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Raymond Muganga, Joanne Bero, Joëlle Quetin-Leclercq, Luc Angenot, Monique Tits, Ange Mouithys-Mickalad, Thierry Franck, Michel Frédérich

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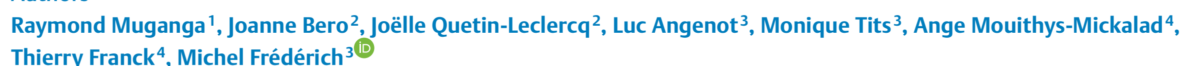
In vitro Antileishmanial, Antitrypanosomal, and Anti-inflammatory-like Activity of *Terminalia mollis* Root Bark

Authors
Raymond Muganga[1], Joanne Bero[2], Joëlle Quetin-Leclercq[2], Luc Angenot[3], Monique Tits[3], Ange Mouthys-Mickalad[4], Thierry Franck[4], Michel Frédérich[3]



Affiliations
1 University of Rwanda, School of Medicine and Pharmacy, Department of Pharmacy, Butare, Rwanda
2 Pharmacognosy Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Bruxelles, Belgium
3 Université de Liège, CIRM, Laboratoire de Pharmacognosie, CHU B36, Liège, Belgium
4 Centre for Oxygen Research and Development (C.O.R.D), Institute of Chemistry B6a, University of Liège

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Correspondence
Prof. Michel Frédérich
Department of Pharmacy, CIRM, Laboratory of Pharmacognosy, University of Liege
Avenue de l'Hôpital 1 B36, B-4000 Liège, Belgium
Phone: + 32 43 66 43 31, Fax: + 32 43 66 43 32
m.frederich@uliege.be

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ABSTRACT

This study aims at determining the *in vitro* antitrypanosomal, antileishmanial, antioxidant, and anti-inflammatory-like activities of *Terminalia mollis* root crude extracts. The antitrypanosomal and antileishmanial activities on *Trypanosoma brucei brucei* (strain 427) and promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) were evaluated *in vitro*. The methanolic root bark extract and standards were profiled by HPLC-PDA, and the majority of compounds identified using literature data. The *in vitro* antioxidant and anti-inflammatory-like activities were determined by evaluating the effect of crude extracts on reactive oxygen species produced by phorbol 12-myristate 13-acetate-stimulated equine neutrophils using lucigenin-enhanced chemiluminescence and on purified equine myeloperoxidase activity measured by specific immunological extraction followed by enzymatic detection. The methanolic, aqueous crude extract, and aqueous crude extract free of tannins exhibited good growth inhibition on *Trypanosoma brucei brucei* (IC_{50} 3.72, 6.05, and 4.45 µg/mL respectively) but were inactive against *Leishmania mexicana mexicana* (IC_{50} > 100 µg/mL). Suramin (IC_{50} 0.11 µg/mL) and amphotericin (IC_{50} 0.11 µg/mL) were used as standard respectively for the antitrypanosomal and antileishmanial activity. Very interesting antioxidant and anti-inflammatory-like activities were observed with 50% hydroethanolic, aqueous crude extracts, and aqueous crude extract free of tannins as well as with pure punicalagin, gallic, and ellagic acid (IC_{50} 0.38–10.51 µg/mL for 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), chemiluminescence, and specific immunological extraction followed by enzymatic detection assays. The study results support traditional medicinal use of the plant for the treatment of parasitical disorders and revealed for the first time the antitrypanosomal potential, anti-inflammatory-like, and antioxidant activity of *Terminalia mollis* root.

Introduction

Although notable progress has occurred in the treatment of trypanosomiasis and leishmaniasis [1, 2], the management and control of these diseases is still a health problem especially in developing countries [2, 3]. New, effective, and safer drugs are needed.

Terminalia mollis M. A. Lawson (Combretaceae) is a Rwandan medicinal plant. The root back of the plant is used to treat fevers, gastrointestinal diseases, diarrhea, gonorrhea, malaria, and other parasitosis [4]. It is already established that some plants may be active against various parasites [5, 6]. This means that the *Terminalia mollis*-based preparation used by traditional healers to treat

