N'allez jamais chez un docteur dont les plantes de la salle d'attente sont mortes

« Erma Bombeck »

To my late Father

To my dear Mother

To my brothers and sisters

To Yvonne and Rayan

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RESUME

Les travaux de recherche de la présente thèse sont divisés en deux parties principales:

- le screening de plantes médicinales rwandaises pour l'activité antiplasmodiale ;
- l'isolement des principes actifs responsables de l'activité antiplasmodiale, l'évaluation de l'activité antiparasitaire et de l'activité anti-inflammatoire des plantes sélectionnées.

Dans la première partie, 13 plantes ont été sélectionnées sur base d'une étude ethnobotanique sur les plantes médicinales utilisées pour traiter la malaria au Rwanda. A partir de ces plantes, 19 échantillons ont été collectés et 46 extraits testés contre *Plasmodium falciparum*. La majorité des extraits testés ont montré une activité *in vitro* dont 16% avec une activité très élevée (IC_{50} < 5 µg/ml). Le test *in vivo* nous a permis de choisir deux plantes, *Zanthoxylum chalybeum* et *Terminalia mollis* pour des analyses plus poussées.

Dans la deuxième partie, tout d'abord, un fractionnement bio-guidé nous a permis d'identifier des composés responsables de l'activité antiplasmodiale de *Z. chalybeum* et de *T. mollis*. Il s'agit respectivement de la nitidine (IC_{50} ± 77 ng/ml) et de l'acide ellagique (IC_{50} ± 175 ng/ml). Ensuite, les deux plantes ont été testées *in vitro* pour l'activité antitrypanosomiale, antileishmaniale, antiamibienne et anti-inflammatoire. *Z. chalybeum* a montré une activité prometteuse sur la leishmaniose, une activité modérée sur le trypanosome, une activité faible sur l'amibe et une activité intéressante sur l'inflammation. *T. mollis* a présenté une activité très élevée sur la leishmaniose, une activité insignifiante sur le trypanosome, une activité modérée sur l'amibe et une activité remarquable sur l'inflammation.

Il est probable que la nitidine, le composé responsable de l'activité antiplasmodial de *Z. chalybeum*, joue également un rôle dans l'activité de la plante sur la leishmaniose et sur l'inflammation et que l'acide ellagique intervient dans l'activité de *T. mollis* sur le trypanosome, l'amibe et l'inflammation. De plus, *T. mollis* contient de l'acide gallique, des punicalagines et leurs dérivés qui contribuent aux propriétés thérapeutiques de la plante notamment au niveau anti-inflammatoire.

En bref, le présent travail a révélé les composés principalement responsables de l'activité antiplasmodiale des plantes sélectionnées, *Z. chalybeum* et *T. mollis* et l'effet de ces deux espèces végétales sur d'autres parasites et sur l'inflammation.

SUMMARY

The work presented in this thesis is divided into two main parts:

- the screening of Rwandan medicinal plants for antiplasmodial activity;
- the isolation of the active ingredients responsible for antiplasmodial activity and the evaluation of antiparasitic as well as anti-inflammatory activity of selected plants.

In the first part, 13 medicinal plants were selected based on an ethnobotanical survey conducted on medicinal plants used in Rwanda to treat malaria. From the selected plants 19 samples were collected and 46 extracts tested against *Plasmodium falciparum*. The majority of the plant extracts analysed exhibited *in vitro* antiplasmodial activity and 16 % presented a high activity (IC_{50} < 5 µg/ml). The *in vivo* assay allowed us to select two plants, *Zanthoxylum chalybeum* and *Terminalia mollis* for further investigations.

In the second part of this work, firstly, a bio-guided fractionation made it possible to identify nitidine (IC_{50} ± 77 ng/ml) and ellagic acid (IC_{50} ± 175 ng/ml) as the main compounds responsible for the antiplasmodial activity of *Z. chalybeum* and *T. mollis*, respectively. Then, the two plants were evaluated *in vitro* for antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activity. *Z. chalybeum* showed a very promising antileishmanial activity, a moderate antitrypanosomal activity, a weak antiamoebic and an interesting anti-inflammatory activity. *T .mollis* presented a very high antitrypanosomal activity, a negligible antileishmanial activity, a moderate antiamoebic and a remarkable anti-inflammatory activity.

Nitidine, the most antiplasmodial compound from *Z. chalybeum*, probably plays a role in the antileishmanial and anti-inflammatory activity of the plant, whereas ellagic acid intervenes in the antitrypanosomal, antiamoebic and anti-inflammatory activity of *T. mollis*. Additionally, *T. mollis* contains gallic acid, punicalagins and their derivatives which contribute to the therapeutic properties of the plant, especially in its anti-inflammatory activity.

Briefly, this work revealed the most active products responsible for the antiplasmodial activity of selected plants, *Z. chalybeum* and *T. mollis* and the effect of these two species on other parasites and on inflammation.

ABBREVIATIONS

ABTS	: 2, 2′-azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid
ACT	: Artemisinin Combination Therapy
CDC	: Centers for Diseases Control and Prevention
CL	: chemiluminescence
CURPHAMETRA	: Centre Universitaire de Recherche sur la Pharmacopée et la Médecine Traditionnelle
DMSO	: dimethlylsulfoxide
FAD	: Food and Drug Administration (USA)
FNRS	: Fonds National de Recherche Scientifique (Belgium)
FxIns.	: precipitate from *Terminalia mollis* fractionnation
FxTMEA	: ethyl acetate fraction of *Terminalia mollis*
$FxTMH_2O$	: aqueous fraction of *Terminalia mollis*
FxTMnH	: n hexane fraction of *Terminalia mollis*
HAT	: Human African Trypanosomiasis
HIV	: Human Immunodeficiency Virus
IV	: intravenous
IC_{50}	: half maximal inhibitory concentration
IL	: interleukin
IM	: intramuscular
IRS	: Indoor Residual Spraying
IRST	: Institut de Recherche Scientifique et Technologique (Rwanda)
LLIN	: Lasting Insecticidal Nets
Lmm	: *Leishmania mexicana mexicana*
MIC	: Minimal Inhibitory Concentration
MOH	: Ministry of Health (Rwanda)
MPO	: myeloperoxidase
MS	: mass spectrometry
MW	: molecular weight
NMCP	: National Malaria Control Program
NMR	: nuclear magnetic resonance
NUR	: National University of Rwanda
PBS	: phosphate buffered saline
PEG	: Polyethylene glycol
PIC	: Project Interuniversitaire de Coopération
PKC	: Protein Kinase C
PKDL	: Post-Kala-azar Dermal Leishmaniasis
PMN	: Polymorphonuclear Neutrophil
PNILP	: Programme National Intégré de Lutte contre le Paludisme
RDC	: Republic Democratic of Congo
REMA	: Rwanda Environment Management Authority
RNA	: Rwanda News Agency

ROS	: Reactive Oxygen Species
SD	: Standard deviation
SIEFED	: Specific Immunological Extraction Followed by Enzymatic Detection
T. b. gambiense	: *Trypanosoma brucei gambiens*
T. b. rhodesiense	: *Trypanosoma brucei rhodensis*
T. mollis	: *Terminalia mollis*
T1A	: First fraction obtained from *Terminalia mollis* fractionation
Tbb	: *Trypanosoma brucei brucei*
TMEA2	: fraction 2 from *Terminalia mollis* fractionation
TMEA3	: fraction 3 from *Terminalia mollis* fractionation
TMEA4	: fraction 4 from *Terminalia mollis* fractionation
TMEt	: 50% hydroethanolic extract of *T. mollis*
TMFT	: fraction free of tannins from *Terminalia mollis*
TMH	: crude aqueous extract of *Terminalia mollis*
TMM	: crude methanolic fraction of *Terminalia mollis*
TNF	: Tumour Necrosis Factor
UNDP	: United Nations Development Program
UNICEF	: United Nations International Children's Emergency Fund
UNR	: Université Nationale du Rwanda
WHO	: World Health Organisation
Z. chalybeum	: *Zanthoxylum chalybeum*
ZA2	: Second aqueous fraction obtained from of *Z. chalybeum*
ZD2	: Second dichloromethane fraction of *Z. chalybeum*
ZP	: precipitate obtained after liquid-liquid fractionation of *Z. chalybeum*
λ	: wavelength

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GENERAL INTRODUCTION

CHAPTER I GENERAL INTRODUCTION

I.1. MALARIA

I.1.1. General description

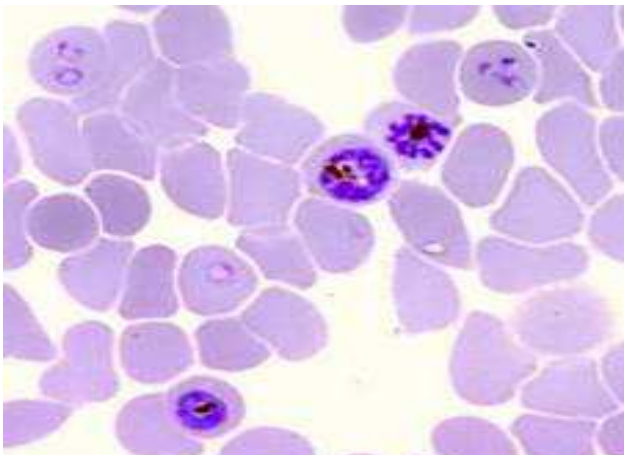

Figure 1.1 *Plasmodium falciparum* in red blood cells (from http://insciences.org/articles.php?tag=Plasmodium%20falciparum)

Malaria is a parasitic disease caused by protozoa called Plasmodium which is transmitted to human by the bite of infected female anopheles mosquitoes. Malaria is the main tropical disease, in 2010 according to WHO, about 3.3 billion people were exposed to it and the highest risk was for people living in sub-Saharan Africa where approximately 81% of cases and 91% of deaths occur mostly among the children population under five years of age and pregnant women (WHOb, 2011). Indeed, in some areas transmission is very high and people may be bitten by infected mosquito several times a day, but the risk may change from one season to another (Ashley et al., 2008).

Figure 1.2 The distribution of malaria in the world (from WHOa, 2011)

This high morbidity and mortality has an important economic and social impact particularly in developing countries. Five species of the genus Plasmodium can cause malaria in human: *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesii* and *Plasmodium falciparum*. *Plasmodium falciparum,* the most dangerous and prominent in Africa, attracts our attention in the present work.

I.1.2. Life cycle of *Plasmodium falciparum*

The life cycle of *Plasmodium falciparum* occurs in the vector mosquitoes and in the vertebrate hosts (human). When a malaria-infected female *Anopheles* mosquito takes her blood meal, it inoculates sporozoites into the human host. The sporozoites invade liver parenchymal cells, multiply and transform into schizonts during the exo-erythrocytic cycle. The hepatic schizonts may burst and release merozoites which enter the bloodstream and quickly infect red blood cells. This blood stage infection, the erythrocytic cycle, is responsible for signs and symptoms of malaria. The parasite passes through different stages from the early red blood cell infective stage, the ring trophozoite to the mature trophozoites and the schizonts which rupture and release new merozoites. Some merozoites invade new red blood cells and amplify the infection. After asexual multiplications a small proportion of merozoites differentiates into male and female gametocytes which are ingested by another female *Anopheles* mosquito during a blood meal to start the mosquito cycle.

During the mosquito cycle, male gametocytes reach the mosquito's stomach and after exflagellation liberate microgametes. Microgametes undergo internal changes, fertilize macrogametes and lead to zygotes. The zygotes develop into motile ookinetes which penetrate epithelial cell of mosquito midgut wall and transform into oocysts. After multiple asexual replications, the oocysts produce sporozoites which invade the mosquito salivary glands from where they will be injected to human during another eventual blood meal.

