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Antioxidant and antiparasitic activities on *Trichostrongylus colubriformis* of the aqueous extract of fresh leaves of *Mitragyna inermis* (Willd.) Kuntze (Rubiaceae)

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Abstract *Trichostrongylus colubriformis* is one of the major parasites that hampers the development of small ruminant breeding in West Africa and particularly in Benin. This parasite is a zoonosis that could infest humans if hygiene measures are not taken in pastures. The aim of the present study is to evaluate *in vitro*, the effect of the aqueous triturated extract of fresh leaves of *Mitragyna inermis* on this parasite. Tests on the motility of adult worms were evaluated at different doses. The antioxidant activity of the extract was also evaluated by two different methods in comparison with vitamin C and acute oral toxicity on Wistars female rats. It appears that the aqueous triturated extract of fresh leaves of *M. inermis* significantly reduced in *vitro the* motility of adult worms of *T. colubriformis* ($p < 0.01$). The extract showed a good capacity to trap the free radical of DPPH (IC_{50} = 6.32 µg/ml) and its capacity to reduce Fe^{3+} and Fe^{2+} ion is remarkable. This could be explained by the high content of tannins, flavonoids… found in the extract. Furthermore, the extract shows a non-toxic effect at a single dose of 2000 mg/kg body weight. These results could explain the use of this plant in traditional medicine in the treatment of parasitic diseases.

Keywords *Mitragyna inermis, Trichostrongylus colubriformis,* antioxidant activity

Introduction

The plant kingdom has a large number of Secondary Metabolites such as polyphenols, flavonoids, condensed tannins which have wide areas of application. These secondary metabolites are much more studied nowadays because of their health benefits or they are used as antioxidants and suspected to be responsible for anthelmintic properties.

Indeed, free radicals, activated oxygen species, oxidative stress and antioxidants have become familiar terms both in the medical world and in the general public [1]. Oxygen, although an essential molecule for life, is potentially toxic

to animal and plant cells. This through the production of free radicals or these reactive derivatives [1-2]. These free radicals in the organism can be supplemented by X-rays or γ, air pollutants (cigarette smoke and industrial contaminants), xenobiotic varieties (toxins, pesticides, herbicides, etc.), excessive alcohol consumption, taking contraceptive pills, immoderate exposure to the sun or to radiation without sufficient protection, the practice of high-level sport [1,3]. All of these are factors that create oxidative damage, which is considered the main cause of many diseases. These include inflammation, cancer, cardiovascular disease or rheumatoid arthritis, atherosclerosis, parasitic diseases (malaria), chronic fatigue syndrome, diabetes, hypertension, kidney disease, early aging, rheumatoid arthritis and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease [1, 3-5]. To defend against oxidative stress, which is a favorable ground for several diseases, medicinal plants with antioxidant properties provide significant support. Thus, in the search for plants with anthelmintic properties, knowledge of antioxidant properties would be an asset in the pharmacological development of traditional medicine.

Indeed, *Haemonchus contortus* and *Trichostronglyus colubriformis* are two major parasitic species of sheep, whose geographical distribution is worldwide, and which are the cause of serious pathologies leading to significant economic losses in breeding [6]. Even if the clinical manifestations of infestation of sheep by *T. colubriformis are* less spectacular than what can be observed with more pathogenic parasites such as *H. contortus*, these two parasites cause parasitic disorders in the health of sheep in Benin and therefore require special attention for the health management of small ruminants. Due to the increasing limits of the effectiveness of synthetic anthelmintics, the use of medicinal plants is the new control method to control the gastrointestinal strongles of small ruminants. In spite of the scientific studies carried out on plants with anthelmintic properties, the knowledge of new anthelmintics derived from medicinal plants is still limited. After evaluating the anthelmintic effect on *H. contortus* of the aqueous triturated extract of *M. inermis* on *H. contortus* [7], the present study proposes a better knowledge of the antioxidant activity and the anthelmintic effect on *T. colubriformis* of the aqueous triturated extract of the fresh leaves of *M. inermis* in order to valorize it.

Material and Method

Quantitative Analyses of Extracts

Quantitation of Total Phenols

Total polyphenols were assayed according to Singleton *et al*. [8], revised method. The standard used is gallic acid. Twenty µl of each of the extracts was taken and dissolved in 100 µl of Folin Ciocalteu reagent. After the incubation for 5 min 80 µl of sodium carbonate (Na_2CO_3) at 75 mg/ml is added. The vortexed mixture is incubated for 2 hours. Absorbance readings were taken with a plate reader at 760 nm.