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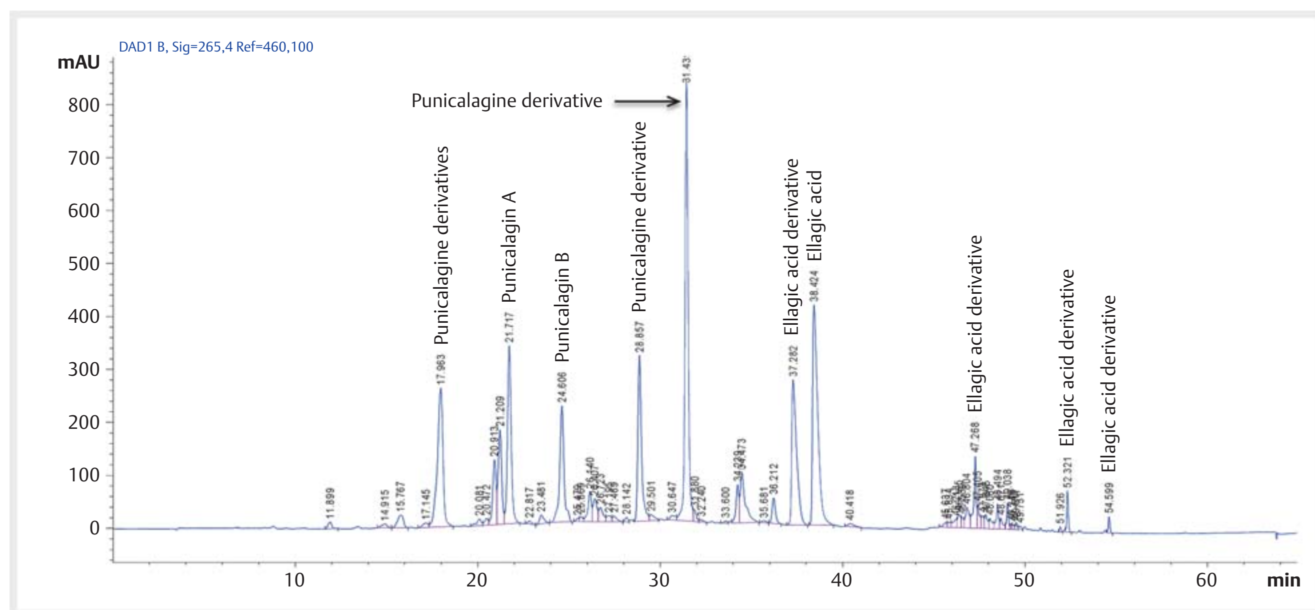

▶ **Fig. 1** HPLC chromatogram of the methanolic root bark extract of T. mollis. An aliquot of the crude extract was dissolved in methanol, and 10 µL injected with a flow rate of 1 mL/min. HPLC-PDA analysis was done using ACN and trifluoroacetic acid (TFA) 0.05 % as mobile phase in gradient mode and the stationary phase was ODS C18 column HYPERSIL 250/4.6 mm (5 µm). Ellagic acid, punicalagin A and B, and their derivatives were identified using standards and literature data.

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malaria and other parasitic diseases (not specified) may eventually be active against other parasitosis. Indeed, punicalagin and gallic acid, both present in the plant, have been reported to have trypanocidal activity [5]. Our previous studies reported the antiplasmodial activity of the plant and the identification of active compounds [7, 8], and it could be interesting to investigate the effect of the same plant on other parasites, such as leishmania and trypanosoma, for which few effective, safe, and affordable medicines are currently available.

As already established, the presence of pathogens in the body leads to inflammation processes. Previous phytochemical study of *T. mollis* reported the presence of polyphenolic compounds in stem bark [9], and the plant exhibited a good antioxidant activity [10]. Polyphenols are known to possess many therapeutic properties such as antitumor, antiviral, and anti-inflammatory [11]. The investigation of the anti-inflammatory effect of *T. mollis* may also be helpful, as this activity may play an important role in reducing tissue damage caused by various pathogens or irritants [12] and thereby prevent various diseases.

In this study, prior to evaluating the potential inhibiting effect of the extracts on the myeloperoxidase (MPO) activity, which plays a crucial role in the inflammation process [13], the total antiradical capacity of different root bark crude extracts were evaluated using the well-known 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [14]. This assay measures the radical scavenging capacity of the extract by reducing the preformed radical cation $ABTS^{\cdot+}$ generated by oxidation of ABTS with sodium persulfate. Although reactive oxygen species (ROS) are produced by normal cellular processes and assist immune function, their excessive production can also cause various diseases and exacerbate inflammation [15]. This may happen when the quantity of ROS exceeds the normal physiological level, which is controlled by the antioxidant defense systems in the body [10]. The extreme production of ROS can be caused by acute inflammation leading to neutrophil degranulation and the MPO release. We investigated the antioxidant and anti-inflammatory-like properties of the plant extracts by evaluating their capacity to reduce ROS produced by stimulated equine neutrophils and their capacity to inhibit MPO activity. MPO is a hemic peroxidase present in the granules of the neutrophils involved in the inflammatory response [16] and playing a major role in acute and chronic inflammation [13].

