

Effect of *in vitro* aqueous extract of fresh cassava leaves (*Manihot esculenta*, Crantz) on the larvae of *Haemonchus contortus* as intestinal parasite of sheep

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Research Article

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Abstract

Animals feeding with cassava is frequent. In the case of sheep, the producer relates consumption to a reduction in the parasite load. The literature has proven the effect of phenolic compounds as an anthelmintic *in vivo*, but no evidence for cyanogenic compounds, also present in all parts of the cassava plant, was found. A controlled *in vitro* bioassay was used to evaluate the aqueous extract of fresh cassava leaves. The efficiency parameter was the imobility of *Haemonchus contortus* larvae at stage L3, also used to evaluate commercial anthelmintics. Culture plates with 100 active L3 larvae per well were used, constituted a replicate of a total of three. It was placed in these wells, water extract of fresh cassava leaves (**FCL**) macerated in water, Ivomec® 0.01% (PCI) as positive control and distilled water as negative one (**NCW**). By considering the immobility of the larvae as a positive anthelmintic effect, the results showed that in NCW all larvae were mobile, while in PCI all 300 larvae were immobile. **FCL** produced a gradient of larval inactivation correlation (r^2 0.996). The best fit equation was $y = -33.39\ln(x) + 40.517$, a logarithmic one, which allowed to calculate the Lethal Concentration (CL) of 3.44 µg.CN-.ml, or 80.0 mg of fresh cassava leaves per milliliter, with performance equivalent to ivermectin. This concentration of free cyanide is compatible with safe consumption of fresh leaves by live weight of sheep. The exact amount of cassava roots, leaves or shoots consumed to provide an effective dose for controlling *Haemonchus contort* should be established *in vivo*. Although phenolic compounds must also be present in the extract, immobility was attributed to cyanogenic compounds since the correlation was proportional to the increase in cyanide concentration. It can be concluded that the consumption of fresh cassava leaves has potential as an anthelmintic agent to be evaluated *in vivo* by feeding sheep and goats. Local use could also add value to the production of fresh cassava leaves, with an average potential production of 2.5 t/ha, available throughout the year, with greater production at the beginning of cultivation and in the summer months. These leaves could be used after the roots harvesting or even in pruning for this purpose. Currently, this volume of good quality protein material is in the field, unused.

Introduction

Parasites are a limiting factor in sheep and goats sheep farming. As Kamaraj & Rahuman (2010), the research of plants with anthelmintic potential has been carried out for years, as alternative controls of parasite populations, especially the gastrointestinal ones, due to the resistance to traditional commercial compounds.

Haemonchus contortus, a hematophagous parasite presents in the abomasum of sheep stands out among the main parasites present in sheep farming, which has been developing parasitic resistance to commercial compounds. This parasite is a potent hematophagous, feeding up to 0.05 ml of blood a day, causing lesions in the inner wall of the abomasum, in addition to anemia, diarrhea, weight loss, edema, supine position, severe weakness, making it a big problem in the production of these animals, as it impacts their performance and can lead to their death (Brik, et al. 2019).

Plant control of sheep parasites has already been considered by several researchers and sheep breeders. Some examples of these plants are *Senegalia gaumeri* (Castanheda-Ramirez, et al. 2017), *Artemisia annua* (Squires, 2010), *Melastoma malabatricum* (Sutsky and Ji, 2019), *Musa paradisiaca* (Marie-Magdeleine et al. 2014); *Leucaena leucocephala* and *Cajanus cajan* (Minatchy et al. 2020). Each plant is rich in compounds known for their anthelmintic potential, such as Condensed Tannins, Total Phenols and Total Tannins (Castañeda-Ramírez, et al. 2017).

