations of 0.625; 1.25; 2.5 and 5 mg mL^{-1}, negative control (distilled water), positive control (albenzadol sulfoxide 0.25 mg mL^{-1} associated with dimethylsulfoxide - DMSO 0.75%) and DMSO control (distilled water with 0.75% DMSO).

To evaluate each treatment, 100 µL of the suspension water and eggs (with 110 eggs), and 400 µL of the respective treatments was added to the culture plates. The plates were incubated in biochemical oxygen demand oven (B.O.D.) at 27°C for 48 h. After, total count of eggs and first stage larvae (L_1), was performed to obtain the hatchability percentage.

To perform the inhibition of larval migration test (Rabel et al., 1994) aqueous solution containing 150 third-stage larvae (L_3) obtained by coproculture in 100 µL of solution was standardized. They were evaluated in quadruplicate, the same aforementioned treatments, except for the positive control treatment, which consisted of 0.01 mg mL^{-1} levamisole hydrochloride associated with 0.75% DMSO.

Microtubes containing 1 mL of the respective treatment solution and 100 µL of L_3 were incubated in B.O.D. at 37°C for two hours, then centrifuged at 6000 revolutions per 3 min. 900 µL was removed and the volume remained as 200 µL.

For test preparation, 24 well culture plates were used, each with a filter opening of 25 µm. In each filter, 1800 µL of each treatment and the remaining 200 µL of the respective wells for each treatment were added.

Plates were again incubated in B.O.D. for two hours at 37°C and after the filters were removed for counting larvae migrated and were retained. Larvae were inactivated with Lugol 5% and the reading was conducted under an optical microscope with 40x magnification.

The migration percentage was calculated by the formula % migration = [Nm / (Nm + Nr)] x 100, where Nm is the number of L_3 larvae migrate through the mesh and Nr is the number of larvae L_3 retained in the mesh. In both tests, the averages were compared by Tukey test at 5% probability by the Statistica software (Stat Soft, 2007).

Determination of total polyphenols content, flavonoids and tannins of ETM

The ETM was diluted in the concentrations 0.25; 0.50; 1; 1.5; 2 and 3 mg mL^{-1} and evaluated in triplicate. For determination of total polyphenols, the method used was the Folin-Ciocalteu. The results were expressed in milligrams of gallic acid per gram of extract. Gallic acid is a precursor of several types of phenolic compounds has simple structure, which is considered standard substance (Stagos et al., 2012).

The dosage of total flavonoids was determined by UV-Vis spectrophotometer according to the methodology of Zhishen et al. (1999), based on the complexation of flavonoids with $AlCl_3$. The results were expressed in milligrams of rutin per gram of extract. The rutin and quercetin, shows the basic structure of flavonoids and can be used as an indirect indicator of flavonoids.

The determination of the content of tannins was according to the methodology of Makkar (1994) adapted by Fagbemi et al. (2005), and the results were expressed in milligrams of tannic acid per gram of dry extract.

Determination of antioxidant activity of ETM

The antioxidant activity of the extract was determined by the ability of H^+ donor for the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), according to Blios (1958). The calculation of the antioxidant activity was performed according to the formula: Antioxidant activity (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100$, where A_{sample} is the absorbance of the samples after 30 min and $A_{control}$ is the absorbance of DPPH; both at 517 nm.

Subsequently, the antioxidant activity was also determined by the iron reduction method (FRAP test) according to Rufino et al. (2006) using solution Trolox 1000 µM as standard. Results were expressed in µM Trolox equivalent per gram of dry extract.

RESULTS AND DISCUSSION

Through the EPG, trichostrongylids eggs were identified. It was possible to verify anthelmintic activity of ETM in inhibiting hatching and larval migration. The results corroborated with other researchers that reported inhibitory action of tanniferous plants on hatchability and migration larvae of nematodes in sheep (Bizimenyera et al., 2006; Maciel et al., 2006; Yoshihara et al., 2014) (Table 1).

For positive controls, albendazole sulfoxide 0.25 mg mL^{-1} showed 100% inhibition of hatching and levamisole hydrochloride 0.01 mg mL^{-1} showed 89% inhibition of larval migration. The control DMSO did not differ ($p<0.05$) from the negative control in hatchability test, but differed in migration test, showing inhibition of 9.60% in hatchability test and 7.14% in migration test.

The anthelmintic activity presented by ETM, probably was due to the condensed tannin content, which in the concentration 3 mg mL^{-1} reached 288.89 mg tannic acid equivalent per gram of extract (Table 2). However, higher concentrations of ETM (> 2.5 mg mL^{-1}) did not differ from NC and DMSO in hatchability test. Thus, these results represent an important perspective for control of nematodes by the consumption of tanniferous plants.