The present study reports the *in vitro* antitrypanosomal, antileishmanial, and anti-inflammatory-like activities of the plant.

Results

HPLC-PDA analysis of the root bark extracts allowed us to identify the presence of ellagic acid, punicalagin A and B, and their derivatives in the sample using standards (▶ **Fig. 1**) and literature data. The major peaks were present in all extracts, but their level was decreasing from the methanolic crude extract to the aqueous crude extract and from the aqueous crude extract to the aqueous extract free of tannins.

Methanolic and aqueous extracts of *T. mollis* showed an interesting antitrypanosomal activity, and the highest activity was observed with the methanolic extract (IC_{50} = 3.73 µg/mL), but they were both inactive towards leishmania (IC_{50} > 100 µg/mL) (▶ **Table 1**).

▸ **Table 1** *In vitro* antitrypanosomal and antileishmanial activities of *T. mollis* root bark extracts. Tests were performed in 3 independent experiments (95 % CI).

Plant extract	Antitrypanosomal activity Tbb (IC_{50}, µg/mL)	Antileishmanial activity Lmm (IC_{50}, µg/mL)
TMM	3.72 ± 2.14	> 100
TMH	6.04 ± 1.06	> 100
TMFT	4.44 ± 2.48	> 100
Ellagic acid	1.92 ± 0.25	18.43 ± 0.41
Suramin	0.11 ± 0.02	ND
Amphotericin B	ND	0.1 ± 0.01

Lmm: *Leishmania mexicana Mexicana*; ND: not determined; Tbb: *Trypanosoma brucei brucei*; TMM: crude methanolic root bark extract of *Terminalia mollis*; TMH: crude aqueous root bark extract of *Terminalia mollis*; TMFT: crude aqueous root bark extract of *Terminalia mollis* free of tannins; For ellagic acid, 1.92 µg/mL is equal to $0.6.10^{-3}$ µM/mL. For suramin, 0.11 µg/mL is equal to $0.84.10^{-4}$ µM/mL. For amphotericn B, 01 µg/mL is equal to $0.11.10^{-3}$ µM/mL.

▸ **Table 2** Summary of IC_{50} (µg/mL) and R^2 values of plant extracts and references on ABTS radical activity, ROS production (CL assay), and MPO activity (SIEFED). Data were calculated as 50 % inhibitory concentration (IC_{50}), n ≥ 3 for ABTS assay, CL, and SIEFED. CL experiments were performed using different batches of equine neutrophils.

	IC_{50} in µg/mL (R^2) (95 % CI)					
	ABTS test		Chemiluminescence		SIEFED technique	
	Sample	Tannins free	Sample	Tannins free	Sample	Tannins free
TMH	4.23 (0.979)	6.76 (0.972)	1.45 (0.962)	0.38 (0.96)	1.99 (0.9562)	2.11 (0.9266)
TEAR	4.01 (0.992)	4.38 (0.978)	3.79 (0.963)	1.32 (0.957)	3.06 (0.820)	4.13 (0.831)
TMEt	2.47	–	1.75 (0.957)	–	1.51	–
E. A.	1.56 (0.986)	–	10.51 (0.896)	–	6.65 (0.926)	–
G. A.	0.89 (0.974)	–	3.5 ± 0.09 (0.897)	–	0.62 (0.928)	–
PUN.	1.77 (0.985)	–	3.71 (0.97)	–	1.74 (0.870)	–

E. A.: Ellagic acid, 0.01 and 10 µg/mL are equal to $0.33.10^{-4}$ and $0.33.10^{-1}$ µM/mL respectively; G. A.: Gallic acid, 0.01 and 10 µg/mL are equal to $0.93.10^{-4}$ and $0.93.10^{-1}$ µM/mL respectively; PUN.: Punicalagins, 0.01 and 10 µg/mL are equal to $0.92.10^{-5}$ and $0.92.10^{-2}$ µM/mL respectively; TEAR: Green tea from Rwanda; TMEt: 50 % hydroethanolic extract of *Terminalia mollis*

The best antioxidant activity was found for 50 % hydroethanolic extract (TMEt) (IC_{50} = 2.44 µg/mL) followed by the crude aqueous extract of green tea from Rwanda (TEAR) (IC_{50} = 4.01 µg/mL) and the crude aqueous extract of *T. mollis* (TMH) (IC_{50} = 4.22 µg/mL) (▸ **Table 2**). Ellagic acid and punicalagin presented a high antioxidant activity (IC_{50} = 1.54 µg/mL and IC_{50} = 1.76 µg/mL), whereas gallic acid IC_{50} was 0.93 µg/mL.