Cassava (*Manihot esculenta*) has also been considered by several authors as an anthelmintic (Sokerya, 2009; Marie-Magdeleine et al, 2010; Al-Rofaai et al (2010); Minatchy et al 2020). Some studies are carried out directly on parasitized animals by inclusion the plant in their feed (Sokerya, 2009; Minatchy et al ,2020) and some others are carried out *in vitro*, observing its effects on eggs, larvae and adults (Marie-Magdeleine et al, 2010; Kamaraj & Rahuman, 2010). These studies have been promising in relation to the anthelmintic effect of cassava and researchers usually attribute this result to the presence of tannins in the plant. However, researchers did not deepen the research on the effect of cyanogenic glycosides such as linamarin and lotaustarin, relevant in the cassava plant (Ravindran, 1995).

When consumed by animals, cyanogenic glycosides are processed through enzymes present in the plant itself and in the cellulosic microorganisms of the rumen and the cyanide is then released into the body, which may or may not trigger an intoxication (Gensa, 2019). The literature proves the tolerance to 2.0 to 6.0 mg HCN.kg⁻¹ of fresh leaves per live weight for ruminants, which is explained by the presence of sulfur-based compounds in their feed, which in contact with HCN forms the thiocyanate which, released in the urine, promotes detoxification (Onwuka et al, 1992).

Sokerya, (2009) obtained promising results regarding the use of fresh leaves and cassava silage with a reduction of eggs present in the animals' feces. In his experiment, the author highlighted the presence of HCN, within the limit for animal consumption, but he does not attribute to cyanide the anthelmintic effect, because the target was the phenolic compounds, and only highlighted the reduction from 585 mg to 170 mg of HCN by the ensiling process.

Due to information gap about the effect water extracted cyanogenic compounds, a protocol *in vitro* was tested to verify the effect of fresh cassava leaves (*Manihot esculenta*) extract on the mobility of L3 larvae of *Haemonchus contortus*, as well as the amount of cyanide needed to reach the Lethal Dose in these circumstances.

Material And Methods.

Obtaining cassava leaves extract: cassava leaves were collected from cassava *Paraguainha*, a traditional and commercial CV, widely used in the Midwest Region of Brazil. The leaves were washed, dried with paper towels and weighed. Then, the leaves were immediately macerated with distilled water, obtaining an extract with a concentration of 80 mg of leaves for each ml.

Cyanide analysis in the cassava leaf extract: the quantification of free cyanide in the extract used the spectrophotometer method described by Brito et. al. (2013).

Obtaining monospecific cultures of *Haemonchus contortus*: the research was carried out with the authorization of the Ethics and Use of Animals Committee of the institution, under protocol n° 013/2012, as follows:

As the technique described by Gordon and Whitlock (1939) a donor (**A**) of *Haemonchus contortus* was select. It was an adult, a one-year-old lamb, naturally infected by nematodes of the Trichostrongylidae family with a count of 23 thousand eggs per gram of feces (EPG). The donor (**A**) was euthanized and immediately its abomasum was extracted, and its internal content was poured into vats and with the aid of spatulas, the adult *H. Contortus* was collected and kept in phosphate buffered saline (PBS) solution and kept at a temperature of 37°C (Witola et al, 2016). To obtain *Haemonchus contortus* eggs, two six-month-old lambs were donors (**B**). Previously both animals were subjected to a parasite purging process, and the absence of gastrointestinal parasites confirmed by coprological examinations exams. The adult helminths collected from the donor lamb (**A**) were introduced into the abomasum of the donor lambs (**B**), according to the surgical technique described by Paiva et al (1999). To confirm the presence of *H. contortus* eggs several coprological examinations were performed from fecal samples collected from the animals 25 days after the procedure. During the entire experimental period, the animals were housed individually in pens, clean of environmental contamination. To avoid external contamination, the pens were cleaned and sanitized daily with detergents and sodium hypochlorite. To obtain the third stage larvae (L3) for the tests, the feces were collected in bags made of cotton fabric specially developed for their collection. Following the guidance of Tissier et al (1975), these collecting bags were fixed for 12 hours directly in the animal's anal region. The material obtained was used to carry out the coprological examinations, following the methodology described by Roberts and O'Sullivan (1950), where feces and vermiculite were mixed in equal volume and placed in cylindrical glass containers with a capacity of 500ml, but only half of the container was used. With the aid of a glass rod, a hole was made in the center of the material in order to provide oxygenation. The container remained partially covered, and a piece of thick string was used over its diameter to ensure that the container to ensure that the opening remained ajar. To keep the culture moist, once a day, distilled water was sprinkled over the material. The containers were labeled with the date and incubated in B.O.D at a temperature of 28°C for 7 days. After the incubation period, the L3 were recovered using the Baermann Technique (Witola et al, 2016).