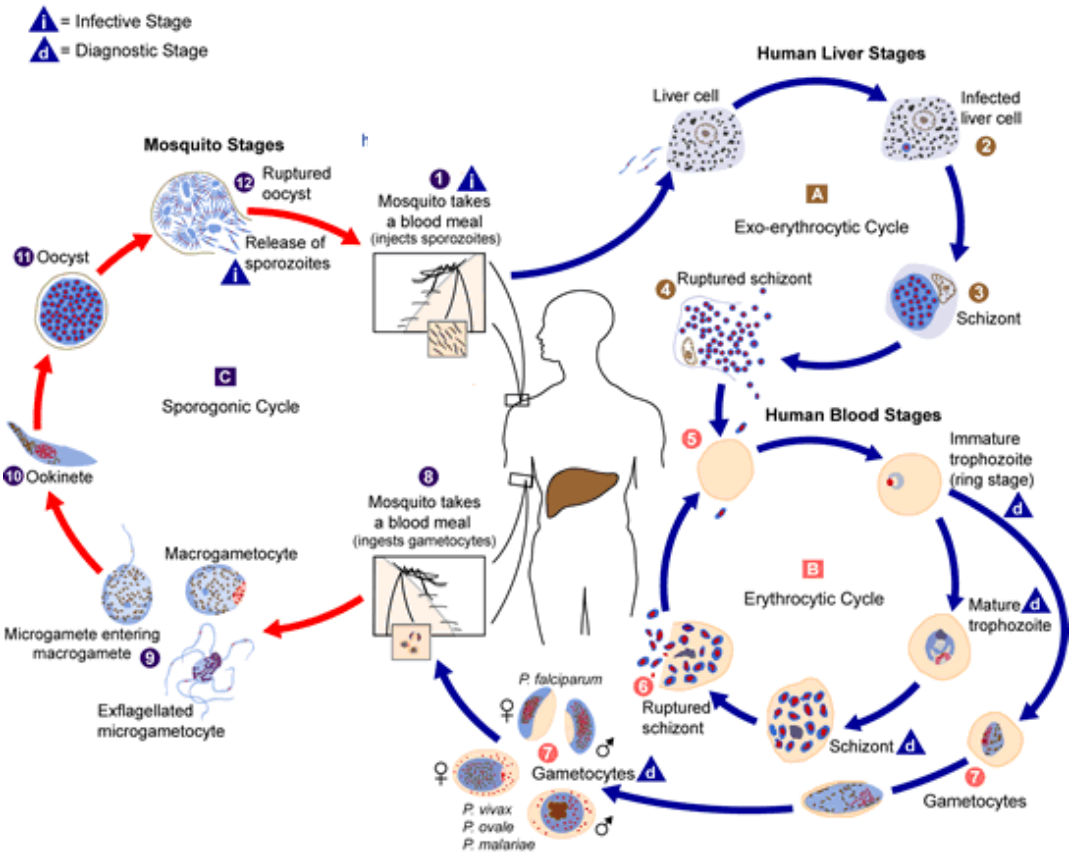

Figure 1.3 Life cycle of *Plasmodium falciparum* (from Centers for Diseases Control and Prevention (CDC) http://www.cdc.gov/malaria/about/biology/index.html)

I.1.3. Diagnosis, treatment and prevention

The signs and symptoms of *Plasmodium falciparum* malaria vary and according to WHO recommended surveillance standards, most infected people have fever which is commonly associated with splenomegaly and anaemia. Other symptoms are headache, back pain, chills, sweating, myalgia, nausea and vomiting but they are not specific to malaria (WHOb, 1999).

Plasmodium falciparum malaria is diagnosed in laboratory using blood smears stained with Giemsa and the parasitaemia is determined under microscope. Antigen detecting rapid diagnostic tests can be also used.

No vaccine is currently available to prevent malaria, even if one developed by GlaxoSmith Kline (GSK), the RTS, S vaccine, is actually in phase III clinical assay (Regules et al., 2011; Sherman et al., 2012). Many drugs can be used to treat malaria but we will focus only on the treatment recommended by the WHO. Since the end of 2010, WHO recommended the use of artemisinin combination therapy (ACT) in many endemic countries and the currently recommended combinations are: artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine, and

dihydroartemisinin plus piperaquine. Chloroquine is only used in some countries such as the Central America (WHOb, 2011). In case of uncomplicated *Plasmodium falciparum* malaria a single dose of primaquine could be used as an anti-gametocyte in addition to ACT (considering the risks of haemolysis in patients with glucose- 6-dehydrogenase (G6PD) deficiency). Severe malaria should be treated with parenteral artesunate followed by a complete course of an effective ACT as soon as the patient can take oral medications. When it is not possible, patients should be alternatively given pre-referral treatment such as artesunate (rectal), quinine (IM), artesunate (IM) or artemether (IM) and referred immediately to an appropriate facility for further treatment. The doses are as follows (WHOc, 2010):

Table 1.1 The recommended doses in the treatment of malaria

UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA	
Drug	**Dose**
Artemeter + lumefantrine	5-14 kg: 1 tablet; 15-24 kg: 2 tablets; 25 - 34 kg: 3 tablets; > 34 kg : 4 tablets in six doses regiments for a period of 3 days. The therapeutic dose range is 1.4–4 mg/kg of artemether and 10–16 mg/kg of lumefantrine
Artesunate + amodiaquine	4 mg/kg/day artesunate and 10 mg/kg/day amodiaquine once a day for 3 days, with a therapeutic dose range between 2–10 mg/kg/day artesunate and 7.5–15 mg/kg/dose amodiaquine
Artesunate + mefloquine	Target dose: 4 mg/kg/day artesunate given once a day for 3 days and 25 mg/kg of mefloquine either split over 2 days as 15 mg/kg and 10 mg/kg or over 3 days as 8.3 mg/kg/day once a day for 3 days. The therapeutic dose range is between 2 – 10 mg/kg/dose/day of artesunate and 7 – 11 g/kg/dose/day of mefloquine
Artesunate + sulfadoxine – pyrimethamine	Target dose: 4 mg/kg/day artesunate given once a day for 3 days and a single administration of 25/1.25 mg/kg sulfadoxine-pyrimethamine on day 1, with a therapeutic dose range between 2 – 10 mg/kg/day artesunate and 25 – 70/1.25 – 3.5 mg/kg sulfadoxine-pyrimethamine
Artesunate + tetracycline or doxycycline or clindamycine	Artesunate (2 mg/kg once a day) plus tetracycline (4 mg/kg four times a day) or doxycycline (3.5 mg/kg once a day) or clindamycin (10 mg/kg twice a day). Any of these combinations should be given for 7 days
SEVERE *PLASMODIUM FALCIPARUM* MALARIA	
Artesunate	Artesunate 2.4 mg/kg (body weight) I.V. or I.M. given on admission (time = 0), then at 12 h and 24 h, then once a day is the recommended treatment. Artemether, or quinine, is an acceptable alternative if parenteral artesunate is not available: artemether 3.2 mg/kg (body weight) I.M. given on admission then 1.6 mg/kg (body weight) per day; or quinine 20 mg salt/kg BW on admission (IV infusion or divided IM injection), then 10 mg/kg (body weight) every 8 h; infusion rate should not exceed 5 mg salt/kg (body weight) per hour.

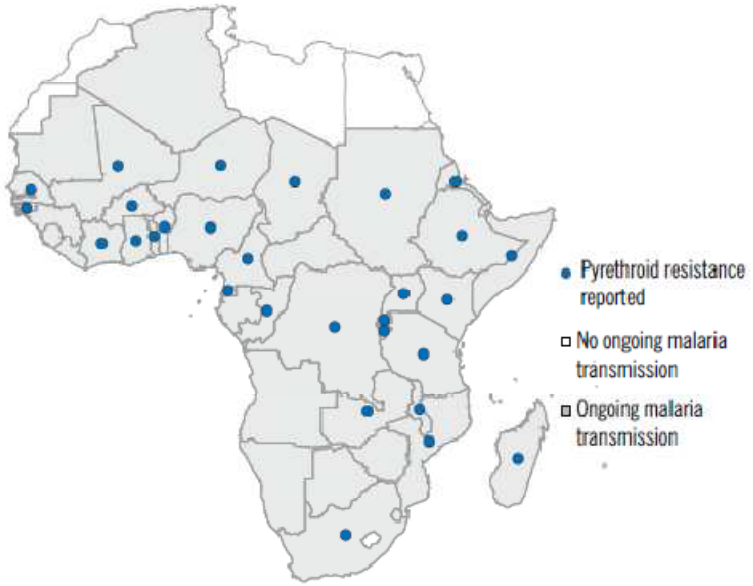

Figure 1.4 Malaria-endemic countries in Africa reporting resistance to pyrethroids in at least one malaria vector in at least one monitoring

The prevention of *Plasmodium falciparum* malaria is based on individual people protection against infective malaria mosquito bites and on the reduction of the intensity of local malaria transmission. For that, the main strategies are:

- The use of Long-Lasting Insecticidal Nets (LLIN): nets that are treated with an insecticide which kills the mosquitoes that touch it, hence protect the person sleeping under the net. This only assures an individual protection as people sleeping outside the net are less protected;
- The use of Indoor Residual Spraying (IRS): residual insecticides are sprayed to the places where many vector species of anopheles mosquito tend to rest after taking a blood meal. This leads to a rapid and effective control of malaria transmission by reducing the local burden of malaria morbidity and mortality.

Another way to prevent malaria transmission is the control of larva but this option is only applicable in few cases easy to identify and in localised breeding sites.

Pyrethroid can be used for LLIN as well as for IRS. However, many cases of pyrethroids resistances were observed especially in Sub-African countries (see Figure 4). The management of this resistance is very important as far as the most endemic countries are concerned.

I.1.4. Malaria case in Rwanda

I.1.4.1. Brief description of the country

Rwanda is a developing country located in East-Central of Africa. It has the highest population density of the continent (area of 26,338 km^2 for a population of approximately 10.7 million) and with an annual population growth rate of 3%. It is a land-locked country surrounded by the Democratic Republic of Congo (West), Tanzania (East), Uganda (North)

and Burundi (South). Commonly known as “Country of thousand hills”, the country relief is mainly marked by steep mountains and deep valleys with an altitude average of 1700 m. The weather is quite stable (annual temperature average around 20°C) with a temperate climate and two rainy seasons separated by a summer period. Rwanda still has to face many challenges after the genocide of Tutsi in 1994 during which an estimated 1 million people died. The government of Rwanda put many efforts to rebuild the country after that tragedy and the health sector is one of its major priorities (PNILPa, 2005).

I.1.4.2. Malaria in Rwanda

Malaria was known in Rwanda since the beginning of 19th century in some areas with low altitude (< 1500 m) and the exploitation of valleys during the colonisation contributed to the spread of the disease (Munyantore, 1989). The most predominant Plasmodium species in Rwanda is *Plasmodium falciparum* (95%) followed by *Plasmodium malaria* (4.5%) and *Plasmodium ovale* (0.5%) (PNILPb, 2005). Efforts were made to fight malaria but in vain because of insufficient financial support. In 1984 there was a surge in morbidity of malaria which was the first cause of mortality in Rwanda (PNILPa, 2005). In 1993, thanks to the cooperation between Belgium and Rwanda, the National Malaria Control Program (NMCP) whose French acronym was PNILP “Programme National Integré de lutte contre le Plasmodium” was created and became operational since 1995. At that time, Rwanda was suffering from *Plasmodium falciparum* malaria resistance to chloroquine which was replaced later by a combination of sulfadoxine-pyrimethamine (sold under the brand name Fansidar®). Between 1982 and 2003 malaria incidence rate in Rwanda increased from 3.5 to 59.94 % and the morbidity raised from 50% in 1998 up to 59.94 % in Rwandan hospitals (PNILPa, 2005).

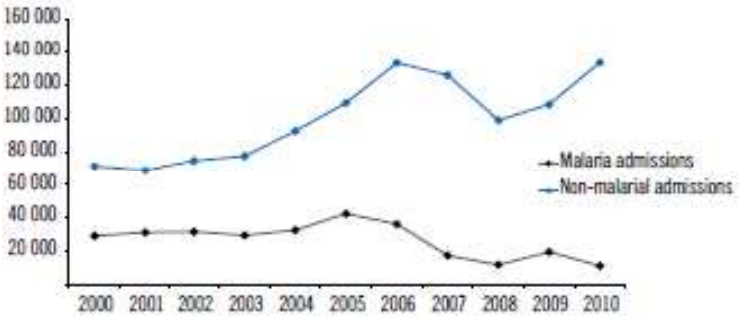

Figure 1.5 Trends in malaria and non-malarial admissions in Rwanda, 2000-2010 (from WHO, 2011)

The NMCP in collaboration with the United Nations International Children's Emergency Fund (UNICEF), the United Nations Development Program (UNDP) and the World Bank through their initiative Roll Back Malaria, attempted to implement different strategies to combat malaria. The NMCP received financial support from different partners among which we can cite the US President’s Malaria Initiative, which launched in

2005 a five years program to quickly reduce malaria-related mortality in Sub-Saharan Africa, Global Fund and WHO. To facilitate malaria control, the country was then divided into different zones according to the level of transmission, disease vector prevalence, altitude and climate. Rwanda is currently divided into 30 administrative districts among which 19 were classified as endemic and the rest as epidemic prone (see Figure 5 below) (USAID, 2010).