Chemiluminescence assay allows the evaluation of the effect of plant extracts and references on the total ROS produced by equine neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA). *T. mollis* aqueous extract and references reduced ROS production in a concentration-dependent manner (▸ **Fig. 2**). A significant inhibition ($p < 0.05$) was found for all concentrations of samples tested (from 0.4 to 40 µg/mL for plant extracts and from 0.01 to 10 µg/mL for pure components). The IC_{50} value of samples free of tannins was slightly lower than those of corresponding extracts with tannins but not significantly ($p < 0.05$) (▸ **Fig. 2** and **Table 2**). All pure compounds tested strongly inhibited the production of ROS; ellagic acid was less active (IC_{50} = 10.6 µg/mL) than punicalagin A & B (IC_{50} = 3.71 µg/mL) and gallic acid (IC_{50} = 3.5 µg/mL) (▸ **Table 2**).

Plant extracts and pure compounds exhibited inhibition of MPO in a dose-dependent manner, as for CL assay. Significant inhibition of MPO was observed for all concentrations tested for plant extracts (▸ **Fig. 3**). The best results were found with hydroethanolic extract (IC_{50} = 1.51 µg/mL) followed by the aqueous crude extract of *T. mollis* (IC_{50} = 1.99 µg/mL) (▸ **Table 2**). Significant inhibition was observed (IC_{50} = 0.62–6.65 µg/mL) for all pure compounds (ellagic acid, punicalagin, and gallic acid) (▸ **Table 2**).

The trypan blue exclusion test showed that the cell viability was ≥ 94 % for both PBS taken as control and plant extracts at final concentration of 200 µg/mL.

Determination of total polyphenol, tannin content and the level of punicalagins, ellagic acid, gallic acid, and derivatives in plant samples (see Supporting Information) indicated that both *T. mollis* root bark and the green tea from Rwanda are rich in polyphenols and tannins.

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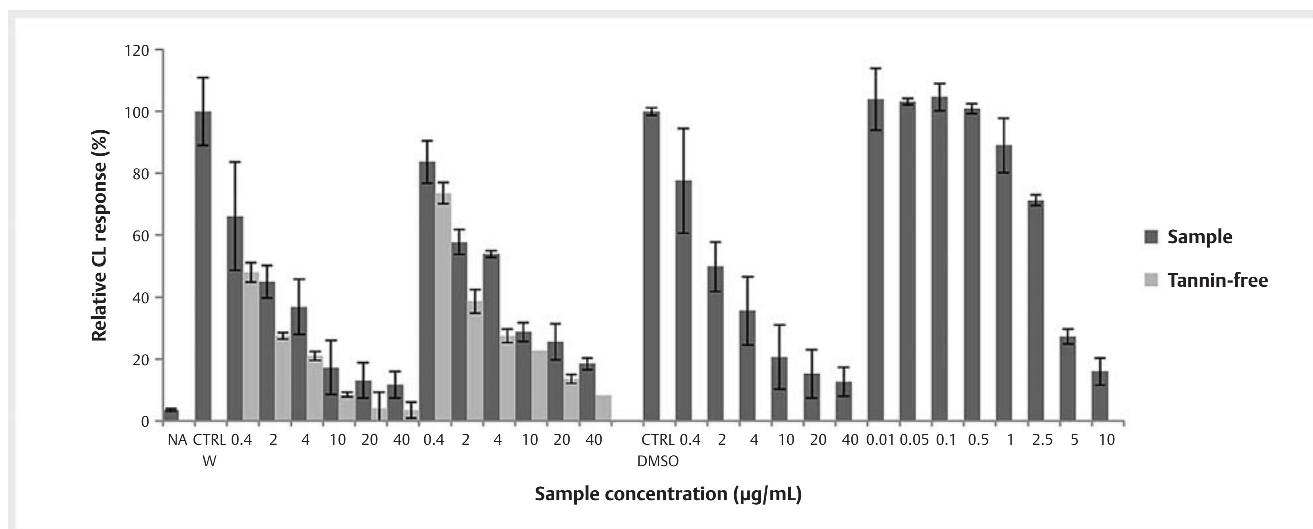

