# In vitro effects of Musa x paradisiaca extracts on four developmental stages of Haemonchus contortus

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## ARTICLE INFO

## ABSTRACT

This study was carried out to evaluate the in vitro effect of Musa x paradisiaca stem and leaf against the parasitic nematode of small ruminants Haemonchus contortus. Three extracts (aqueous, methanolic and/ or dichloromethane) of Musa x paradisiaca stem and leaf were tested in vitro on four developmental stages of H. contortus using egg hatch assay (EHA), larval development assay (LDA), L3 migration inhibition assay (LMI) and adult worm motility assay (AWM). The highly significant ($P < 0.0001$) ability to stop larval development (inhibition >67% for each extract) and the negative effect of the dichloromethane extract of leaf on adult worm motility (43% of inhibition of motility after 24 h of incubation) compared to the negative controls, suggest anthelmintic properties of Musa x paradisiaca stem and leaf against H. contortus. The active principles responsible for the activity could be secondary metabolites such as terpenoid and flavonoid compounds present in the leaf and stem of the plant.

## 1. Introduction

Gastrointestinal nematodes (GIN), especially *Haemonchus contortus*, had been classified as a major health and welfare problem for small ruminants particularly in the tropics (Aumont et al., 1997; Chandrawathani et al., 1999; Geerts and Dorny, 1996; Hoste et al., 2005; Jabbar et al., 2006; Wolstenholme et al., 2004). Such parasites impair animal health and welfare and productivity since gastrointestinal nematodes infection results in increased death rate and poor growth and reproduction (Aumont et al., 1997; Coop et al., 1982; Dakkak, 1995; Hoste et al., 2005). To date, the usual mode of control of gastrointestinal parasitism relies on the repeated use of chemical anthelmintic drugs. However, the worldwide emergence of drug resistance (Aumont et al., 1997; Jabbar et al., 2006; Jackson and Coop, 2000; Wolstenholme et al., 2004) in GIN populations motivates investigation into alternative approaches. Phytotherapy is one of these alternative approaches currently explored (Athanasiadou and Kyriazakis, 2004; Githiori et al., 2006; Wolstenholme et al., 2004). Phytotherapy is mainly based on the use of preparations of leaves and seeds and the effects are attributed to plant secondary metabolites. *Musa x paradisiaca* is a plant of medicinal interest in human medicine in the Caribbean (TRAMIL, 1999; Longuefosse, 2003): the leaf and stem are used

to treat diarrhoea; the stem is good for asthenia and wounds, and the leaf for the treatment of inflammation, headache and rheumatism. Previous studies (TRAMIL, 1999) reported that *M. Paradisiaca* had antimicrobial and healing activities. Nevertheless, only a few studies have reported on the efficacy of this plant against nematodes. Sharma et al. (1971) tested aqueous extracts of *M. Paradisiaca* root on adult *H. contortus* and found a significant activity of 5% at the concentration of 1 in 25 of a 100 g/L root extract. Batatinha et al. (2005) tested the in vitro effect of aqueous extracts of leaves of *Musa cavendishii* Linn. on *Strongyloidea* larvae of goats and found a reduction of development of more than 95% at the concentration of 130.6 mg/ml. *Musa acuminata* was tested in vivo on goats experimentally infected with *H. contortus* (Vieira et al., 1999) and it did not significantly reduce the number of adult worms.

The possible secondary metabolites with an anthelmintic activity of *M. paradisiaca* are yet to be identified. As tannin compounds are a part of the metabolites of this plant (TRAMIL, 1999), they should be considered as potential anthelmintics. Nevertheless, other compounds might not be excluded. Secondary metabolites may be extracted from the raw plant material by different solvents (water, dichloromethane, and methanol according to the polarity of the molecules to be extracted (Balansard et al., 1991). For example hot water will extract heterosides, iridoids and tannins; methanol will extract tannins, catechins, terpenoids and alkaloids, and a more apolar solvent such as dichloromethane will extract semi polar and more apolar compounds (Balansard et al., 1991).

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Several methods are commonly used for testing nematicidal activity of both chemical drugs and plant extracts. Amongst them, *in vitro* assays revealed to be relevant and cheaper than *in vivo* methods. The Egg Hatch Assay (EHA), (Hubert and Kerboeuf, 1984) and the larval development assay (LDA), (Hubert & Kerboeuf, 1992) are currently used for the detection of anthelmintic resistance in GIN (Coles et al., 1992), using positive control as albendazole which has a specific activity on both development stages. Albendazole causes degenerative alterations in the tegument and intestinal cells of the worm by binding to the colchicine–sensitive site of tubulin, thus inhibiting its polymerisation or assembly into microtubules. The larval migration inhibition assay (LMI), (Rabel et al., 1994; Wagland et al., 1992) and the adult worm motility assay (AWM), (Hounzangbe-Adote et al., 2005) allow a more realistic evaluation of the *in vivo* nematicidal activity. For these two assays the positive control used is the levamisole which activity targets the adult and L3 larvae because of its agonistic activity on the nicotinic acetylcholine receptors in nematode muscles. These four tests are based on the hypothesis that a nematicidal activity observed *in vitro* would be indicative for a potential *in vivo* activity.

The current study aimed to explore the anthelmintic properties of the banana tree against the gastrointestinal parasite *H. contortus,* and to enquire on the phytochemical compounds involved in the activity. Several *in vitro* tests were performed with several plant extracts, to increase the chances of detecting an anthelmintic activity.