Sheathing and larval motility test of third stage larvae: L3 stage larvae were sheathed in 0.15% sodium hypochlorite solution at 37 °C for 5 min (Almeida, 2018). After the larvae were washed five times in sucrose solution (12%) in the centrifugation process to remove the remains of the sheaths and possible dead larvae (Paiva et al, 2001). The number of larvae by ml was estimated by diluting L3 in distilled water using the counting of larvae under an optical microscope. To perform the *in vitro* bioassay, the larval motility test described by Hubert and Kerboeuf (1984) and Preston et al (2015) was used, with adaptations. The unsheathed L3 larvae were added to culture plates of 12 wells (100 L3 per well), following by the serial concentrations of 5.2 to 80 mg of cassava leaves.ml⁻¹, each well was adjusted

with distilled water for a total volume of 2 ml in each. A 0.01% in distilled water of Merial Ivomec® was used as a positive control (**FCL**) and distilled water as a negative control (**NCW**). Subsequently, the plates were homogenized for 3 minutes and incubated at 28°C in a bacterial incubator for 24 hours. After this period, with the aid of a stereomicroscope, the mobile (**viable**) and immobile (**inviable**) larvae were counted. All bioassays were performed in triplicate. The motility index was calculated for each group of different concentrations of positive and negative control, applying the **Equation I**:

$$\text{Motility \%} = \frac{(\text{n}^{\circ} \text{immob. L3})}{\text{Total n}^{\circ} \text{ of L3 in the treatment}} \times 100 \quad \text{Equation I}$$

Where:

Unviable as considered the immobile L3 larvae

Viable as considered the mobile L3 larvae

Statistical analysis: the lethal concentration (LC) of fresh leaves extract from *Manihot esculenta*, able to paralyze all larvae, was estimated from the results, where the concentrations were transformed into Log10. Data from the larval motility index were compared using ANOVA and Tukey test, where the significance level adopted was 0.05%. All analyzes were performed using the BioEstat® program.

Results And Discussion

Sheep farmers believe from their experience and observation that there is a reduction in the load of intestinal parasites eggs, including *Haemonchus contortus* in animals feed with cassava. The researchers decided to investigate the hypothesis that this reduction is due to the presence of free cyanide in fresh cassava leaf extract that was established at the same time as sampling was done for the mobility test of L3 larvae of *H. contortus*.

Table 1 presents the results of the controlled bioassay, in which 100 isolated L3 stage larvae (in triplicate) were subjected to variations in the concentration of extract of fresh cassava leaves in water. The aqueous extract of fresh cassava leaves, as elaborated, showed a concentration range of 0.22 to 3.44 µg of CN⁻ per ml.

Table 1 – Effect of fresh cassava extract and cyanide concentration on the motility of a hundred L3 larvae of *H. contortus* (Average of 3 repetitions).

Mass concentration of fresh cassava leaves in the water extract	CN ⁻ Concentration	Number of mobile larvae per repetition			Mobility reduction
		mg x ml ⁻¹	µg x ml ⁻¹	R1	R2
5,2	0,22	95	92	94	93,67
10,0	0,43	70	65	60	65,00
20,0	0,86	40	50	48	46,00 c
40,0	1,72	20	25	22	22,34 d
80,0	3,44	0	0	0	0,00 e
Ivomec® (FCL)	0,01%	04	03	0	2,34 e
Distilled water NCW)	0,00	98	99	96	97,66 24a