Quantitation of flavonoid

The total flavonoid content of plant extracts can be estimated by the aluminum trichloride ($AlCl_3$) method. Quercetin is used as a reference compound in order to realize the calibration curve. 100 µl of an $AlCl_3$ solution (2%) was taken and 100 µl of the sample was added. Absorbance readings are taken with a plate reader at 415 nm after a 10 min incubation.

Quantitation of condensed tannins

The method used to determine the tannin content is that of vanillin sulfuric acid. To 50 µl of each sample or standard, 100 µl of the solution of vanillin (4%) in methanol, 1 ml of concentrated hydrochloric acid and 2 ml of methanol are added. The mixture is incubated for 15 min and the absorbance is read at 500 nm. Catechin is used as a standard. This is the method used by Heimler *et al.* [9].

Calculation of the content

The levels of secondary metabolites are determined by the following formula:

$$T = \frac{C * Vr}{Vp * Cp}$$

T = Content of the compounds; C = Concentration obtained from the calibration curve; Vr = Reaction volume; Vp = Volume of extract sampled and Cp = Concentration of the sampled extract solution.

Antioxidant activity of plant extracts

It was determined by two different methods. The different methods are distinguished by the oxidizing silver used, the substrate used, the methodology used and the potential interactions of the sample with the reaction medium [10].

DPPH (2.2-diphenyl-1-picrylhydrazyl) method

Preparation of solutions to be tested

The test solutions were prepared in 10 tubes in a geometric dilution ranging from 1 mg/ml to 0.0019 mg/ml and 200 µg/ml to 0.39 µg/ml for ascorbic acid.

DPPH was prepared at a concentration of 0.04 µg/ml.

Operating mode

1 ml of test extract was mixed with 4 ml of the 0.04 µg/ml DPPH ethanolic solution. After homogenization, the mixture was incubated for 30 minutes in the dark and the absorbance was read at 517 nm using a spectrophotometer against a blank (control containing all reagents except the test extract). Each test was repeated twice. This is the method described by Lamien-Meda *et al*, [11].

The inhibitory concentration IC_{50} is an estimate of antioxidant activity. It represents the dose of the sample that neutralizes 50% of DPPH free radicals. The IC_{50} value is determined by extrapolation across from the concentration-dependent absorbance regression line (C).

The EC_{50} defines as the effective concentration of the substrate that causes 50% loss of DPPH activity, takes into consideration the concentration of DPPH in the reaction medium. It is the expression of the inhibitory concentration IC_{50} per milligram of DPPH. The lower the concentration, the higher antioxidant activity.

The Anti-Radical Power (APR) is inversely proportional to the EC_{50} ($APR = 1/EC_{50}$).

Reducing power by the FRAP method

2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1% aqueous solution) are added to 1 ml of the test solution (extract or ascorbic acid) and the mixture is incubated for 30 min at 50°C in a tepid bath.

After incubation and addition of 2.5 ml trichloroacetic acid (10%), the entire mixture was then centrifuged for 10 min at 3000 rpm. To 2.5 ml of the supernatant 2.5 ml distilled water and 0.5 ml aqueous $FeCl_3$ solution (0.1%) were added.

Absorbances were read at 700 nm against a calibration curve obtained from ascorbic acid (0-200 mg/l). This is the method of Hinneburg *et al.* [12] used by Bakasso, [13].

The reducing power was expressed in ascorbic acid equivalents (ACE) (mmol ascorbic acid/g dry extract) considering 1 mM equals FRAP of 1 ml of the dry extract according to the following formula:

$$C = \frac{c \times D}{M \times Ci}$$

C = concentration of reducing compounds in mol EAA/g dry weight;

c = concentration of the sample read; D = dilution factor of the stock solution;

Ci = concentration of the stock solution; M = molar mass of ascorbic acid (176.1g)

Adult worm motility test

Collection of adult worms

After slaughter of the animal artificially infested with *H. contortus*, the abomasum was removed and the contents poured into physiological fluid previously prepared by dissolving 9 g of NaCl in 1 liter of water (9% NaCl). The worms were then recovered and placed in physiological liquid at 37°C.

Motility inhibition technique for adult worms

The extract solutions to be tested are prepared with PBS in six different concentrations (75, 150, 300, 600, 1200 and 2400 µg/ml). The collected worms with good motility are each placed in 1 ml of physiological fluid in 24-well NUNC plate wells and placed in an oven at 37°C. After one hour, 800 µl of physiological fluid is removed and replaced by the extracts to be tested. A negative control (PBS buffer) and a positive control (levamisole 250 µg/ml in PBS) are also formed. The test is repeated six times for each of the concentrations and for the controls. The inhibition of adult worm motility in the treatments performed is used as the criterion for anthelmintic activity. After the worms are brought into contact with the extracts, motility is observed under a magnifying glass every 6 hours. The observation stops when the immobility of all the worms contained in the PBS is observed. This is the method of Hounzangbé *et al,* [14].