▸ **Fig. 2** Inhibitory effects of *T. mollis*, green tea extracts, and gallic acid on the chemiluminescence response produced by PMA-stimulated equine neutrophils. Aqueous extracts of *T. mollis* and green tea were dissolved in pure water, hydroethanolic extract, and gallic acid dissolved in DMSO and tested at least in triplicate at final concentrations of 0.4, 2, 4, 10, 20, and 40 µg/mL for plant extracts and 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL for gallic acid. The percentage of inhibition was calculated versus the control pure water or DMSO taken as 100 %. All data were expressed as means ± SD, n ≥ 3 and experiments were performed using different batches of neutrophils.

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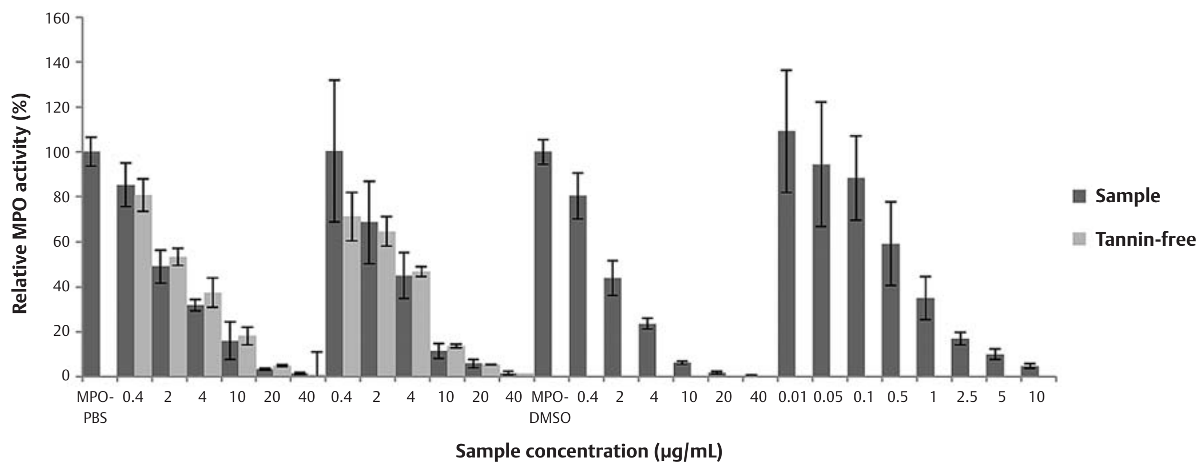
Discussion

Plant root bark sample was extracted with different solvents, and a decoction was performed with water to mimic the method of preparation used by traditional healers as previously described [7]. The extraction yield obtained for different crude extracts was similar to that early reported [7]. The identification of punicalagin, ellagic acid, and their derivatives in *T. mollis* were already reported in our previous studies [8] and were the major compounds in the aqueous crude extracts but relatively at low level compared to the methanolic one. This can be explained by the fact that the solubility of ellagic acid in water is low [17]. The crude extract free of tannins contains ellagic acid and its derivatives but not punicalagin and its derivatives. Indeed, punicalagin and its derivatives are ellagitannins, which are retained by hide powder used to prepare this extract. The antitrypanosomal activity is already established for *Terminalia* species [18]. In this study, ellagic acid showed a good antitrypanosomal activity (IC_{50} = 1.92 µg/mL), but this activity was not significantly different from that obtained from the crude extract (▸ **Table 1**), suggesting that the plant possesses other active compounds. Moreover, all analyzed extracts (methanolic, aqueous, and aqueous extract free of tannins) were active against *Trypanosoma brucei brucei* (Tbb), and HPLC-PDA analysis showed that they all contain ellagic acid, punicalagins, and their derivatives as major compounds of the plant. Punicalagin and gallic acid (also present in the plant) have been previously reported to have trypanocidal activity [3, 19], meaning that those compounds may act as antitrypanosomal by synergism. However, the antitrypanosomal activity of *T. mollis* could be attributed to urolithins, intestinal microbial metabolites from ellagic acid and punicalagin [20]. Indeed, urolithins are benzopyranones [21], which are cystein protease inhibitors, and their antitrypasomal activity was already reported [22–24].