Bizimenyera et al. (2006) reported a 100% inhibition of hatching of larvae of *Trichostrongylus colubriformis* with 25 mg mL^{-1} of extracts from different parts of *Beltophorum africanum*. Maciel et al. (2006) worked with the same plant at the same concentration and found that the ethanolic extract of the leaves inhibited 100% hatching larvae of *Haemonchus contortus*, while the hexane extract at the concentration 50 mg mL^{-1} inhibited only 16.92% of hatching. According to the authors, the mechanism of the anthelmintic action still had to be determined. However, it could be due to tannins, since the extracts with tannins removed exhibited slightly less activity than the crude extracts.

Table 1. Arithmetical average of hatching and migration of third-stage larvae inhibition of gastrointestinal nematodes of sheep percentage and their standard deviation in treatment.

Treatment (mg mL^{-1})	Hatchability inibition (%)*	Larval migration inibition (%)*
ETM 0.625	11.69^{b} ± 2.85	11.39^{d} ± 1.19
ETM 1.25	19.66^{b} ± 0.45	11.63^{cd} ± 1.76
ETM 2.5	15.63^{bc} ± 5.44	17.04^{bc} ± 0.75
ETM 5	8.65^{c} ± 1.24	17.66^{b} ± 1.35
NC – Distilled water	5.37^{c} ± 0.75	4.81^{c} ± 0.40
DMSO Control	5.27^{c} ± 3.04	6.53^{de} ± 1.03
PC – Albendazol sulfoxide	100^{a} ± 0	NT
PC – Levamisole hydrochloride	NT	88.93^{a} ± 4.29

ETM: Hydroalcoholic extract of buckwheat at 10%; NC: negative control; DMSO control: distilled water with 0.75% DMSO; PC: positive control; NT: not tested; *Means followed by the same letter in the column do not differ significantly by Tukey test at 5% of probability.

Table 2. Mean values for the total polyphenols, condensed tannins and flavonoids for different concentrations in mg mL^{-1} of hydroalcoholic buckwheat extract at 10% (ETM).

Concentration ETM (mg mL^{-1})	Polyphenols (mg equiv. gallic acid)*	Tannins (mg equiv. tannic acid)*	Flavonoids (mg equiv. rutin)*
0.25	19.18^{c} ± 4.70	**	0.57^{d} ± 0.00
0.5	38.31^{b} ± 1.54	17.23^{e} ± 3.56	13.62^{c} ± 4.36
1	53.00^{a} ± 1.33	70.44^{d} ± 7.05	19.19^{bc} ± 2.18
1.5	57.04^{a} ± 1.07	126.15^{c} ± 12.79	22.95^{b} ± 0.95
2	50.99^{a} ± 3.13	176.15^{b} ± 13.12	25.07^{ab} ± 0.71
3	54.33^{a} ± 0.68	288.89^{a} ± 10.21	31.00^{a} ± 1.72
CV (%)	30.18	71.37	54.16

*Per gram of extract. Means followed by the same letter in the column do not differ significantly by Tukey test at 5% of probability. **Value out of the standard curve. CV: coefficient of variation.

Table 3. Mean values for the antioxidant activity of ETM by the methods of the donor capacity of H+ to the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reduced iron (FRAP test).

Concentration ETM (mg mL^{-1})	Antioxidant activity (AA%)*	Antioxidant activity (µM equiv. Trolox p/ g of ETM - FRAP)*
1.5	21.59^{c} ± 0.59	452.14^{a} ± 41.28
2	27.22^{b} ± 0.05	467.05^{a} ± 17.07
3	38.71^{a} ± 1.06	468.12^{a} ± 31.70
CV (%)	26.00	6.15

*Means followed by the same letter in the column do not differ significantly by Tukey test at 5% of probability. CV: coefficient of variation.

Alonso-Diaz et al. (2008) found that the four tanniferous plant extract (*Acacia pennatula, Lysiloma latisiliquum, Piscidia piscipula* and *Leucaena leucocephala*) in the concentrations 1.2 mg mL^{-1} inhibited 49.1 to 63.8% migration of larvae L_3 of *H. contortus*. All four plant extracts interfered with the process of L3 exsheathment, which might be involved as a mechanism of action of tannins on *H. contortus* larvae. Yoshihara et al. (2014) required 100 mg mL^{-1} of a commercial extract of *Acacia mearnsii* for 97.1% inhibition of larval migration. In addition to the anthelmintic action, the extract caused ultrastructural changes in adult parasites *H. contortus* after contact *in vitro* and *in vivo* (Yoshihara et al., 2015). For flavonoids and tannins, the highest levels were found in the concentration of 3 mg mL^{-1}. For polyphenols, concentrations with higher levels were 1; 1.5; 2 and 3 mg mL^{-1}, which did not differ (Table 2).