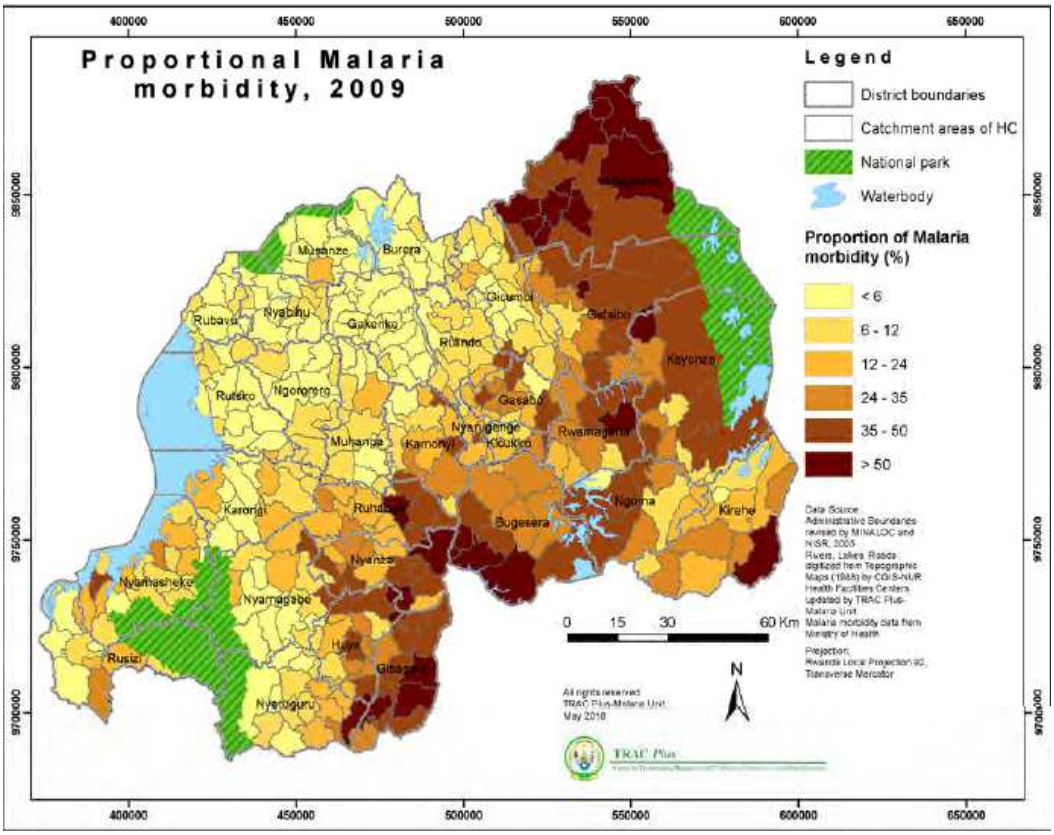

Figure 1.6 Proportional morbidity (% of outpatient visits attributed to malaria) of confirmed and presumed malaria cases by district in 2009 (from USAID, 2010)

With the full support from government and WHO, NMCP has set up a strategic plan to effectively prevent, control and treat malaria. Because of the rise of sulfadoxine-pyrimethamine resistance, WHO recommends the use of Artemisinin-based combination therapy (ACT) and the one used in Rwanda is artemether-lumefantrine. ACT are available, long lasting insecticidal treated nets were distributed since 2005 (up to 81% of entire population), health insurance scheme was introduced in the whole country since 2004, training sessions were organised nationwide for community health workers and diagnosis and treatment are accessible to everyone affected by malaria. Consequently, in 2010, Rwanda experienced a reduction of approximately 74 and 55 % for the number of confirmed malaria cases among outpatients of all ages and malaria deaths respectively. Those reductions were

even greater for children under 5 years old (WHOb, 2011). It appears clearly that Rwanda is on the right track to reach the objectives of its 2020 vision as well as global health millennium goals. However, efforts are still needed to maintain an effective surveillance system to prevent and to treat malaria properly as the risk of *Plasmodium falciparum* resistance to available drugs and insecticides is still high. In this context, the search for new anti-malarial agents still remains a priority.

I.2. TRYPANOSOMIASIS

I.2.1. General description

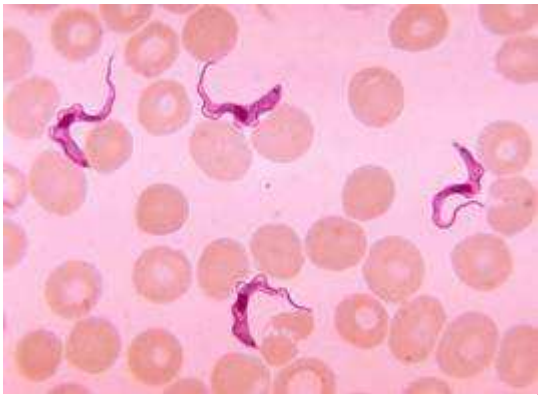

Figure 1.7 Bloodstream *Trypanosoma brucei*

Trypanosomiasis is a parasitic disease caused by trypanosoma, which is a genus of parasitic flagellate protozoa of the family Trypanosomatidae, transmitted to the vertebrate bloodstream, lymph, and spinal fluid by certain insects and often causing diseases in humans and in domesticated animals. Trypanosoma was found for the first time in trout blood by Valentin and in 1845 it was observed for the first time in mammalian (in blood of mole and field mouse) by Gros (Wery, 1995). Many trypanosoma species exist, some are able to affect human and to cause some diseases:

- *Trypanosoma brucei gambiense* found in West Africa, responsible for chronic sleeping sickness or human African trypanosomiasis, it is transmitted to humans by *Glossina palpalis* (Tsetse fly);
- *Trypanosoma brucei rhodensiense* commonly found in Eastern African countries, vector is *Glossina morsitans* and it causes acute sleeping sickness;
- *Trypanosoma cruzi*, found in South and Central America, the parasite is zoonotic, mainly transmitted by blood-feeding with *Rhodnius prolixus* but can be transmitted to human through contaminated blood transfusion (Salem et al., 2006) to cause American trypanosomiasis or chagas disease.

Some trypanosome species cause animal trypanosomiasis such as *Trypanosoma brucei brucei* and *trypanosoma vivax* (cattle, antelope, horses and camels), *Trypanosoma equiperdum* (horses and donkeys). In this section we will focus only on human African trypanosomiasis.

I.2.2. Human African trypanosomiasis

I.2.2.1. Description

As earlier mentioned, human African trypanosomiasis (HAT) also called sleeping sickness, is caused by two flagellated protozoa, *Trypanosoma brucei rhodensiense* (in East and South Africa) and *Trypanosome brucei gambiense* (in West and Central Africa) (Hoet et al., 2004; Salem et al., 2006). As mentioned above, the disease is transmitted to human by the bloodsucking *Glossina palpalis* called tsetse fly. Since 1970 this disease has increased dramatically and caused then a significant health and economic problems in Sub Sahara areas because of limited heath systems. Up to 40 000 cases per year and as many deaths were reported by the 1990s (Jung and Elain et al., 2008). Is it estimated that HAT is endemic in over 30 African countries and in some countries such as Angola, the Democratic Republic of Congo, Uganda and South Soudan more than 60 million people reached epidemic proportions (see figure 1) and about 300 000 to 500 000 undiagnosed cases were reported. According to WHO report, *T. b. gambiense* is endemic in 24 countries of west and central Africa and causes more than 90% of reported cases of sleeping sickness. *T. b. rhodesiense* is endemic in 13 countries of eastern and southern Africa, representing less than 10% of reported cases. The African Region has the largest proportion of reported cases (90%) and the Eastern Mediterranean Region the remaining 10% (WHOb, 2010). For the first time, new cases of HAT has dropped below 10 000 in 50 years. Indeed, in 2009, there were 9 878 reported cases compared with more than 17 600 in 2004 and almost 38 000 only in 1998 (WHOb, 2010).

I.2.2.2. Life cycle and transmission

Tsetse fly ingests bloodstream trypomastigotes from an infected mammalian host during a blood meal. The parasites enter the digestive tract of the fly and during the following 3 to 5 weeks undergo multiplication and morphologic changes as well as migration into the salivary glands, transform into epimastigotes and then into infective metacyclic trypomastigotes. The cycle in the fly is rarely finished in the field and only about 0.1 % of flies reach the mature infection phase (Brun et al., 2010). When the tsetse fly bit another mammalian host during a blood meal, it injects trypomastigotes in the skin. The injected metacyclic trypomastigotes transform into bloodstream trypomastigotes, which are carried to other sites via the lymphatic system and multiply by binary fission in various body fluid. During this stage (1 to 3 weeks after inoculation) some general symptoms like bout of fever, headaches and joint pains appear. Then the trypanosome may eventually migrate to intestinal space to cause

endarteritis. In the last stage of the disease trypanosoma may pass through the blood-brain barrier and reach the central nervous system resulting in the progressive neurological deterioration and probably encephalopathy (Jung and Elain et al., 2008).

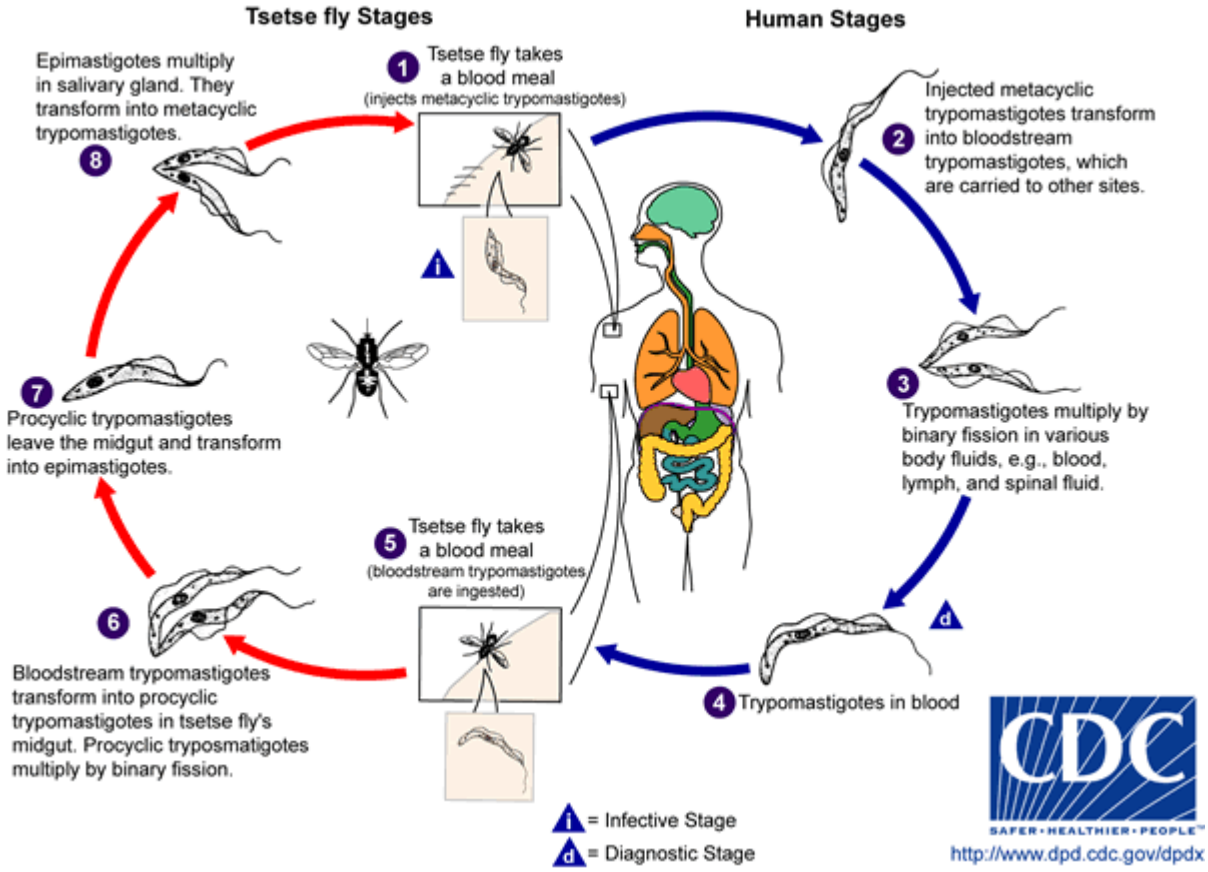

Figure 1.8 Life cycle of *T. brucei gambiense* and *T. brucei rhodesiense* (from Centers for Diseases Control and Prevention (CDC) http://www.cdc.gov/parasites/sleepingsickness/biology.html)

I.2.2.3. Diagnosis, treatment and prevention

Diagnosis is very important but requires some specific biochemical analysis and qualified staff and those requirements are lacking in most rural areas where the parasite is endemic. The diagnosis of this disease is done by confirming the presence of the parasite in any biological fluid, usually in the blood and lymph system. The main challenge is the absence of specific symptoms during the first stage of the disease as well as the lack of sensitivity of the parasitological methods available. The diagnosis is then very difficult and sometimes symptoms observed from patient are mis-interpreted as malaria or influenza (Hoet et al., 2004). So far, the only available serological test for screening is Card Agglutination Trypanosomiasis Test (CATT), used exclusively for *T .b. gambiense* infections and for establishing suspicion of infection. Parasitological tests determine the presence of trypanosomes in the patient to confirm the infection. Even if trypanosome is present in any

body fluid, the parasitaemia could be to low (especially in the gambiense form of the disease) to be detectable by available parasitological methods which have a low sensitivity. This means that a negative parasitological result in the presence of a positive serological test does not necessarily prove the absence of infection. Then tests must be repeated a couple of times to complete diagnosis (WHO).