T. mollis root bark extracts were not active against leishmania. However, ellagic acid and punicalagin (2 ellagitannins present in this plant) have weak antileishmanial activity. Indeed, ellagic acid was moderately active (IC_{50} = 18.43 µg/mL) (▸ **Table 1**), and punicalagin has been previously reported to have a moderate antileishmanial activity (IC_{50} > 10 µg/mL) [3]. In contrast, gallic acid is known to have a good leishmanicidal activity [25]. Based on our results, *T. mollis* is not recommended to treat leishmania even though it contains ellagic acid active against this parasitosis. According to a Rwandan traditional healer, a *T. mollis*-based preparation is used to treat "gastrointestinal disorders". The term "gastrointestinal disorders" may describe for traditional healers various diseases such as parasitosis or digestive manifestations, the second most common cause of complications of trypanosomiasis [26] that *T. mollis* extract can treat. As already mentioned above, the presence of those parasites in the body leads to an inflammation process. Therefore, the possible enzyme MPO inhibition activity of the plant could be of additional value as MPO is involved in redox processes that occur at the inflammation sites.

T. mollis root bark extracts and green tea aqueous extract (used as a reference) displayed an interesting total antioxidant activity on the ABTS test (IC_{50} < 10 µg/mL), and comparable radical scavenging activity was observed for the 2 extracts (results not shown). Ellagic acid and punicalagin exhibited an interesting antioxidant activity, and comparable antioxidant activity has been previously reported [26]. *T. mollis* crude aqueous extract free of tannins, for which the level of the major constituents was low compared to other crude extracts analyzed, also showed a promising antioxidant activity because the plant contains not only ellagitannins but also ellagic acid and some condensed tannins such



► **Fig. 3** Effect of plant extracts and gallic acid on MPO activity measured by SIEFED. Samples were analyzed at least in triplicate at final concentrations of 0.4, 2, 4, 10, 20, and 40 µg/mL, and MPO was used at a final concentration of 25 ng/mL. The percentage of inhibition was calculated for each sample concentration versus MPO-PBS or MPO-DMSO taken as 100 % (mean ± SD, n ≥ 3).

as catechin, epicatechin, gallocatechin, and epigallocatechin [9] as well as gallic acid and derivatives not retained by hide powder.

Considering the molecular weight of the compounds, punicalagin is the most potent antioxidant compound, and its high activity probably results from the presence of 16 phenol functions per molecule [27].

The anti-inflammatory activity of gallic acid, ellagic acid, and punicalagin is already established [28, 29], but it is the first time that the anti-inflammatory-like activity of punicalagin and ellagic acid has been proven on MPO. As already mentioned above, the root bark of *T. mollis* contains gallic acid, ellagic acid, punicalagins, and their derivatives, which may be primarily responsible for the anti-inflammatory-like activity of the plant. Indeed, those compounds, especially punicalagin and gallic acid, presented strong radical scavenging and modulatory activities on the oxidant response of stimulated neutrophils and myeloperoxidase, a key cell and enzyme involved in inflammatory response. Nevertheless, urolithins especially urolithin B, the final catabolized product, for which a strong antioxidant and anti-inflammatory activity is already known [30], plays a key role in the anti-inflammatory-like activity observed from *T. mollis* root bark extract. The cell effect does not seem to be linked to a toxic effect. The trypan blue exclusion test showed that the cell viability was ≥ 94 % for both PBS taken as control and plant extracts at final concentration of 200 µg/mL, demonstrating the absence of cytotoxicity towards neutrophils. It should be noticed that other aspects of the inflammation process should be also considered, as the present study explored only some of them.

Many researchers agree about the role of pro-inflammatory cytokines in malarial disease [31]. Ellagic acid, the major antiplasmodial constituent of *T. mollis* [8], may also act on the release of pro-inflammatory cytokines. Unfortunately, we did not explore this hypothesis. Trypanosomiasis disease is associated with inflammation [32], and the fact that the plant has both anti-trypanosmiasis and anti-inflammatory-like activity would constitute an advantage. Inflammation is a complex biological response, and only some aspects concerning the oxidant response of PMNs (polymorphonuclear leukocytes) i. e. the capacity to reduce ROS generated by stimulated neutrophils and the potentiality to inhibit MPO were considered in the course of this study. Indeed, previous studies suggested that these polyphenols, the most abundant bioactive compounds in some other plants like pomegranate, could be considered in the treatment of inflammation [33–36]. Further studies are recommended to clarify this point.