Acute oral toxicity test

The study of the acute toxicity of the three extracts of the plant *M. inermis* was conducted according to the method described in OCDE [15].

Conduct of animals

The animal material consists of six (6) Wistars female rats weighing between 150 g and 200 g divided into two batches of three each, five days before the administration of the extract, in order for them to acclimatize to the laboratory conditions. The rats are randomly selected, individually marked to identify them in their cages.

Experimental device

Determination of acute toxicity was conducted using the 2000 mg/kg body weight limit test. The animals were fasted on the day before the administration of the extracts. They are deprived of food but not water. After this period of fasting, the animals are weighed and the extract is administered to them using a gastric tube in a single dose of 2000 mg/kg body weight. The first batch (or control batch) received distilled water, the second batch received the extract orally. Immediately after the administration of the extracts, the animals continued to be deprived of food for 3 to 4 hours. After this period, they were fed and put under observation for 14 days. They were also weighed on the 7th and 14th days after the administration of the extracts. During this period, the different behaviors of the rabbits such as agitation, diarrhea, convulsion, drowsiness, visual disturbance, hair loss, trembling and mobility disorder were observed.

Blood sampling and hematological and biochemical analyses

The animals were anesthetized with chloroform and blood was drawn from the retro-orbital sinus using 75 x 1.5 mm capillary tubes and distributed into a dry tube and a tube containing the anticoagulant ethylene diamine tetra acetic acid (EDTA).

Blood counts and red blood cell constants were determined using an automated system from the blood in the EDTA tube. Biochemical parameters were determined in serum.

Determination of organ weight

Animals anesthetized with chloroform at the end of the experiment were sacrificed and some vital organs (liver, kidneys, spleen, heart, lungs) were quickly removed and weighed. An assessment of the macroscopic aspect of the different organs was also made by direct visual observation.

Statistical analysis

Results were presented as mean ± standard deviation. The Excel spreadsheet was used to calculate the means and standard deviations of larval migration and to generate the illustrative graphs. The different values were integrated into a custom-made two-criteria repeated analysis of variance model. The comparison of the means for the different

tests was done using the SNK procedure that runs the Student Newman and Keuls test using the agricolae package of the R software (R Core Team 2013). The differences are considered significant at the 5% threshold.

Results and Discussion

Content of the secondary metabolites dosed

The content of some secondary metabolites was determined in the aqueous triturated extract of the leaves of the plant. The plant is well rich in total polyphenol with a content of 938.15 ± 0.004 mg equivalent of gallic acid per g of extract. The flavonoid content is 35.51 ± 0.0003 mg equivalent of quercetin per g of extract and the condensed tannin content is 20.23 ±003 mg equivalent of catechin per g of extract.

Antioxidant activity by the FRAP method

By this method, the concentrations were determined using a calibration curve established with ascorbic acid and the values are expressed in mmol ascorbic acid equivalents/g extract (EAA). The higher the concentration is the greater the reducing power. It appears that the aqueous triturated extract of the leaves of *M. inermis* has a reducing power of 0.57 at a concentration of 0.5 mg/ml. Vitamin C has a reducing power of 5.79 at a concentration of 50 µg/ml.

The FRAP (Ferric reducing antioxidant power) method is based on the ability of extracts to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) Hinneburg *et al.* [12]. The ferric ion $Fe^{3+}$is the most active of the metals most often found in foods of plant or animal origin. The reducing power of an extract with respect to this ion can be considered as an indicator of its antioxidant activity [16].

Antioxidant activity by the DPPH method

Table 1: Antiradicalar activity of aqueous extract

Samples	IC_{50} (µg/ml)	EC_{50}(µg/mg)	APR
Aqueous extract	6.32	238.18	0.004198
Vitamin C	48.65	1824.83	0.000547

With the DPPH method, the triturated extract of *M. inermis* seems to have a strong antioxidant capacity respectively. This is the trapping of the free radical of *Diphenylpicrylhydrazyl* (purple in soluton) by compounds in the extract that can yield a hydrogen atom, leading to a decrease in the purple coloration characteristic of the appearance of *Diphenylpicrylhydrazine (*yellow in solution) [17-18].