The treatment of HAT is complicated because of its cost and the lack of efficient drugs. So far, the only commercial drug available for the treatment of advanced stage sleeping sickness cases is Melarsoprol (Arsobal®). However, in the last year some endemic countries like Angola, the Democratic Republic of Congo, Sudan and Uganda reported some cases of Melarsoprol treatment failure with a rate of over 20% (WHOa, 1999). *T. b. rhodesiense* resistance to pentamine and to eflornithine was also observed in laboratory (Croft et al., 1997). Table 1.2. provides the recommended treatment of HAT according to World Health Organisation (1999) and CDC (2010) (CDC, 2010; WHOa, 1999).

To prevent HAT transmission, two principal strategies can be used:

- Reduction of human reservoir of infection by finding and treating individual cases. This requires a surveillance system. For instance, suspected or endemic villages should provide village-based data before treatment.
- Reduction of man-fly contact using adapted vector control (tsetse fly). Various methods may be used including the use of tsetse fly traps and insecticides (ground or aerial spraying) (Wery, 1995; WHOb, 1999).

Table 1.2 The recommended treatment of HAT (WHO, 1999; CDC, 2010)

Name of the drug	Indication	Dosage	Most frequent adverse reactions
Pentamidine isethionate	Early stage of HAT due to *T. b. gambiense* infections	4 mg base per kg body weight daily or every other day by intramuscular injections (7 to 10)	Generally reversible: hypotension, abdominal pain, vertigo, hypersalivation and mild nephrotoxicity
Suramine sodium	Early stage of HAT due to *T. b. rhodesiense i*nfections	Adult dosage: 1 g per a single weekly intramuscular injection for 6 weeks Pedriatic dosage: 20 mg/kg intravenous on days 1, 3, 5, 14 and 21	nausea, vomiting, urticaria and less often renal damage and exfoliative dermatitis
Melarsoprol (Arsobal®)	Advanced stage of HAT, when the central nervous system is affected, in both *T. b. gambiense* and *T. b. rhodesiense* infections	2-3.6 mg per kg body weight by strict intravenous injections (3 series of 3) given with a 7 to 10 day rest period between each series	reactive encephalopathy (the most serious) myocardial damage, hypertension and exfoliative dermatitis
Eflornithine (Ornidyl®)	late stage of HAT due *T. b. gambiense* infections. Suitable in patients where melarsoprol has failed	400 mg per kg body weight evenly divided in 4 daily intravenous infusions (every 6 hours) during 7 or 14 days	Reversible: diarrhoea, anaemia, thrombocytopenia, vomiting and fever

I.2.2.4. Trypanosomiasis in Rwanda

In 2009, only sporadic cases of trypanosome were reported in Rwanda and transmission of the disease seems to have stopped (see Figure 2). However, there are still some areas where tsetse flies, the vectors of HAT are still present like in National Park of Akagera in Eastern Province of Rwanda and according to WHO (2011), Rwanda is a part of endemic country even if no updated data about trypanosomiasis incidence are available (WHOa, 2011). Furthermore, Rwanda is the most densely populated country in Africa (Ndaruhuye et al., 2009) and people are looking for some available areas where they can cultivate, hunt or fish. They don't hesitate to venture in the National Park of Akagera where tsetse flies can easily transmit the disease. Recently, a project to protect the whole Akagera National Park started and will hopefully contribute to the prevention of the disease. It must be pointed out also that

countries bordering Rwanda, particularly the Democratic Republic of Congo (RDC) and Uganda are affected (WHOb, 2010); meaning that the risk is still there. Rwanda is sadly exposed to both types of African Trypanosomias, *T. b. gambience* present in RDC and Uganda and *T. b. rhodensis* in Uganda. Rwandan refugees from neighbouring countries could also bring back the parasite and another risk of HAT contamination looms large if appropriate prevention strategies are not implemented.

I.3. LEISHMANIASIS

I.3.1. General Description

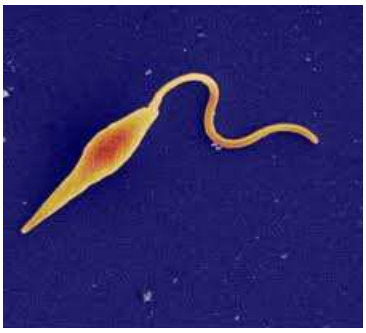

Figure1.9 Lieshmania

Leishmaniasis is a parasitic disease caused by twenty pathogenic *Leishmania species* and transmitted through the bites of infected female sand fly *Phlebotomus* and *Lutzomyia*. Leishmania is a flagellate protozoan which belongs to Trypanosomatidae family and parasites the tissues of vertebrates causing different types of the disease. Cutaneous form is the most common (skin ulcers usually form on exposed areas, such as the face, arms and legs) and nearly 2% of patients of cutaneous form may develop a mucosal leishmaniasis which occurs some months or years after apparition of cutaneous lesions (Bonaventura et al., 2006). It can provoke progressive lesions that can partially or totally destroy the mucous membranes of the nose, mouth and throat cavities and surrounding tissues. The most severe form is visceral leishmaniasis, also known as kala azar, which attacks various internal organs (spleen and liver) and can be fatal within two years if untreated. Rare is post kala azar dermal leishmaniasis which is a chronic cutaneous leishmaniasis appearing in some patients prio-affected by visceral leishmaniasis. Another form of leishmaniasis, difficult to treat, is diffuse cutaneous leishmaniasis which produces disseminated and chronic skin lesions.

According to WHO, leishmaniasis is one of the most neglected diseases in the world, it is prevalent in 88 countries mainly in rural areas, and about 350 million people are at risk to get contaminated (WHOa, 2010). Approximately more than 1.6 million new cases occur annually but only about 600,000 are reported. From 1.6 million estimated cases about 500,000 cases are visceral mostly (90%) in Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan and 1.1 million cutaneous mainly in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru,

Saudi Arabia, Sudan and the Syrian Arab Republic or mucocutaneous mostly in Brazil, Peru and the Plurinational State of Bolivia (WHOb, 2010).

I.3.2. Life cycle and transmission

The life cycle of leishmania has two stages, the sand fly stage which is a flagellated promostigote stage and the human stage, a non-flagellated amastigote stage.

Human stage: during a blood meal, infected sand fly injects into the skin promostigotes which are phagocytised by neutrophils present at the bite site. The promostigotes are released by neutrophils and then consumed by macrophages, histocytes and monocytes of different organs where they transform into amastigotes, the tissue stage of the parasite. Amastigote multiply by simple division in cell (including macrophage) of various tissues. The destruction of host cell provokes the dissemination of the parasite into blood and lymph system and the parasite are again phagocytised by new cells.

Sand fly stage: during another blood meal, sand fly ingests infected macrophages. After ingestion of the parasite, amastigotes migrate in the midgut and transform into promastigotes which after division reach the anterior midgut and foregut. From there, another human stage will start if the infected sand fly takes blood meal and injects promastigotes into the skin of a mammilia host.

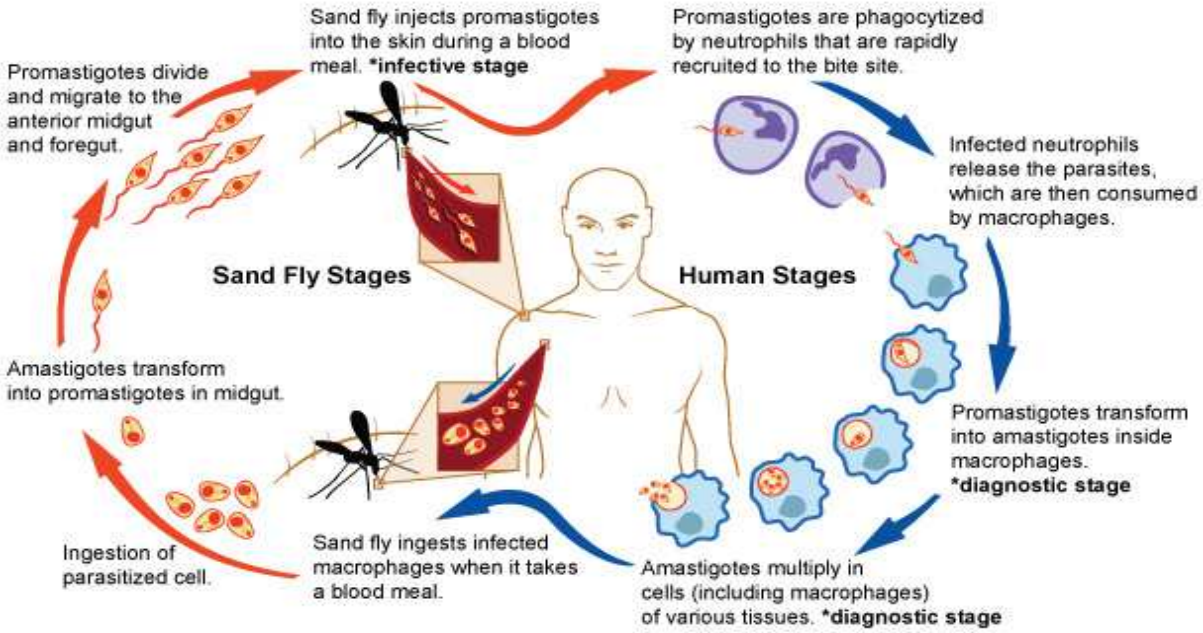

Figure 1.10 Life cycle of leishmania (from National Institute of Allergy and Infectious Diseases (NIAID))

The transmission can be zoonotic (rodent or dog) or anthroponotic for example by blood transfusions or contaminated needles or rarely from a pregnant woman to her baby.

I.3.3. Diagnosis, treatment and prevention

The signs and symptoms of visceral leishmaniasis are quite similar to chronic malaria, schistosomiasis or other systemic infection's ones. Similarly, cutaneous leishmaniasis symptoms may mimic that of staphylococcal or streptococcal skin infections or mycobacyterial ulcer, leprosies, etc. It is also difficult to distinguish from mucocutaneous leishamaniasis cases to, for example, allergic rhinitis, deep mycoses, lymphoma infections (WHOa, 2010). In case of cutaneous leishmaniasis diagnosis may be done by parasitological test from the lesion. A microscopic examination of stained smear can detect the presence of intracellular amastigotes in macrophage. According to CDC, visceral leishmaniasis may be diagnosed by taking a blood sample and/or taking a biopsy from the bone marrow to show the parasite. Diagnosis of cutaneous leishmaniasis will require a small biopsy or scraping of the ulcer whereas a biopsy of the affected tissues is needed to diagnose mucocutaneous leishmaniasis. Biopsy samples are examined using microscopy, culture or other methods to isolate the parasite and identify the specific kind of *Leishmania* causing the ulcer.

The treatment of leishmaniasis is given only after confirmation of the disease, patients should consult with their primary health care provider and some may be recommended to look for specialist, such as a dermatologist or infectious disease specialist. The treatment depends on the form of the disease and sometimes the disease may not require treatment. As recommended by WHO, the treatment regimen of leishmaniasis must follow national and regional guidelines to avoid drug resistance. In endemic countries drug policy, therapeutic decision will be based on the individual benefit-risk ratio, health service setting, public health consideration and on the availability of anti-leishmanial drugs. Indeed, it has already been reported that the infecting species and geographical region affect the efficacy of treatment (WHOa, 2010). Special attention is paid on African endemic countries and the recommended treatment regimens for each form of leishmaniasis are given below. Antileishmanial medicines and treatment regimens are qualified by grade of evidence as follows:

****: evidence found with at least one properly designed randomized controlled trial

*** : evidence observed from well-designed trials without randomization

**: opinions of respected authorities based on clinical experience, descriptive studies or reports of experts committees

* : expert opinion without consistent or conclusive studies (WHOa, 2010).

Visceral leishmaniasis caused by *Leishmania donovani* in East Africa (Ethiopia, Eritrea, Kenya, Somalia, Sudan and Uganda) and Yemen

1. Combination: pentavalent antimonials (20 mg +/kg per day intramuscularly or intravenously) + paromomycin (15 mg (11 mg base) per kg body weight per day intramuscularly) for 17 days ****
2. Pentavalent antimonials: 20 mg /kg per day intramuscularly or intravenously for 30 days ****
3. Amphotericin B deoxycholate: 0.75–1 mg/kg per day by infusion, daily or on alternate days, for 15–20 doses ****
4. Miltefosine orally for children aged 2–11 years, 2.5 mg/kg per day; for people aged 12 years and < 25 kg body weight, 50 mg/day; 25–50 kg body weight, 100 mg/day; > 50 kg body weight, 150 mg/day; orally for 28 days for 28 days****
5. Liposomal amphotericin B: 3–5 mg/kg per daily dose by infusion given over 6–10 days up to a total dose of 30 mg/kg***.

In some special cases like pregnancy or lactation and leishmania-HIV coinfection, we can use preferentially Amphotericin B deoxycholate and lipid formulations.

Post-kala-azar dermal leishmaniasis (PKDL) in East Africa

1. Pentavalent antimonials: 20 mg/kg per day intramuscularly or intravenously for 30–60 days, when indicated**
2. Liposomal amphotericin B: 2.5 mg/kg per day by infusion for 20 days, when indicated**.