The very high antitrypanosomal activity of *T. mollis* recommends this plant as a good candidate for the development of a standardized plant extract in the treatment of trypanosomiasis. Punicalagins, ellagic acid, gallic acid, and derivatives may be the major active constituents. This study revealed for the first time the antitrypanosomal and anti-inflammatory-like activity of *T. mollis*.

Materials and Methods

Plant material

Root bark samples of *T. mollis* were collected from the Eastern Province of Rwanda in October 2010. The botanical identity of the plant specimen was confirmed by Professor Elmar Robbrecht (National Botanic Garden of Belgium). After authentication, a voucher number (BR0000005087167) was attributed to the sample. Vouchers were deposited at the Rwandan National Herbarium (Butare) and at the National Botanic Garden of Belgium (Meise). The samples were treated as previously reported [7]. Commercial green tea was obtained from Tilman Pharmaceutical plant, and Rwandan green tea was purchased from Rwanda Mountain Tea Rubaya-Nyabihu Tea Factories.

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Preparation of samples

Methanolic, 50% hydro-ethanolic extract, and aqueous crude extracts of *T. mollis* were prepared as previously reported [7]. Briefly, each organic extract was obtained by macerating 5 g of powdered plant sample 3 times in 25 mL of solvent for 30 min under constant shaking at room temperature. After filtration, the extracts were evaporated to dryness under reduced pressure with a rotary evaporator. For the aqueous extract, 2 g of powdered plant material was decocted for 1 h using 100 mL of distilled water. The mixture was filtered, and the filtrate freeze-dried to obtain the dried crude aqueous extracts. All extracts obtained were weighed and their yield calculated. Samples free of tannins were prepared according to European pharmacopeia 8.5 [37]. Plant material was powdered to a size of 250 µm; around 1 g was placed in 150 mL of distilled water and boiled in water-bath at 100 °C for 30 min. After cooling, the mixture was filled to 250 mL with pure water then filtrated. Hide powder was added to an aliquot and the mixture shaken for 1 hour at room temperature. After filtration, the filtrate obtained was freeze-dried to give a crude aqueous extract free of tannins. The extraction yield was 21.70%, 36.20%, 36.75%, 3.43%, and 5.58% for methanolic, aqueous, 50% hydro-ethanolic, *T. mollis* crude aqueous free of tannins, and green tea aqueous extract free of tannins respectively.

HPLC analysis

HPLC-PDA analysis was conducted using ODS C18 column HYPERSIL 250/4.6 mm (5 µm) (Alltech) and ACN and trifluoroacetic acid (TFA) 0.05% in gradient mode respectively as stationary and mobile phase. The flow rate was 1 mL/min, and the injection volume was 10 µL.

In vitro antitrypanosomal and antileishmanial assays

The bloodstream forms of Tbb strain 427 (Molteno Institute for Research in Parasitology) were cultured in HMI9 medium (Sigma-Aldrich), which contains 10% heat-inactivated FBS (Sigma-Aldrich), and incubated at 37 °C in a humidified atmosphere with 5% CO_2, as already described [38, 39]. The strain MHOM/BZ/84/BEL46 of *Leishmania mexicana mexicana* (Lmm) in promastigote forms (BRC-Leishmania) were cultivated *in vitro* in a semi-defined medium (SDM-79) (Sigma-Aldrich) [38], supplemented with 15% heat-inactivated FBS and incubated at 28 °C in a humidified atmosphere with 5% CO_2.

The *in vitro* antitrypanosomal and antileishmanial activity of plant extracts were evaluated as previously described [40]. Three extracts were tested: methanol, aqueous, and aqueous crude extract free of tannins as well as ellagic acid, analytical standard grade (Alfa Aesar). Suramin (99%) and amphotericin B (± 80%) (Sigma-Aldrich) were used as positive controls for antitrypanosomal and antileishmanial activity respectively. Plant extracts, ellagic acid, and positive controls were prepared as described [40] and tested in triplicate. The results were expressed as the mean IC_{50} (the concentration of a product that can reduce the level of parasitaemia to 50%).

Antioxidant and anti-inflammatory-like activity

The total antiradical capacity of crude extracts was determined using $ABTS^{\cdot+}$ (Sigma-Aldrich) radical cation decolorization method [14]. Three crude extracts (hydro-ethanolic, aqueous, and aqueous crude extract free of tannins) were prepared at different concentrations in water and in DMSO, analytical standard grade (Sigma-Aldrich). Gallic acid (analytical standard grade, Sigma-Aldrich), ellagic acid (analytical standard grade, Alfa Aesar), and a mixture of punicalagin A (30.7%) & B (65.6) (Chromadex) were used as standards, and methanol was used as a blank sample. An aliquot of 20 µL of standard solutions (at different concentrations) and plant extracts adjusted to 0.4, 2, 4, 10, 20, and 40 µg/mL final concentration were treated as detailed elsewhere [14], and the percentage decrease of absorbance was calculated, assuming that the absorbance of the solvent (pure water or DMSO) is equal to 100%, and IC_{50} calculated.