(*): Equal letters in the columns did not differ at the 0.5% level

The results confirmed the toxic effect over the sheathed larvae of *H. contortus*, an effect proportional to the concentration of extract of fresh cassava leaves. From the data presented in **Table 1**, a curve was fitted relating the mobility of L3 larvae with the concentration of the cassava leaves extract. The curve that best fit the results allowed was the logarithmic equation $y = -33.39\ln(x) + 40.517$, with $R^2=0.996$ (**Figure 1**), with a positive and significative correlation on the effect of increased immobility of larvae and the increasing free cyanide concentration in the cassava leaves extract. The equation highlights that the greatest effect was caused between 0.0 and 0.5 µg of CN x ml⁻¹, when about 50% of L3 larvae lost mobility. With the increase in free cyanide content from this concentration, the curve becomes less accentuated, but it is not asymptotic before affecting the total of larvae, equating to the control of the commercial product based on ivermectin, which had an immediate impact on the mobility of the larvae.

The equation allowed us to calculate the dose that may cause total paralysis a hundred L3 larvae (DL₁₀₀) as occurred with approximately 3.5 µg of CN ml⁻¹, corresponding to 80 mg of cassava leaves.ml⁻¹ or 80g.l⁻¹. This result agrees with the results from Sokerya, (2009) that the consumption of cassava leaves can reduce the number of intestinal parasites in sheep but may contradict the author's conclusion that a dose of 170 mg of HCN per kg can be innocuous as anthelmintic. Onwuka et al, (1992) and Sokerya, (2009) reported the need to use a 246 to 248 mg100 g of cyanide to achieve similar effects.

It is noteworthy that cassava leaves may vary in cyanide content depending on the cultivar or variety, age and time of year (Ravindran, 1995). The results obtained in this experiment partially agree with those of Suteky and Ji (2019) with 47.87% of the larvae immobilized with 12.5 mg by ml of cassava leaves extract, but this difference can be attributed to the fact that the authors were based on larval development and not on larval motility.

Al-Rofaai et al (2012) reported an efficiency of 57.33% on L3 larvae by an extract of 12.5 mg of cassava leaves.ml⁻¹, which would be near to 22mg to achieve total larvae immobility. Although even less than the 80mg we founded to immobilize all of the L3 larvae, it is necessary to consider that the experiment did not eliminate the effect of the solvents used in the preparation of the cassava leaf extract. The author used solvents to evaluate, in addition to tannins, alkaloids, flavonoids, steroids and phenols. The solvents used were hexane, chloroform, ethyl acetate and methanol (80%), precisely the one that presented the best effect against the larvae, so it impossible to separate the cyanide effect from the effect of the solvents used.

The same use of solvents was founded in several other studies carried out to evaluate the anthelmintic action of cassava extract. Marie- Magdeleine et al (2010), before processing the extract, removed the cyanogen glycosides and then dehydrated the extract. The resulting powder was then diluted with three different extraction solvents, dichloromethane, methane and water, because the author focusses the action of tannins on the parasites.

In our experiment, we used only water, because the objective was to evaluate cassava leaves in its natural way, with minimal chemical interventions, so that we could have the closest scenario to the process of ingesting the plant leaves in the animal's rumen, guaranteeing the presence of all its compounds even those that are not soluble in water.

In several studies carried out to evaluate the anthelmintic action of cassava leaves extract, the authors use several solvents for the extraction, having in mind to study the effect of phenolic compounds. Al-Rofaai et al (2012) evaluated tannins and phenols, substances known for their antiparasitic effects, not only against *H. contortus*, but also on other nematode species. With the same purpose, alkaloids, flavonoids, steroids and phenols were also evaluated (Sokerya 2009; Marie-Magdeleine et al, 2010; Al-Rofaai et al, 2012; Suteky and Ji, 2019; Constant, 2020). Marie-Magdeleine et al (2010), for evaluating the effect of tannins on the parasites, took care to remove the cyanogen glycosides, and the resulting powder from the process was diluted with dichloromethane, methane and water.