Cutaneous leishmaniasis

Local therapy

Leishmania major:

- 15% paromomycin plus 12% methylbenzethonium chloride ointment twice daily for up to 20 days****;
- Intralesional antimonials, 1–5 ml per session plus cryotherapy (liquid nitrogen: – 195 °C), both every 3–7 days (1–5 sessions)****
- Thermotherapy: one or two applications of localized heat (50 °C for 30 seconds) ****

- intralesional antimonials or cryotherapy independently, as above.

Leishmania tropica, Leishmania aethiopica and *Leishmania infantum*

- 15% paromomycin/12% methylbenzethonium chloride ointment, as above*
- intralesional antimonials plus cryotherapy, as above *
- thermotherapy, as above****
- intralesional antimonials, alone, as above***
- cryotherapy, alone, as above**

Systemic therapy

Leishmania major

- fluconazole, 200 mg oral daily for 6 weeks****
- pentavalent antimonials, 20 mg/kg per day intramuscularly or intravenously for 10–20 days*
- pentavalent antimonials, 20 mg/kg per day intramuscularly or intravenously plus pentoxyfylline, 400 mg three times a day for 10–20 days****

Leishmania tropica and *Leishmania infantum*

- pentavalent antimonials, 20 mg /kg per day intramuscularly or intravenously for 10–20 days*
- pentavalent antimonials, 15–20 mg/kg per day intramuscularly or intravenously for 15 days plus oral allopurinol 20 mg/kg for 30 days, to treat leishmaniasis recidivans caused by *Leishmania tropica***

Leishmania aethiopica

Pentavalent antimonials 20 mg/kg per day intramuscularly or intravenously plus paromomycin, 15 mg (11 mg base)/kg per day intramuscularly for 60 days or longer to treat diffuse cutaneous leishmaniasis**.

Mucocutaneaous leishmaniasis

All species

- pentavalent antimonials: 20 mg/kg per day intramuscularly or intravenously for 30 days**

- pentavalent antimonials: as above plus oral pentoxifylline at 400 mg/8 h for 30 days****
- amphotericin B deoxycholate: 0.7–1 mg/kg by infusion every other day up to 25–45 doses**
- liposomal amphotericin B: 2–3 mg/kg daily by infusion up to a total dose of 40–60 mg/kg**

Note that there is little data available on the therapy of mucocutaneaos leishmaniasis due to *Leishmania aethiopica.*

No vaccine for general use against leishmaniases is available but usually strong immunity was observed after recovering from infection meaning that there is a possibility to find a reliable vaccine. Moreover, one vaccine, Leish-111f + MPL-SE, is being evaluated for the immunotherapy of PKDL in the Sudan, in phase- 1‑2 trials in Peru and in a phase-1 trial in India. Another option to get immunity is leishmanization where live *Leishmania major* promastigotes from a fresh culture are inoculated into the derma but it is not recommended in normal conditions and should be used for research (WHOa, 2010).

The prevention of leishmaniasis infection is based on the control of reservoir hosts (WHOa, 2010). Individual protection from sand fly bites as well as the continual case detection, surveillance, adequate treatment and prevention of reinfection are highly recommended. Furthermore, in some endemic areas, human constitutes an important reservoir for visceral leishmaniasis and some parasites such as *Leishmania tropica* depend on humans to survive. WHO and CDC recommend the use of insecticide-treated bed nets or insect repellent to exposed skin.

I.3.4. Leishmaniasis in Rwanda

Similar cases of leishmaniasis are known in Rwanda, unfortunately there is no official data about the prevalence of the disease. Considering that the spread of HIV infection (pandemic in Rwanda (MOH, 2011) makes people susceptible to visceral leishmaniasis (Alvar et al., 2008) we may say that the degree of risk in Rwanda is high. Furthermore, there is no national disease control plan to address fight against neglected tropical diseases such leishmaniasis in Rwanda (http://cghed.ei.columbia.edu/?id=projects_ntd).

I.4. AMOEBIASIS

I.4.1. General description

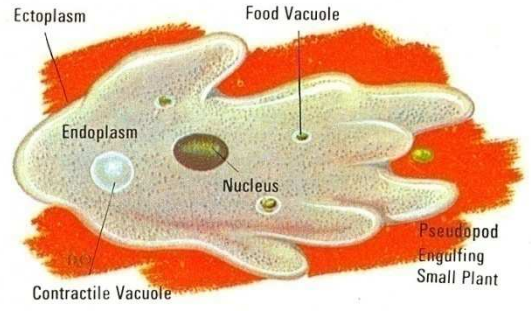

Figure 1.11 *E. histolytica*

Amebiasis is a parasitic and infectious disease caused by a protozoan called *Entamoeba histolytica*. People affected by amebiasis may have a wide range of symptoms, including diarrhea, fever, and cramps. The disease may also affect the intestines, liver, or other parts of the body. Invasive amoebiasis is the second most common cause of mortality due to parasitic infections in the world (Stanley et al., 2003) whereas amoebic colitis amoebic abscesses are the third leading cause of death from parasitic diseases worldwide (Salle et al., 2007). It is estimated that more than 280 million people are infected (Upcroft et al., 2001). The disease is worldwide distributed, but the high rate is found in tropical areas especially in developing countries as a result of poor sanitation where, according to WHO, the disease has a prevalence of 50 % of the general population and causes more than 100.000 deaths every year.

Entamoeba hystolytica is a pathogenic amoeba, associated with intestinal and extraintestinal infections and is one of the main protozoal pathogens of the human intestinal tract (Jung et al., 2008). Mobile trophozoites of *Entamoeba hystolytica* were found for the first time in 1875 at Saint Petersbourg by Lösch from the faeces of a farmer with acute dysentery. The parasite may occur in two forms either in infective form, cyst or in invasive form, trophozoite. Trophozoites are sensitive to outside body conditions whereas cysts can survive weeks in external milieu. Most of the time, the parasite affects human and primates but can attack dogs and cats.

I.4.2. Life cycle and transmission

Human consumes water or food contaminated by cysts which migrate to small intestine where they transform in feeding stage, trophozoite. Trophozoites invade large intestine of the host and multiply by binary fission. Some parasites will stay colonizing the lumen or invade the wall of the intestine and multiply in tissue and this continual invasion may cause both

superficial and deep lesions. Amoeba may enter portal venous or lymphatic system and then reach other organs such as liver, brain, lungs, spleen, kidney, genital organs, urinary track, and skin. The most affected organ is the liver where hepatic abscesses appear but other abscess of the lungs and brain were observed (Virk, 2008). Other parasites after multiplication by binary fission transform in cysts which will exit the body transported by faecal matter (Wery, 1995). *Entamoeba histolytica* transmission will be done by cysts present in water contaminated with faeces or food. Most of the time human is infected after ingestion of contaminated water or food but amoebiasis can be spread from amoebic person-to-person contact for example for people who have anal intercourse. Only human and some non human primates constitute the natural reservoirs (Virk, 2008).

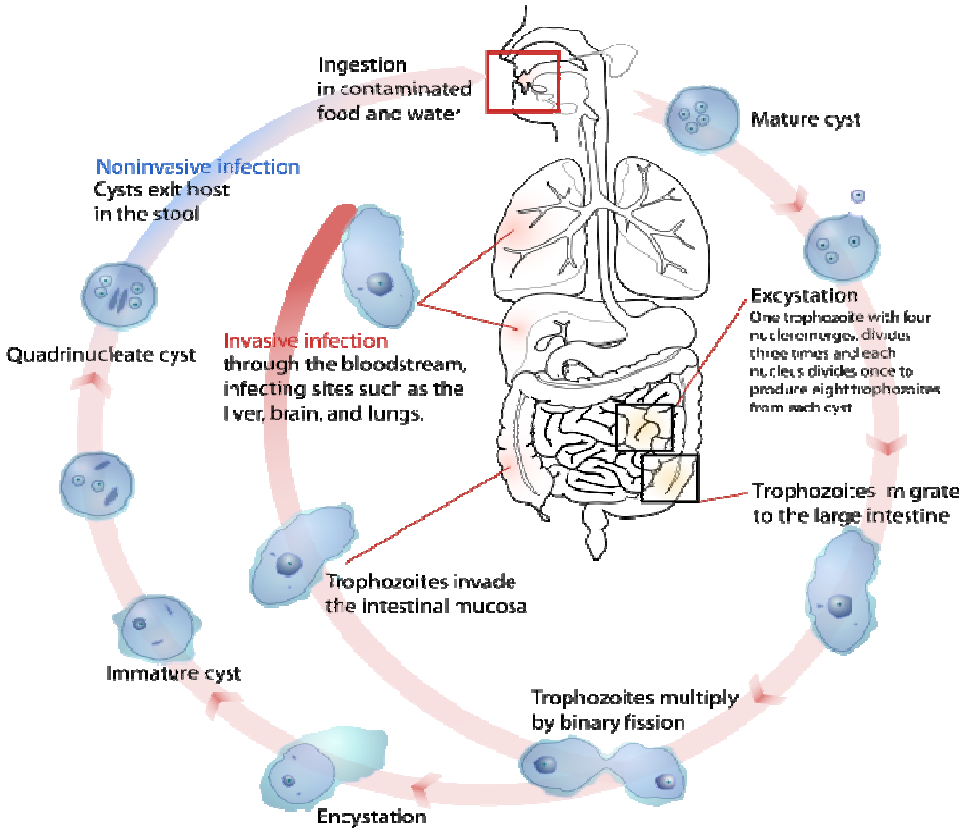

Figure 1.12 Generalized life cycle of *Entamoeba histolytica* (from LadyofHats, 2008)

I.4.3. Diagnosis, treatment and prevention

At early stage of amoebiasis infection, most people do not present any symptom (in case for example of luminal amoebiasis e.i. asymptomatic infection) but some symptoms may be observed one week after contamination. It is estimated that only 10 – 20 % of people infected by the disease present symptoms (Roy et al., 2012). Symptoms vary from mild diarrhoea

(lower abdominal pain or cramps, diarrhoea, fatigue, excessive gas, rectal pain etc.) to severe dysentery (bloody stools eventually with signs of hypovolemia, fever, vomiting etc.). The symptoms of intestinal amoebiasis are dysentery, colitis, appendicitis, toxic megacolon and amebomas. In case of chronic disease, colitis, weight lost and intermittent abdominal pain may be observed (Virk, 2008).

Intestinal amebiasis is commonly diagnosed by examination of the stool by identification of trophozoites and cysts in fresh faeces but it may be difficult to distinguish them from non-pathogenic amoeba species like *Entamoeba dispar* or faecal leukocytes. Then it is recommended to repeat the exam at least two times. Other specific methods exist, for example the use *Entamoeba histolytica*-specific antigen or other immunologic tests but they are not widely available.

Liver abscesses diagnosis may be done using blood test (level of liver enzymes), radiographic studies or serological tests. Some enzyme immunoassay kits for *Entamoeba histolytica* antibody or antigen detection are more useful in this case, but there are only available in developed countries like United States (Virk, 2008).

The most common drug for the treatment of amebiasis is metronidazole, a derivative of 5-nitromidazole. However, this drug has variable efficiency, serious side effects such as headache, nausea, dry mouth, metallic taste, neurotoxic effects which are manifested as incoordination, dizziness, convulsions and ataxia (Kapoor et al., 1999). Some metronidazole failures were already observed (Virk, 2008). Tinidazole, a structural analogue of metronidazole approved by US Food and Drug Administration (FDA) has fewer side effects and is also indicated in some countries such USA (Virk, 2008). There are other non-imidazole drugs such as nitazoxanide, paramomycin and niridazole which are effective against amoeba but all those drugs are less active against the cyst of *Entamoeba histolytica* (Nagpal et al., 2012). The treatment with metronidazole or tinidazole should be followed by a luminal agent such as iodoquinol or paromomycin because it is less active against intestinal forms (Wery, 1995). The recommended doses are:

a) *Intestinal amoebiasis*
   - Metronidazole:
     - Adults: 2 g orally per day in 2 – 3 doses for 5 – 10 days
     - Children: 35 – 50 mg/kg orally per day in 3 doses for 5 - 10 days

- Tinidazole:
  - Adults: 2 g orally per day, single dose for 3 days
  - Children > 3 years old: 50 mg/kg orally per day (up to 2 g) for 3 – 5 days

Amoebic colitis should be treated by metronidazole and a luminal agent such paromomycin or iodoquinol to avoid colonisation (Gilbert et al., 2010; Stanley et al., 2003). The dose for paromomycin is 25-35 mg/kg per day divided into three doses for seven days.

b) *Amoebic liver abscess*
   - Metronidazole: 750-800 mg three times daily for 5-10 days
   - Tinidazole: 2 g daily for five days (Ximénez et al., 2011).

No vaccine or recommended chemoprophilaxis is available. However, amoebiasis infection may be prevented by improving personal hygiene practice. It is then recommended to wash hands before eating food or after going to toilet, to consume clean and safe water (boiled and or filtered water) and food, to prevent the contamination of water supply with faeces, to avoid sexual practices involving contact with faeces etc.