Isolation of equine neutrophils

Horse blood was drawn from the jugular vein of healthy horses fed and bred in identical conditions (Department of Clinical Sciences, Anesthesiology and Equine Surgery, University of Liège, Belgium) using ethylenediaminetetraacetic acid (Sigma-Aldrich) as anticoagulant. Neutrophils were isolated using a discontinuous percoll density gradient as previously reported [41]. For each batch of neutrophils, 60 mL of blood from 1 horse was used. The neutrophils were carefully collected, washed with 2 volumes of physiological saline solution, and resuspended in 20 mM PBS (Sigma-Aldrich) (at pH 7.4 with 137 mM NaCl and 2.7 mM KCl). The cells were used within 4 hours, and each experiment was repeated at least twice with different batches of neutrophils.

Measurement of the ROS produced by neutrophils activated with PMA CL assay)

The level of ROS produced by activated neutrophils was determined by lucigenin-enhanced chemiluminescence using a method adapted from Benbarek and collaborators [42]. *T. mollis* and green tea at final concentrations of 0.4, 2, 4, 10, 20, and 40 µg/mL were analyzed. Also, gallic acid, ellagic acid, and a mixture of punicalagin A & B, initially dissolved in DMSO, were used as references at final concentration of 0.01, 0.05, 0.1, 0.5, 1, 2, and 4 µg/mL. PMA (Sigma-Aldrich)-stimulated neutrophils incubated with PBS containing 1% of the vehicle (DMSO or pure water) instead of plant extracts were used as control and taken as 100% of CL response. The percentages of inhibition for all samples were calculated in relation to the control.

Viability test

This test was carried out to verify if the decrease of chemiluminescence response was not due to the toxicity of the samples analyzed. The trypan bleu (ICN Biomedicals Inc) exclusion test was used [43]. Samples at a final concentration of 20 and 40 µg/mL for plant extract and 2 and 4 µg/mL for references were incubated for 40 min with unstimulated neutrophils (10^6 cells) in PBS. After incubation, the percentage of viability was microscopically estimated for each sample in triplicate. Unstimulated neutrophils with DMSO or water were used as control.

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Measurement of active MPO by specific immunological extraction followed by enzymatic detection method (SIEFED)

The measurement of active MPO was done by specific immunological extraction followed by enzymatic detection (SIEFED), as already reported [44]. MPO (Sigma-Aldrich) in a diluted buffer with 1% DMSO or pure water was used as a control and taken as 100% MPO activity, and the percentages of inhibition were calculated for all samples in relation to the control.

Statistical analysis

Each experiment was run at least 2 times. For antiparasitic and anti-inflammatory-like activity, each measure was repeated at least 2 times ($n \geq 6$). Statistical analysis was carried out using GraphPad Prism 5.04 (GraphPad Software). The IC_{50} values were calculated after converting the concentrations into their decimal logarithm and applying the function "log (inhibitor) versus normalized response-variable slope". All results are expressed as mean ± standard deviation (SD) and, where applicable, p value < 0.05 is considered as significant.

Supporting Information

Details regarding HPLC analysis of *T. mollis*, ABTS test for all samples, CL, and SIEFED for pure compounds (ellagic acid, punicalagin, and gallic acid) are available as Supporting Information.

Contributors' Statement

Conception and design of the work: J. Quetin-Leclercq, M. Frédérich, L. Angenot, M. Tits, R. Muganga, A. Mouithys-Mickalad; data collection: R. Muganga, J. Bero, T. Franck, L. Angenot, M. Tits; analysis and interpretation of the data: R. Muganga, J. Bero, T. Franck, A. Mouithys-Mickalad, M. Tits, L.Angenot, M. Frédérich, J. Quetin-Leclercq; statistical analysis: T. Franck, A. Mouithys, J. Bero, R. Muganga; drafting the manuscript: R. Muganga, M. Frédérich, J. Quetin-Leclercq, J. Bero; critical revision of the manuscript: M. Frédérich, M. Tits, L. Angenot, T. Franck, A. Mouithys-Mickalad, J. Quetin-Leclercq.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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