Another experiment carried out in Malaysia by Sokerya (2009) explores the potential of cyanide present in cassava leaves, although focusing on goats and not sheep. The author evaluated the anthelmintic effects of cassava feed in animals contaminated with *Haemonchus contortus*, but used the cassava plant in different ways such as fresh, ensiled and dehydrated leaves. The count of eggs present in the feces of these animals was done after a period of time after ingestion and showed positive results in all groups in which the animals were fed with cassava leaves, but mainly in the consumption of fresh leaves. The authors justifies that they did not carry out the detoxification of cassava by drying, as carried out by Marie-Magdeleine et al (2010), precisely because the objective was to relate the forms of feeding the animals with cassava and the respective responses on the parasite load. However, the authors emphasizes that the risk of poisoning the animal by cyanide should not be neglected, which is why they measured the amount of HCN present in the plants used, which presented 585mg of CN⁻ per kg in fresh leaves and 170mg of CN⁻ per kg in silage made with the same leaves. The author also highlighted that

the values found fall within the tolerance limits for ruminants, which is 2 to 6 mg/kg of the animal's live weight, according to Onwuka et al, (1992), reducing the risk of intoxication in these animals.

Although our bioassay makes it quite clear that the cyanide present in cassava leaves, and not only its phenolic compounds, has a direct action against *H. contortus* larvae, these results should be evaluated and adjusted by feed the sheep with fresh leaves.

The comparison of results with the literature highlighted the lack of literature that presents results of anthelmintic potential of cassava leaves evaluated by aqueous extract or that highlight the cyanogenic compounds of cassava, although the literature that points out the risks of cyanide consumption for human and animal health is very abundant.

The review also highlighted that the focus of the anthelmintic use of cassava leaves for feed sheep and goats has always been hypothesized by the effect of the phenolic compounds present, such as tannins, as they are reported in the literature for their antiparasitic effects, not just in *H. contortus*, as well as in other nematode species. Also in this aspect, it would be important to compare, in the same *in vitro* assay, the effect of cyanogenic and phenolic compounds, with and without the use of solvents.

On the other hand, the research started from the principle that, if the anthelmintic effect is proven, the use of leaves is easier and more feasible, since it is not necessary to use the roots, which have commercial value, or the branches, which are used as planting material. Leaves can be obtained without interruption during all year or all the summer in some south regions of South America, using pruning, as the literature has confirmed that up to 3 pruning's over 12 months of cultivation does not affect root yields.

If cassava leaves contain compounds from the phenolic group, such as tannins and saponins, in addition to cyanogenic glycosides (Tao et al, 2019), it also has a high nutritional value (Pereira et al, 2018). The use of cassava leaves by feeding of sheep or goats is possible (Vilpoux et al. 2013) and may also be a powerful tool and can diversifying its antiparasitic power and avoid the occurrence of resistance to the present anthelmintic principles.

This type of local use could also add value to the production of cassava leaves, as assessed by Sagrilo et al. (2001) in weight of leaves harvested in commercial cassava plantations for starch extraction, with 12 and 24 months of cultivation with the 5 main cultivars planted. The authors found an average production of fresh leaves 2.4 ton. ha⁻¹, ranging from 1.8 to 7.5 ton. ha⁻¹, available throughout the year, with higher production in the beginning of cultivation and in the summer months in Brazil (September to May). Currently this volume of good quality protein material is in the field, without use.

Conclusions

Considering that it is impossible to rule out the antiparasitic effect of phenolics and even though the investigation of the effect of cyanogen compounds from cassava leaves was never approached in depth, the bioassay was planned with a different concept and perspective in the control of endoparasites, as an

aqueous extract and targeting *H. contortus* larvae mobility as an index of parasitosis and as a target for the evaluation of anthelmintic substances.

With this experimental design, it was possible to conclude that the aqueous extract of fresh cassava leaves was able to immediately inhibit the movement of a hundred L3 larvae (DL100) of *H. contortus* as occurred with near 3.5 µg of CN⁻.ml⁻¹, corresponding to 80 mg of leaves by ml⁻¹ or 80g by liter.