I.4.4. Amoebiasis in Rwanda

There is no official or reliable updated information about amoebiasis in Rwanda. However, Rwanda as well as other tropical developing countries has high prevalence of the disease. According to the local Malaria control program, intestinal parasitosis was the third cause of morbidity in Rwanda in 2004 (Figure 13). Even if more efforts were made to ameliorate health care systems especially after the 1994 Tutsi genocide, Rwanda suffers again from inadequate sanitation in some rural areas. Indeed, in 2004, the use of public sanitation service was estimated at 28.6 % only (PNILPa, 2005). Many cases of heavy transmission and continual infection are very common. It should be also noted that many people do not consult and resort to self-medication with a risk of drug resistance. Moreover, people from rural areas prefer to use medicinal plants which are more available and more affordable meaning that the real incidence of amoebiasis is even higher than officially reported.

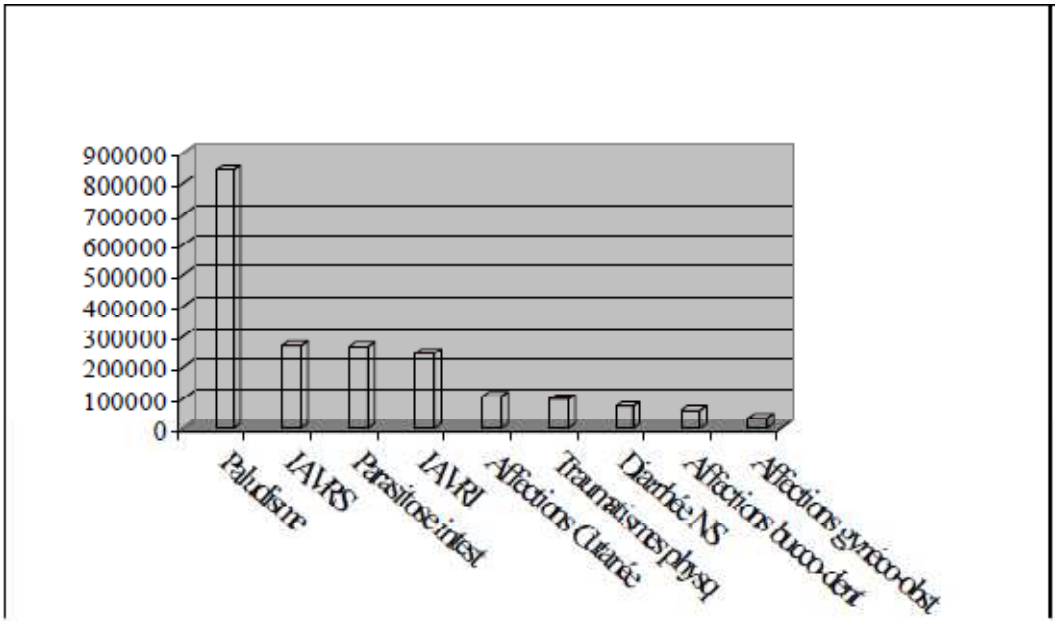

Figure 1.13 The causes of morbidity in Rwanda in 2004 (from PNILP, 2005)

IAVRS (Infection Aiguës des Voies Respiratoire Supérieures): Acute upper respiratory tract infections.

IAVRI (Infection Aiguës des Voies Respiratoire Inférieures) : Acute lower respiratory tract infections.

I.5. INFLAMMATION

I.5.1. Overview

Inflammation is fundamentally a protective and complex response mainly from blood vessels and leukocytes (white blood cells), designed to rid the organism of both the initial cause of cell injury (e.g., pathogens, damaged cells, or irritants) and the consequences of such injury (e.g., necrotic cells and tissues). Mediators and circulating leukocytes, as well as tissue phagocytes that are derived from circulating cells are the major defenders of our body against foreign invaders. The presence of mediators and leukocytes in the blood gives them the ability to go to any site where they may be needed. Because invaders such as pathogens and necrotic cells are typically present in tissues, outside the circulation, it implies that the circulating cells and mediators have to be rapidly recruited to these extra-vascular sites. The inflammatory response coordinates the reactions of vessels, leukocytes, and plasma mediators to achieve this goal. In the practice of medicine, the importance of inflammation is that it can sometimes be inappropriately set of or poorly regulated, and then becomes a

pathophysiologic basis to many disorders (Kumar et al., 2009; Serhan et al., 2010). The inflammation may be a cause of morbidity and mortality (Revillard et al., 2001).

The inflammation is characterized by the increase of blood flow to the tissue concerned, temperature rise, redness, swelling and pain (Revillard et al., 2001) and can be classified as acute or chronic event. Acute inflammation is the initial response of the body to harmful stimuli and is mainly characterised by the exudation of fluid and increase movement of plasma proteins and immigration of leukocytes (mainly polymorphonuclear neutrophil leukocytes) from the blood into the damaged tissues. Chronic inflammation is defined morphologically by the presence of lymphocytes, macrophages and plasma cells in the tissues and may persist for a long period. It is also associated with the proliferation of blood vessels, fibrosis and damaged tissues (Serhan et al., 2010).

Inflammation is a biological response in tissues and various types of cells such as neutrophils, mast cell, eosinophils, basophils, lymphocytes, macrophages and fibroblasts intervene in this complex reaction. Among those cells implicated in inflammation, polymorphonuclear neutrophil leukocytes play a specific role.

I.5.2. Polymorphonuclear neutrophil leukocytes

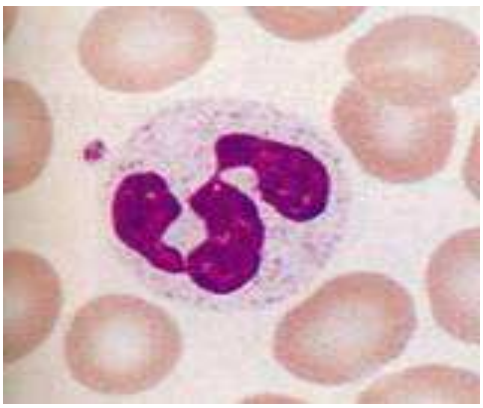

Figure 1.14 Polymorphonuclear neutrophils (from www.paolobellavite.it/neutrophil.html)

Polymorphonuclear neutrophil leukocytes (PMN) also called neutrophils are the most numerous leukocytes present in peripheral blood (90% of cells and 60 – 70 % of leukocytes in the blood) (Lévy et al., 2001). As part of innate immune response, PMN act as the first line of defence against pathogens. The presence of PMNs remains one of the most consistent markers of inflammation as they play a major role in inflammatory response (Firestein et al., 2008; Serhan et al., 2010). PMNs have various functions such as motility, phagocytosis, and bactericidal activity (Walrand et al., 2004) by their ability to generate reactive oxygen species (ROS) such as superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), Hypochlorous acid (HOCl) and nitric oxide ($NO^{\cdot}$) via an activation cascade of several enzymes such us NO synthase, NADPH oxidase and myeloperoxidase (MPO). Moreover, those cells are also specialized in the release of granular enzymes having proteolytic (elastase, colagenase) or oxidant (MPO) activities and also mediators of

inflammation (cytokines, bioactive lipids) which will contribute to the destruction of pathogen agents. Figure 1.15 below illustrates the main elements involved in the inflammatory response of PMNs.

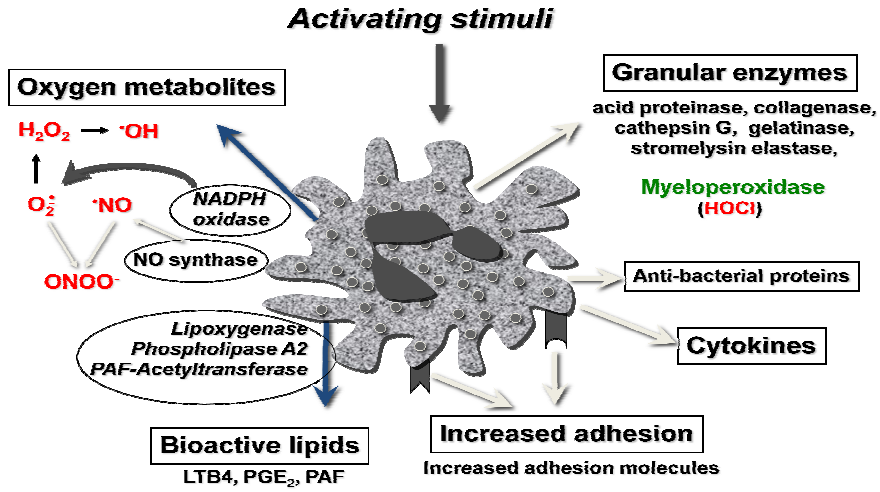

Figure1.15 Neutrophil, a key cell involved in inflammation and immune response (Franck et al., 2009)

I.5.3. NADPH oxidase

NADPH oxidase is multiprotein enzyme with membrane components (gp 91phox and p22 phox (gp: glycoprotein; p: protein; phox: phagocyte oxidase) and cytosolic ones (p40phox, p47phox, p67phox). Once PMN is activated by triggering agents such as agonists (Protein Kinase C: PKC), the cytosolic component undergo phosphorylation, migrate through the plasma membrane and associate to membrane components to became a multiprotein enzyme.

This enzyme will reduce oxygen into superoxide anion ($O_2^{\cdot-}$) (see Figure 1.16) from which other ROS will be generated (see Figure 1.15) either by spontaneous superoxide dismutation dismutase-enhanced dismutation of superoxide anion, yielding hydrogen peroxide (H_2O_2) the substrate of myeloperoxidase (MPO) (Bokoch et al., 2002).

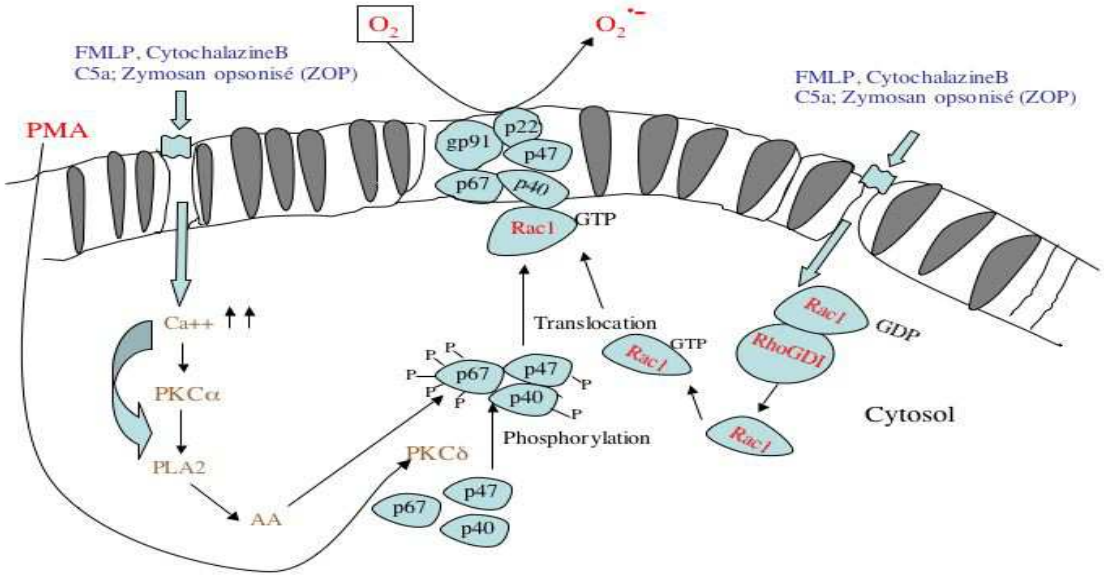

Figure 1.16 Assembling of NADPH oxidase (Mouithys-Mickalad, 2007)
PMA: Phorbol 12-Myristate 13-Acetate

I.5.4. NO synthase

Nitric oxide synthase (NOS) is a key enzyme for the production of $NO^{\bullet}$ which is implicated in physiological and pathological events such as intra-uterine development, cirrhosis, etc. $NO^{\bullet}$ can combine with $O_2^{-\bullet}$ to produce $ONOO^-$ very reactive and responsible for the majority of toxic effects from $NO^{\bullet}$ (Massion et al., 2002).

I.5.5. Myeloperoxidase

Myeloperoxidase (MPO) is a haemic enzyme from primary granules, plentifully released during degranulation of neutrophils and in lesser extent by some monocytes and some type of macrophages. MPO with the membranous NADPH oxidase, generates ROS and participates then in innate immunity (Arnhold, 2004). The excessive concentration of MPO in tissues and in plasma indicates a huge activity of neutrophils (Serteyn et al., 2003). However, elevated concentrations of MPO in patients contribute to tissue damage resulting in significant risk for initialisation and propagation of acute and chronic vascular inflammation diseases. MPO is considered as a biomarker of inflammatory diseases such as cardiovascular events including atherosclerosis (Hazen et al., 1996), cancer (Winterbourn, 1985) (chronic inflammation) and sepsis (acute inflammation) (Kumara et al., 2004). As this enzyme is implicated in various

diseases, it has recently attracted many researchers especially in the evaluation of new products capacity to modulate the MPO activity.