Anthelmintic activity

Table 2: Extract effect on the motility of adult worms

	Concentrations (µg/ml)	6h	24h	48h
PBS	d0	0	0	25
Levamisol	250	25	100	100
Aqueous extract	75	50	25	100
	150	50	50	75
	300	100	100	100
	600	25	50	100
	1200	50	75	100
	2400	0	0	50

Exposure of adult worms of *T. colubriformis to the* aqueous extract of the fresh leaves of *M. inermis* showed that already at 6 hours, total inhibition was observed with the adult worms at the dose of 300 µg/ml whereas this inhibition was total with the positive reference control at 24 hours. At 48h, apart from the PBS, the negative reference control, only the 150 µg/ml and 2400 µg/ml doses of the aqueous extract could not inhibit all the adult worms.

Acute oral toxicity of the aqueous extract of fresh leaves

Variation in rabbit body weight during the observation period

After 14 days of observation, no statistical difference in body weight between the treatment and control groups was observed ($p > 0.05$) (Table 3).

Table 3: Body weights of control rabbits and those who received the extracts

	Day 1	Day 7	Day 14
Aqueous extract	152 ± 14a	161,66 ± 19,55a	166,33 ± 19,11a
Control	153,66 ± 6,22a	163,33 ± 19,77a	171 ± 20,66a

The same letters indicate that the values are not significantly different at the 5% threshold (SNK, R software).

Organ weights from control and extract-treated rabbits

Macroscopic observation of the internal organs revealed no abnormalities. Comparison of the different values shows that there is no significant difference between the control and treated groups ($p > 0.05$) (table 4).

Table 4: Organ weights harvested from rabbits at the end of the 14 days of observation

	Liver	Lungs	Kidneys	Heart	Rate
Aqueous extract	5.48 ± 0.60a	1.53 ± 0.04a	1.4 ± 0.2a	0.5 ± 0.02a	0.31 ± 0.02a
Control	5.19 ± 0.44a	1.82 ± 0.44a	1.41 ± 0.16a	0.63 ± 0.05a	0.37 ± 0.04a

The same letters indicate that the values are not significantly different at the 5% threshold (SNK, R software).

Biochemical analyses

No significant changes in biochemical parameters such as urea, BUN and TGP were found ($p > 0.05$). However, a considerable decrease in creatine of extemporaneous and hydroethanol extracts was recorded compared to the control lot (Table 5).

Table 5: Biochemical parameters of rabies on Day 14

	Urea	Creatine	TGO	TGP
Aqueous extract	0.6 ± 0.08a	4.7 ± 0.26b	268.76 ± 31.58a	125.18 ± 8.61a
Control	0.64 ± 0.03a	6.16 ± 0.64a	266.26 ± 75.42a	104.7 ± 33.09a

Different letters indicate a significant difference in values at ($p < 0.05$; SNK, R software). TGO: Transaminase Glutamate Oxoloacetate TGP: Transaminase Glutamate Pyruvate.

Hematological analyses

The results show that there is no significant change in hematological parameters ($p > 0.05$) (Table 6).

Table 6: Hematological parameters of rabies on Day 14

Hematological parameters	Control	Aqueous extract
White blood cells	10.2 ± 3.82a	10.66 ± 0.63a
Red blood cells	6.86 ± 2.57a	7.3 ± 0.35a
Hemoglobin concentration	11.07 ± 4.15a	11.77 ± 0.58a
Hematocrit	41.93 ± 15.72a	42.70 ± 0.36a
Average globular volume	65.7 ± 24.64a	65.25 ± 1.51a
Mean corpuscular hemoglobin level	18.88 ± 7.08a	18.72 ± 1.13a
Mean corpuscular hemoglobin concentration	27.19 ± 10.19a	27.26 ± 0.64a
Blood Platelets	781.4 ± 293.02a	722.4 ± 21.97a

Different letters indicate a significant difference in values at ($p < 0.05$; SNK, R software).

Discussion

In ruminants, the most prevalent parasitic nematodes of the digestive tract are commonly referred to as gastrointestinal strongles (GIs). The latter are a major source of pasture pathology that can lead to significant production losses [19]. GIs are small round worms, about 10 mm long and 0.15 mm in diameter, which parasitize farm animals and are responsible for gastrointestinal strongylosis in sheep and goats. Control of parasitic helminths, including nematodes, is considered an essential component of herd health management. Within nematodes, ovine

gastrointestinal strongylosis is a widely prevalent pathology [6]. Among SGI species, *H. contortus* and *T. colubriformis* cause the most adverse consequences in small ruminants and are of particular interest in parasitology. After a study on the efficacy of the aqueous triturated extract of fresh leaves [7] and the acetonic and methanolic extracts of the leaf powder [20] of *M. inermis* on *H. contortus* with interesting results, the present work was devoted to the evaluation of the efficacy of the triturated extract of fresh leaves on *T. colubriformis* in order to promote this plant in the manufacture of an antiparasitic phytomedicament. Statistical analyses showed that the aqueous extract of the fresh leaves of *M. inermis* inhibited the motility of the adult worms of *T. colubriformis* as well as the positive reference (levamisol), and this inhibition varied compared to the negative reference control ($p < 0.01$). It also varied with dose ($p < 0.01$) and time of observation ($p < 0.01$) and the 300 µg/ml dose appears to be more effective (Table 2).