## 2. Materials and methods

### 2.1. Plant extracts

Plant materials were collected in Guadeloupe (French West Indies). The *M. paradisiaca* leaves and stem were first lyophilised and then ground. Three different extractions were performed on leaf either with water, methanol ($CH_3OH$) or dichloromethane ($CH_2Cl_2$), in order to extract most of the polar and semi-polar molecules depending on the solvent. Two different extractions with water and methanol were performed on stem.

First, 50 g of ground leaf or stem were extracted with 1 L of hot distilled water: 500 ml of boiling water mixed with the powder, simmered and filtered through a Büchner, and then 500 ml of boiling water mixed with the plant organ residue and simmered again. The filtrates were collected together and lyophilised to obtain a powdered extract.

Then, 50 g of each ground organ were lixiviated by 500 ml of solvent (methanol or dichloromethane): the ground plant organ was moistened with 150 ml of solvent to cover it, and then let to maceration for 3 h sheltered from light. Thereafter, the filtrate was removed while the rest of the solvent (350 ml) was poured on the residue. The filtrates were collected together and evaporated at low pressure at 40 °C.

All the extracts (aqueous, methanolic and dichloromethane) were stored, in the dark in desiccators, at 22 °C.

### 2.2. In vitro anthelmintic assays

The anthelmintic efficacy tests of the different extracts of each organ of *M. paradisiaca* on the different life-cycle stages of *H. contortus*, were performed using four different procedures.

For each assay, the four parasitic stages were obtained from faeces and abomasums of Black Belly donor lambs which were experimentally infected by an oral administration of a pure 10 000 *H. contortus* third stage larvae (L3) suspension.

### 2.2.1. Egg hatch assay

The *in vitro* anthelmintic activity of the three extracts of the two plant organs on the egg hatching of *H. contortus* was carried out according to a modification of the method used for testing anthelmintic resistance (Assis et al., 2003). Eggs were extracted from the faeces of the donor sheep according to the method described by Hubert and Kerboeuf (1984).

After crushing the faeces in water, and successive siftings (500, 250, 125, 63, 50 and 30 μm sieves), eggs were collected and centrifuged for 10 min at 2000 rpm. The supernatant was removed and a NaCl solution (density 1.2) was added. After homogenisation, the mixture was centrifuged for 15 min at 3000 rpm. The ring with eggs was then extracted by pouring it on a 32 μm sieve and then by abundant washing first with distilled, and then with sterilised water. The egg suspension with a concentration of 400 eggs per ml, was distributed in 24-well plates (0.5 ml per well). Concentrations of plant extracts (2400, 1200, 600 and 300 μg/ml), and albendazole (5, 2.5 and 1.25 mg/ml, used as positive control) were diluted in PBS (0.1 M phosphate, 0.05 M NaCl; pH 7.2) buffer and were added to the wells (0.5 ml per well). In addition, PBS negative controls were also included in the assay. Five replicates were run per concentration.

After 48 h incubation at 25 °C, egg hatching was stopped by adding Lugol's iodine solution. The number of L1 larvae and egg per well was then counted using a reverse microscope (at 100× magnification). The percentage of hatched eggs was determined using the ratio: number of L1/(number of egg + number of L1).

### 2.2.2. Larval development assay

The objective of this assay was to test the *in vitro* efficacy of the extracts to inhibit the larval development of *H. contortus* from the L1 stage to the infective L3 stage. The assay used was a modification (Assis et al., 2003) of the technique described by Hubert and Kerboeuf (1992).

L1–L2 stages larvae were obtained after culture of eggs extracted from the faeces of the donor lambs. The eggs were incubated at room temperature for 48 h in 24-multiwell plates (0.5 ml per well with a concentration of 400 eggs/ml). After hatching, 70 μl of culture medium (for one plate: 10 μg of autolytic yeast extract, 1.5 ml of distilled water and 170 μl of Earle's balanced solution, stabilised to pH = 7.2 with a $NaHCO_3$ solution at 50 g/L) was added to each one of the wells, followed by either each concentration of extracts (2400, 1200, 600 and 300 μg/ml), albendazole positive control (5, 2.5 and 1.25 mg/ml), all diluted in PBS buffer, and PBS negative control (0.5 ml per well and five replicates for each dose). All dilutions were made in PBS.

The larvae were incubated for 8 days at room temperature (25 °C), in order to permit the larval development from the first stage to the third infective stage. Thereafter, several drops of a Lugol's iodine solution were added and the number of larvae was counted using inverted microscope (at 40× magnification) by separating L3 infective larvae from L1 to L2 larvae. The percentage of development was calculated as the ratio: number of L3/total number of larvae per well.

### 2.2.3. Larval migration inhibition assay

This test is aimed at evaluating the anthelmintic effect of the extracts on the migration capacity of the infective larvae L3. This test was performed according to Rabel et al. (1994) in Hounzangbe-Adote et al. (2005).

*H. contortus* L3 were obtained by larval culture from the faeces of donor lambs. Eggs reached the L3 stage after 10 days. The L3 were then collected by sedimentation using Baermann's devices. The anthelmintic effect of each extract was tested using 150, 300, 600, 1200 and 2400 μg of extract/ml PBS with 1000 L3 per dose. Negative (in PBS only) and positive controls (levamisole at 1.25, 2.5 and 5 mg/ml) were also prepared in PBS and incorporated in the assay. Five replicates were run for each plant extract and for the controls. After 3 h of incubation at room temperature (25 °C),