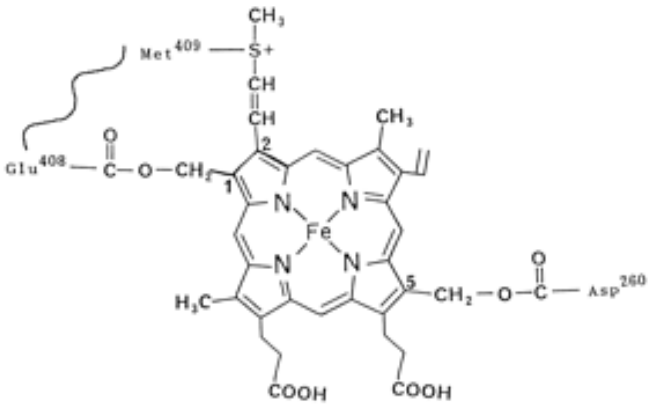

Figure 1.17 Structure of human MPO (heme part) (Andersson et al., 1996)

For this purpose, a new and original method called SIEFED (Specific Immunological Extraction Followed by Enzymatic Detection) can be used to specifically detect the activity of equine MPO and to assess the direct interaction of some substances with the enzyme (Franck et al., 2006). The Figure 1.18 below describes the production of ROS from MPO.

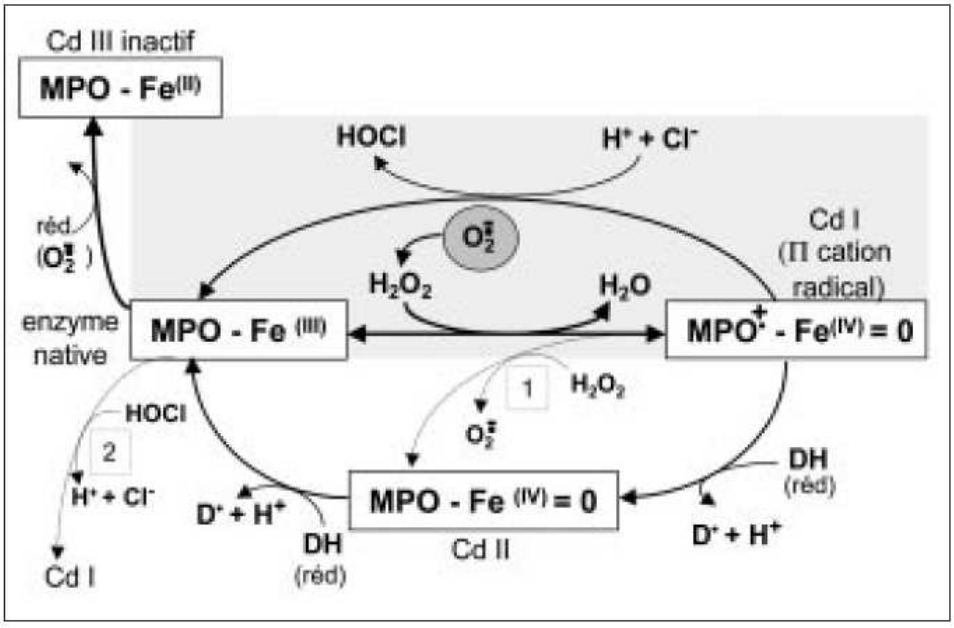

Figure 1.18 Steps of enzyme activity of MPO and the production of ROS. (Serteyn et al., 2003). Cd: compound; réd: reduction reaction

The use of equine MPO gives quite similar results as human MPO even if each enzyme has its own specificities (Deby-Dupont et al., 1998; Mathy-Hartert et al., 1998; Pincemail et al., 1991).

I.5.6. Mediators of inflammation

The mediators which intervene in the inflammation process come from either cells (histamine, serotonin, prostanglandins, leukotrienes, reactive oxygen species, cytokines etc...) or from plasma (some complement fragments, proteases activated during coagulation, etc...). In the present study we will focus on the reactive oxygen species and cytokines which are closely related to our work.

I.5.7. Reactive oxygen species (ROS)

As a consequence of neutrophils exposure to pathogens or other natural stimuli, oxygen-derived reactive species can be released. Within PMNs, the main source of ROS is NADPH oxidase. The production of ROS depends on the activation of NADPH oxidase which reduces molecular oxygen to superoxide anion radical one of the main substrates for myeloperoxidase. As described above, different enzymes are implicated in the production of ROS (Deby-Dupont et al., 1999). The figure 1.19 below illustrates their role.

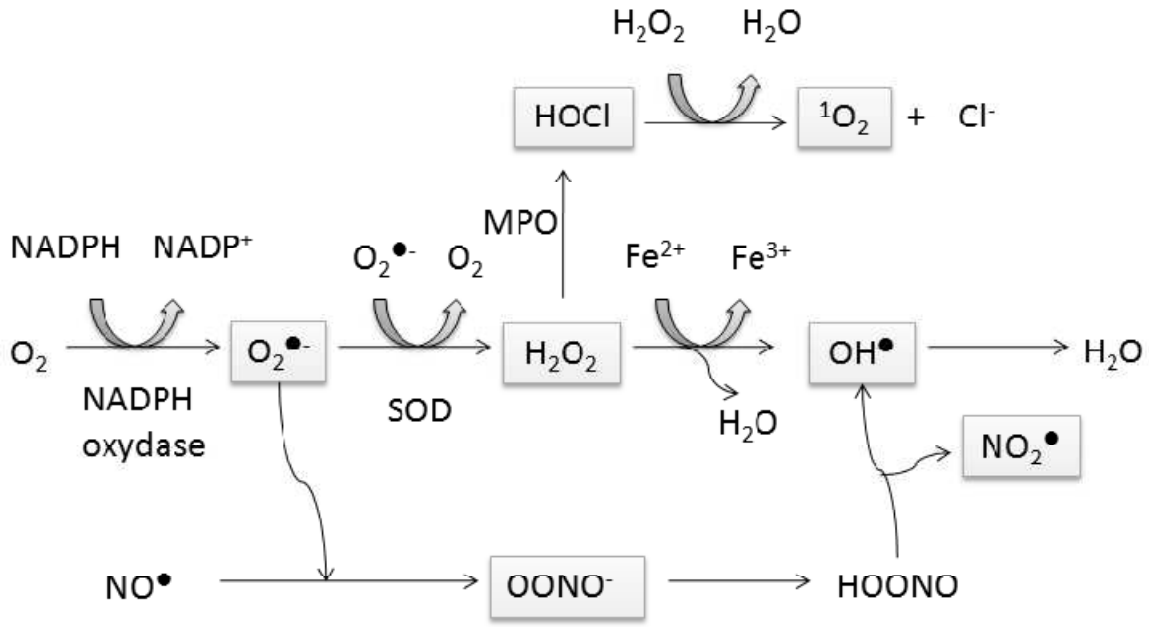

Figure 1.19 Products generated by neutrophils oxidation activity via NADPH oxidase, myeloperoxidase and NO synthase activity (Derochette, 2011)

Considering the key role of ROS in the inflammation process, the decrease of ROS production is an important parameter to study new anti-inflammatory agents. The anti-inflammatory capacity of a sample such as a plant extract can be evaluated in vitro by measuring its potentiality to reduce ROS produced by stimulated equine neutrophils using lucigenin-enhanced chemiluminescence (CL). The stimulation of neutrophils *in vitro* can be obtained by phorbol 12-myristate 13-acetate (PMA) (see Figure 1.16).

I.5.8. Cytokines

Cytokines are soluble proteins or glycoproteins produced by leukocytes and in many cases by other cell types (Fitzgerald et al., 2001). They have many roles; most cytokines play a key role as triggers and regulators of immune responses such as innate immunity and/or the inflammation response. Therefore, they constitute very interesting targets for new therapeutic strategies. However, since more than 90 cytokines and cytokines receptors have been identified, the selection and identification of which cytokines are involved in the development and pathogenesis of a particular disease is a crucial step. Cytokines are involved in many diseases such as inflammatory diseases, bone disorders, metabolic diseases etc (Adkinson et al., 2008; Kopf et al., 2010). The following few lines will briefly deal with the role of inflammatory cytokines in systemic infectious diseases especially malaria.

There has been a great deal of discussion about the mechanism of the disease caused by *Plasmodium falciparum* (malaria). On one hand, some researchers argue the hypothesis based on the concept of insufficient oxygen reaching vital organs. On other hand, other researchers' hypothesis is based on cytokine, in which excessive release of pro-inflammatory cytokines are the primary driving force of malaria disease and ultimately, death. However, it seems clear and many researchers agreed that cytokines such as Tumour Necrosis Factor (TNFα) and interleukin-1 (IL-1) are the essential cytokines in systemic diseases caused by infectious agents (Clark et al., 2006). Recently, there has been an increasing interest on cytokines such as TNFα that are produced during the early stage of inflammation (Kopf et al., 2010). We hope that further researches focusing on the role of cytokine in pathophysiology of malaria and other parasitic diseases will contribute to the development of new drugs.

I.6. THESIS OBJECTIVES

During the past decade, undivided attention has been given to malaria from both political and scientific communities. However, falciparum malaria is still a major health problem

especially in subtropical countries such as Rwanda. In Rwanda, *Plasmodium falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine was observed and the use of long-lasting insecticidal nets to prevent the disease is no longer safe as the resistance to the insecticides pyrethroids was already reported. Consequently, it is imperative to look for new anti-malarial drugs, and medicinal plants constitute an interesting source.

The present work consists of the phytochemical investigation and evaluation of the antiplasmodial activity of Rwandese medicinal plants used in the treatment of malaria. This work involves the investigation of the antitrypanosomal, antileishmanial, antiamoebic as well as the anti-inflammatory activity of selected plants. To reach the aim of our thesis, the followed steps were done:

1. Ethnobotanical survey of medicinal plants used in Rwanda to treat malaria;
2. Preparation of crude extracts from selected plants;
3. *In vitro* and *in vivo* screening of the antiplasmodial activity of crude extracts;
4. Determination of plant extracts selectivity for antiplasmodial activity;
5. Bioguided fractionation and structure determination of active ingredients;
6. Evaluation of the antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activity of selected plants.

1.7. THESIS OUTLINE

The composition of the present thesis consists of two main parts. The first compartment deals with the evaluation of the antiplasmodial activity of medicinal plants used to treat malaria in Rwanda and the second consists of the identification of the active ingredients and the determination of the antiplasmodial, antitrypanosomal antileishmanial, amoebic and anti-inflammatory activity of selected plants. The first part, preceded by a general introduction (Chapter 1) is divided in two chapters: Chapter 2 provides the ethnobotanical survey on the medicinal plants used in Rwanda to treat malaria while Chapter 3 deals with the *in vitro* and *in vivo* evaluation of the antiplasmodial activity of selected plants. The second part has two chapters: Chapter 4 which focuses on the antiparasitic and anti-inflammatory activity of *Terminalia mollis* and Chapter 5 which provides the antiparasitic and anti-inflammatory activity of *Zanthoxylum chalybeum*. Chapter 6 forms the general discussion and Chapter 7 deals with conclusions and perspectives of the study.

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PART ONE

PART ONE

The first part of this dissertation compiles all results from the screening of Rwandan medicinal plants for antiplasmodial activity. It is divided in two chapters:

1) Chapter II which deals with the ethnobotanical survey of medinal plants used in the treatment of malaria;
2) Chapter III which reports the results from the *in vitro* and *in vivo* evaluation of the antiplasmodial activity of selected plants.

CHAPTER II. RWANDAN MEDICINAL PLANTS USED IN THE TREATMENT OF MALARIA

Although Rwanda is a small country, it has a rich biodiversity of flora. Ethnobotanocal research is a crucial step in the screening of medicinal plant for their beneficial properties. For this purpose, in collaboration with Rwandan Institute of Scientific and Technological Research, a list of traditional healers and their corresponding address was established. Each participant was interviewed individually. All ethnobotanical and ethnopharmacological data obtained were compiled and analysed based on literature. The results from this study are presented below as article under submission.

Abstract

Concerned about the search for new antimalarial drugs, an ethnobotanical survey was carried out in Rwanda. The aim of the study was to identify Rwandan medicinal plants used in the treatment of malaria through a questionnaire addressed to local traditional healers. As results, 111 folkloric preparations were inventoried mainly as mixtures of various plants and only 35 (31.7%) were used alone. From the most cited and based on literature review, 13 plants were selected and 19 plants samples collected for further antiplasmodial activity evaluation.