T. colubriformis is a parasite of the small intestine of small ruminants that is 5 to 6 mm long. Females are not very prolific (200 eggs per day). *T. colubriformis* is chymivorous, its infestation in the host causes atrophy of intestinal villi and lesions of catarrhal or exudative congestive enteritis. It is responsible, in the acute form, for profuse diarrhoea, dehydration and mortality in lambs and, in the chronic form, for intermittent diarrhoea and reduced production in adults; associated with progressive weight loss and some cases of mortality during severe infestations. The dangerousness of this parasite (*T. colubriformis*) may also be due to the fact that it is a zoonosis and its strong presence in farming systems still constitutes a risk for the population. The effectiveness of the extract in inhibiting the motility of adult worms is a great asset in the fight against the proliferation of this parasite. This observed effect of the extract can be linked to the chemical group suspected to be responsible for anthelmintic properties such as polyphenols [21], flavonoids [22-23] and especially condensed tannins [24-25]; all of which have a high content in the plant extract. The antioxidant power of this extract would constitute a favorable asset for the reinforcement of its *in vivo* activity.

Indeed, oxygen although being an essential element for the life of aerobic organisms, 3 to 5% of this inspired oxygen is at the origin of oxygenated free radicals [26]. Also called reactive oxygen species, oxygenated free radicals are highly toxic. They are also generated by exposure to ionizing radiation, organic pollutants, the intake of certain medications, etc [26]. In case of excess in the organism, free radicals attack cell membranes, proteins, collagen microfibrils, nucleic acids of chromosomes and especially causes mutations within the DNA creating a series of abnormalities including the risk of cancer. They are also responsible for oxidative stress [27]. Although it is not a disease in itself, oxidative stress thus constitutes a favourable ground for the development of various pathologies [1]. It constitutes an imbalance in favour of pro-oxidants [28] between free radicals and enzymatic and non-enzymatic antioxidants. The antioxidant power of this extract could therefore strengthen the organism *in vivo* in its defense against oxidative stress while remaining anthelmintic. Another beneficial effect of this extract is its safety obtained *in vivo* after the evaluation of acute oral toxicity.

In general, the aqueous triturated extract of fresh leaves of *M. inermis*, administered orally at a dose of 2000 mg/Kg body weight to Wistars strain rabbits did not cause any abnormal behaviour or deaths in the animals during the 14 days of observation**.** Also, no adverse effects such as convulsions, agitation, diarrhea, tremors, breathing difficulties and weight loss were observed. Therefore, it follows that the extract is not toxic at the dose of 2000 mg/kg body weight on rabbits of Wistars strains. The absence of cyanogenic derivatives in the crushed extract of fresh leaves of *M. inermis* and the non-toxicity of the extract on brine shrimp larvae [7], confirm the safety of the extract on Wistars rabbits. These results corroborate those of Timothy *et al.* [29] who obtained a lethal dose of 2000 and 1587.5 mg/kg body weight respectively after evaluation of the toxicity of the aqueous and ethanolic extracts on Swiss rats. They are also similar to those of Monjanel-Mouterde *et al*. [30] who showed that the hydroethanolic extract of *M. inermis* would be toxic at a dose higher than 5000 mg/kg body weight. The extract, presenting no danger to human and animal health, could thus be used in traditional medicine for the manufacture of phytomedicines.

Conclusion

From the present work, it appears that the aqueous triturated extract of fresh leaves of *M. inermis* inhibits the motility of the adult worms of *T. colubriformis* and this grace to the content of the extract in total polyphenol,

flavonoids and condensed tannins. In addition, the non-toxicity of the extract on the Wistars female rats and its ability to strengthen the body's antioxidant defenses through its ability to trap the free radical of DPPH and reduce the Fe^{3+} ion to Fe^{2+} ion, seems to be a major issue to preserve the health of humans and animals. Further studies are needed to determine the exact nature of the active ingredient in this extract.

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