The results obtained are promising but should be complemented with direct feeding tests with fresh, hay and dry leaves, accompanied by dosing the free cyanide, which is generated by the rumen digestive process and circulates with the blood to reach the intestinal parasites, but also to study the effect on hematophagous ectoparasites such as ticks. Tests should be also carried out with a longer duration to verify the possibility of resistance to cassava compounds occurring over time. Only after these tests will rural producers recommend the use of cassava leaves as a natural anthelmintic medicine.

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Declarations

CONFLICT OF INTEREST DECLARATION I, Ana Flávia Lopes Medeiros, author responsible for submitting the manuscript entitled (Effect of in vitro aqueous extract of fresh cassava leaves (*Manihot esculenta*, Crantz) on the larvae of *Haemonchus contortus* as intestinal parasite of sheep.) and all co-authors who here are presented, we declare that WE DO NOT OWN, CONFLICT OF INTEREST of order: (x) folks, (x) commercial, (x) academic, (x) political (x) financial in the manuscript.

Figures

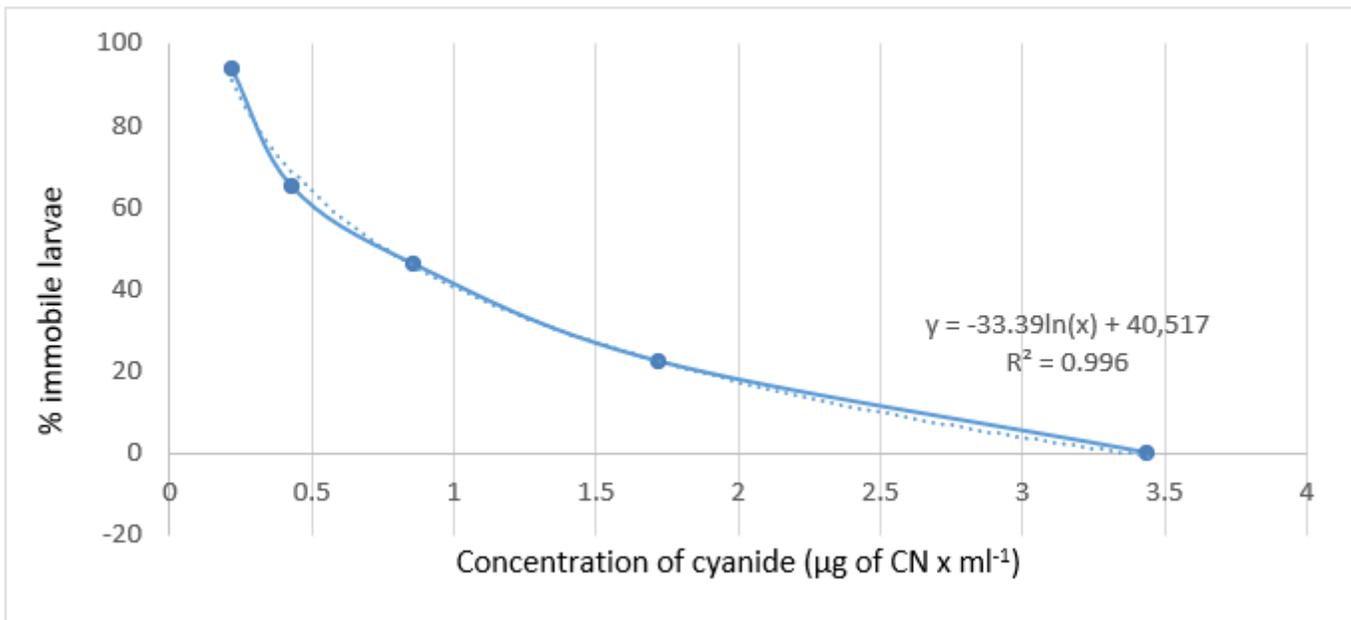


Figure 1

Effect of concentration of CN^- in cassava (*Manihot esculenta*) fresh cassava leaves extract leaves ($\text{mg} \cdot \text{ml}^{-1}$) and the reduced motility of L3 larvae of *H. contortus* provided (average of 3 repetitions). Discontinuous line: obtained values; Continuous line: fitted equation