ETM showed antioxidant activity (AA%) at all tested concentrations, reaching 38.71% at the concentration of 3 mg mL^{-1} (Table 3). The $IC_{50\%}$ corresponds to 3.83 mg

mL^{-1}. For iron reduction test (FRAP), ETM reached 468.12 µM Trolox equivalent per gram of extract at the concentration of 3 mg mL^{-1}.

According to Sun and Ho (2005), buckwheat presents an effective antioxidant activity as compared to the natural antioxidants, and natural antioxidants may have the potential to prevent lipid oxidation of food. Karamac (2010) found that the antioxidant activity of tannins fraction of phenolic compounds from buckwheat showed $IC_{50\%}$ corresponding to 0.019 mg mL^{-1}.

The author concluded that comparing the antioxidant activity of tannin fractions from buckwheat with the literature data concerning the antioxidant activity of fractions isolated from other plants, leads to the conclusion that buckwheat fractions are strong antioxidants. The highest values found by Karamac (2010) for the antioxidant activity of buckwheat in relation to this study were due to the different way of extraction. The author obtained the antioxidant activity of isolated fractions of tannins (from seeds and groats), whereas the present study showed the hydroalcoholic extract of ground seeds.

The primary antioxidants in the buckwheat are rutin, quercetin and hyperin, and the bran and husk have from 2 to 7 times more antioxidant activity than grasses such as oats, tricicale and barley (Morishita et al., 2007; Holasova et al., 2002; Zdunczyk et al., 2006). The antioxidant activity of methanol extracts to 80% in different cultures presented the following order, considering the highest to lowest: buckwheat, barley, oats, wheat, rye (Zielinski and Kozlowska, 2000). Considering the antioxidants in buckwheat, there may be benefits in using this crop in animal feed due to the fact that it reduces the production of free radicals and can help prevent lipid oxidation, which increases the shelf life of the meat (Lima Júnior et al., 2013).

Inglett et al. (2010 and 2011) evaluated antioxidant activity in buckwheat extracts with water, hydroalcoholic solution 50% ethanol and 100% ethanol, using microwave irradiation or water bath for 15 min at different temperatures (23 to 150°C). Regardless of the heat source, higher antioxidant activities was found in 100% ethanol extract at 100 and 150°C: 5.61 and 5.73 µmol Trolox equivalent per gram of extract, respectively.

Rutin is a useful bioflavonoid in the treatment of various medical conditions, mainly due to its antioxidant action (Karamac, 2010). Flavonoids may have antimicrobial activity due to the disruption and destruction of microbial membranes by forming complexes of the bacterial wall with soluble proteins (Soldera et al., 2010). This represents additional advantage considering the possibility of the occurrence of secondary bacterial infections to gastrointestinal parasites. Likewise, the antioxidant activity of flavonoids is important due to the control of the production of free radicals (Sousa et al., 2007). The highest concentration of rutin in buckwheat is found in the leaves and flowers (Vojtíšková et al., 2012).

In addition to direct anthelmintic action that ETM presents, buckwheat in animal feed can still provide indirect action due to immune stimuli that protein diet provide favoring combating of worms. Protein supplementation determines reduction of eggs per gram of faeces values (Veloso et al., 2004; Igarashi et al., 2013).

At 51 days after sowing, Klein et al. (2010) found crude protein contents (CP) in a late and early cultivar of buckwheat to be, respectively 17.27% and 15%. These values are superior to commonly used fodder grasses. Italiano and Neto (2006) found 11.53% of CP in Andropogon, 10.58% in Tanzania and 9.15% in Tifton-85. Gorgen (2013) found in a buckwheat cultivar, crude protein levels at 47 and 57 days respectively, with 23.8 and 14.7%. Alencastro (2014) found in the cultivars tested at 50 and 70 days, 12.73 and 11.13% CP, respectively. The lower level is due to the fact that the advance of maturity of buckwheat causes reduction in crude protein, and the highest levels are observed in the vegetative stage, reducing after flowering. The result of the analysis of buckwheat forage is promising for animal feed, mainly for dairy cattle in the fall season with problem of lack of forage (Klein et al., 2010).

Additionally, buckwheat has a high content of lysine, an essential amino acid deficient in most cereals, demonstrating potential in animal feed (Joshi and Padora, 1991; Kunachowicz et al., 1996). Buckwheat can also act as functional forage manipulating ruminal fermentation and providing reduction in methane formation by 12% (Amelchanka et al., 2010; Leiber et al., 2012).

Conclusion

For the conditions evaluated in this study, it is concluded that anthelmintic and antioxidant activity, demonstrate the potential of ETM in parasitological control of sheep.

Conflict of Interests

The authors have not declared any conflict of interests.

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