Key worlds: ethnobotany, malaria, Rwandan medicinal plants, traditional healers, voucher specimen

II.1 INTRODUCTION

Despite the strenuous efforts of the international health community, malaria remains a major cause of illness and death especially in tropical countries (WHOb, 2011). As it is the case in many Sub-tropical areas, malaria in Rwanda is not only a serious health problem but also an important economical challenge (Ettling et al., 1991; Sagara et al., 2009; Tabuti, 2008; UN,

2011). The government of Rwanda through National Malarial Control Program and his partners committed to eradicated malaria in the country by using the recommended artemisinin based combination therapies (ACTs), insecticide-treated bed-nets and health education (Fanello et al., 2007; USAID, 2010). However, an increase in malaria cases locally observed in 2009 may again happen and recently, it has been reported that malaria affects one out of six children under five years of age at Butare, one of the endemic areas in Southern Province of Rwanda (Gahutu et al., 2011). Therefore, new approaches for development of new drugs to treat and to prevent malaria will be of interest and medicinal plants constitute an incomparable source (Ginsburg et al., 2011). Moreover, medicinal plants play an important role in health care in Rwanda; it is estimated that between 60 and 80 % of the population use to initially consult a traditional healer before going to health care or hospital facilities (Balick et al., 1996; Barnes et al., 1996). Several medicinal plants have been used to cure malaria in Rwanda and continue to serve especially in rural areas, even if artemeter + lumefantrine (Coartem®), the recommended ACT is available (RNA, 2009; WHO, 2008). However, traditional medicine practitioners in Rwanda are not allowed to treat malaria and they are then afraid to talk about the plants they are secretly using to cure this disease. As a part of the search for new antimalarial drugs an ethnomedicinal survey was conducted and this study is reporting the results of that investigation.

II.2. METHODOLOGY

II.2.1. Study area

The investigation on the traditional medicinal plants used to treat malaria in Rwanda was conducted in the whole country. As the origin of many drugs discovered is plants used in traditional medicine (Cotton et al., 1996; Ginsburg et al., 2011; Matu et al., 2003; Njoroge et al., 2006), the approach used in this study is based also on traditional medicine. Traditional healers from Nyagatare, Kayonza, Rwamagana and Nyamata (Eastern Province), Musanze (Nothern Province), Kigali (Kigali Province), Muhanga, Huye and Nyamagabe (Southern Province), Rubavu, Karongi and Rusizi (Western Provinces) were contacted and interviewed. The areas of collection are shown in Figure 2.1.

II.2.2. Data collection

The study was conducted between April and October 2007. Targeting medicinal plants used to cure malaria for which modern therapy is recommended by local ministry of health and WHO, it was not easy to get the needed information from traditional healers who are supposed to follow the policy. Therefore, all ethnobotanical information was obtained from traditional healers who are already in contact with the local Institute of Scientific and Technological Research whose French acronym is IRST, through his Phytomedicine and Life Sciences Research Programme, the former "CURPHAMETRA". Those traditional healers are ready to collaborate and freely give all needed information about the plants they are using. Data were collected through a survey employing interviews based on a checklist of the following questions: the name of traditional healer, known signs and symptoms of malaria, the name of the plant and the part(s) used to cure the disease, the mode of preparation, doses, side effects and contraindications. All data collected were analysed and compared to available literature review. Voucher specimens of botanic species of our interest were collected, identified by a specialist and deposited at National Herbarium (Butare).

II.2.3. Collection of the plants

Plants were selected according to their frequency of use in different regions, their use in the most endemic regions, the fact that they are used alone (not in combination with other preparations) and the fact that the plant has not yet been or poorly studied for antiplasmodial activity.

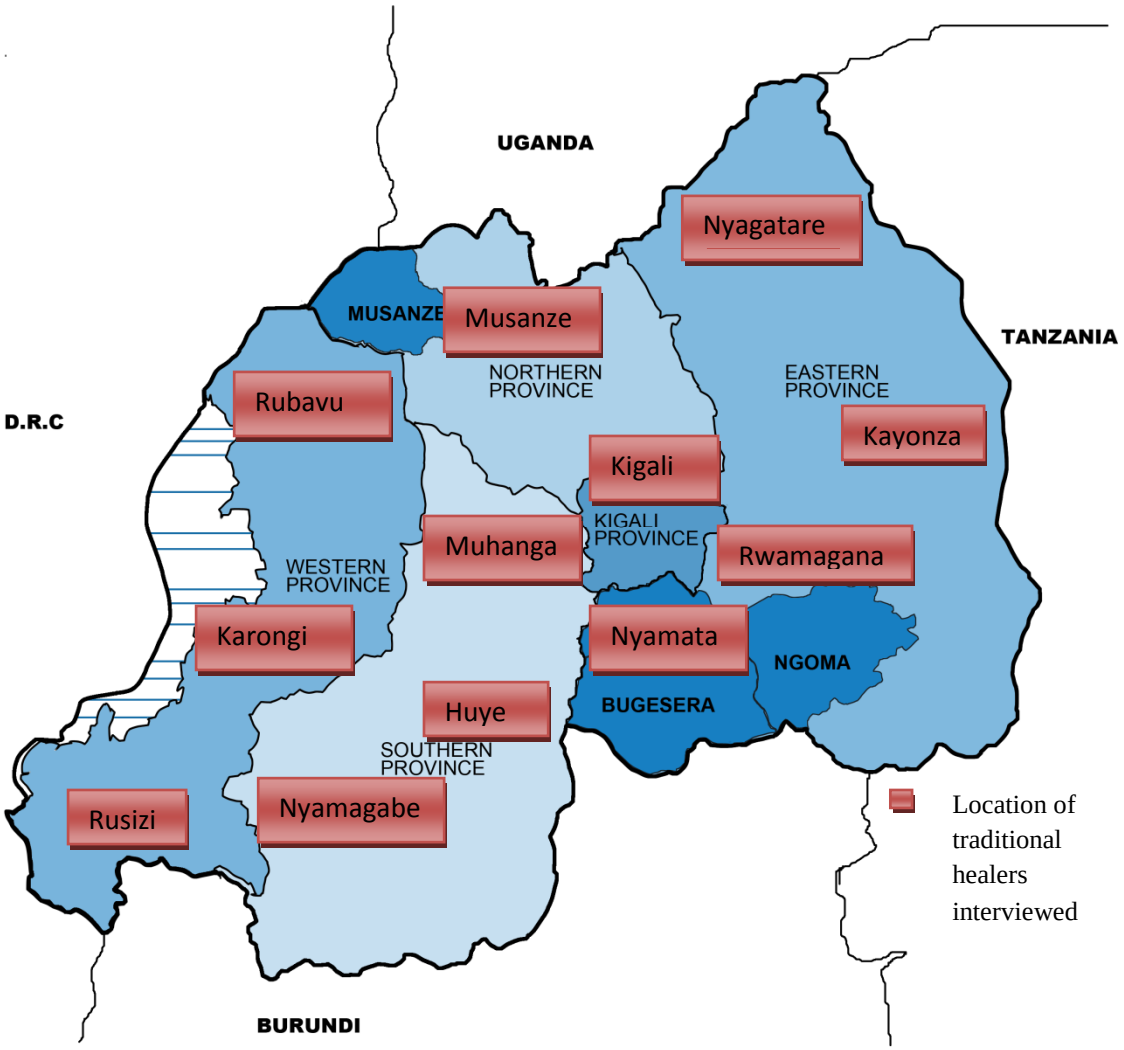

Figure 2.1 Map of Rwandan, provinces and residences of traditional healers interviewed (adapted from http://www.rwandaworks.com)

II.3. RESULTS AND DISCUSSION

A total number of 74 traditional medicine practitioners were interviewed and as recommended by the ministry of health in Rwanda, the majority (almost 99 %) were members of an association or cooperative. The number of men and women was quite similar (53 and 47% respectively for men and women). Fever, headache and asthenia were the common known symptoms of malaria but most of the respondents did not distinguish between malaria and fever. Only 3 traditional healers (< 5 %) claimed that they used to treat patients with malaria infection already confirmed by a health centre. Rwandan traditional healers use mainly a mixture of various plants to treat malaria. Indeed, among 111 different folkloric preparations recorded as malaria remedies, only 35 plants (31.7%) were used alone.

However, we found that some medicine plants used alone by some traditional healers are also used in combination with other plants by their colleagues. It means that, traditional healers use a combination of plants either to reinforce the existing activity of one plant or to hide the real active plant. Nevertheless, some plants are used for their antipyretic activity (example *Polygala luteoviridis*), for their potent diarrheic or emetic activity or just they have many beneficial properties. Plants with emetic and diarrheic activity like *Rumex sp*, and *Solanecio mannii* are used because some traditional medicine practitioners believed that malaria parasite can be eliminated by vomiting or by diarrhea. Some plants such as *Aloe dawei*, *Moringa oleifera* are used for their therapeutic activity or nutritional values (Fahey, 2005) and then are often added in many preparations. The majority of traditional healers does not have enough information about the biology of parasite and try to treat the disease considering the whole body. Based on available literature review on Rwandan medicinal plants, 78 new plants used for the treatment of malaria were found. This may be explained by the fact that new traditional healers came in Rwanda from neighboring countries after 1994 Tutsi-genocide and some vernacular names given by traditional healers were from neighboring countries. This is the case of *Zanthoxylum chalybeum* at Rusizi County where traditional healers call the plant "igugu", a Burundian name, whereas the vernacular name of the plant in Kinyarwanda is "intareyirungu". It was also noticed that some plants coming from aboard are cultivated in Rwanda and used to treat malaria. This is the case of *Azadirachta indica*, *Artemisia annua*, from Asia and *Cinchona officinalis* already known for their antiplasmodial activity (Druilhe et al., 1988; Prakash et al., 2002; Tonk et al., 2006). Different vernacular names were attributed to one plant even in the same area. For instance, *Tithonia diversifolia*'s vernacular names are "Cyimbazi", "Icyicamahirwe", "Igitwelve". Contrarily, some authors attributed for one vernacular name different scientific names which vary from known synonyms. This case concerns "Umunyuragisaka" called, *Dodonaea viscosa* (L) Jacq. or *Solanum terminale* Forssk. (Bloesch et al., 2009; Rwangabo, 1993) but also *Blepharispermum pubescens* S. Moore (Desouter, 1991).

Table 2.1 Plants commonly used for the treatment of malaria in Rwanda and their non exhaustive ethnobotanical information

Scientific and family name	Local vernacular name	Part used	Frequency (%)	Ethnobotanical information
Conyza aegyptiaca (L.) Aiton ASTERACEAE	Wambuba	Leaf	36	**Used alone**: 1) A decoction of powdered dried leaves is made with water. The solution obtained is filtered and one cup is given three times a day. 2) An infusion is made with a handful of the leaf and 1 liter of water and a cup is administered orally three times a day. **In association with *Aloe dawei* & *Clerondendrum fuscum*:** 3 leaves of *C.aegyptiaca* + ½ leaf of *A.dawei* + many leaves of *C. fuscum* are crushed, added to 1 liter of water. The mixture is left to boil and filtered. A sip of the filtrate is given 3 times a day. The preparation may be taken during 3 days in case of high fever and headaches. **In association with *Prunus africana* and *Tetradenia riparia***: a handful of the leaf of each plant is grinded and 1 teaspoon of the obtained powder is added into one liter of water. A half cup of the solution is administered orally.
Microglossa pyrifolia (LAM.) O.Kunte ASTERACEAE	Umunyuragisaka	Leaf	31	**Used alone**: a decoction is made with 2 handfuls of the leaf and 1 liter of water. The solution obtained is filtered and approximately 250 ml of the solution is orally given per day during 2 days. Alcohol is strictly avoided. **In association with *Solanecio mannii* and *Cassia didymobotrya***: a handful of the leaf is crushed and extracted with ± 75 ml. After filtration two and four tablespoons per day during 2-3 days for children and adults respectively. Milk should be avoided. **In association with *Tetradenia riparia***: grinded leaves are extracted with water and one cup is given 3 times a day for adult (a total volume of 1.5 l is enough for adult). For child (at least 10 years of age) 3

				teaspoons per day are orally administered.
Cinchona officinalis L. RUBIACEAE	Ikinini (Quinquina)	Leaf & stem bark	26	**Used alone**: 2 tablespoons of powdered stem bark is extracted with one cup of water and the filtered decoction obtained administered 2 times a day. The leaf is also used but it is less active. **In association** (see other preparations below).
Vernonia amygdalina Del. ASTERACEAE	Umubirizi	Leaf	26	**In association** see other preparations.
Tetradenia riparia (Hochst) Codd LAMIACEAE	Umuravumba	Leaf	22	**Used alone**: An infusion is made with a handful of the leaf and 1 liter of water and one tablespoon is orally administered three times a day. A child (under 7 years of age) may receive a quarter of the adult dose. **In association:** see other preparations
Solanecio mannii (Hook.f.) Jeffrey ASTERACEAE	Umutagara	Leaf	16	**Used alone**: A decoction is made with 10 leaves and ± 200 ml of water. A half of cup 2 – 3 per day. The solution obtained is given with enough quantity of porridge to provoke vomiting. **In association with *Mitragyna rubrostipulata* and *Gomphocarpus semilunatus***: A mixture of five leaves of *S. manii* and five leaves of *M. rubrostipulata* plus and a handful of *C. semilunatus* is grinded and extracted with ± 70 ml of water. A half of the solution obtained is added to 500 ml of banana wine and then used for 2 days. **In association with *Microglossa pyrifolia* and *Cassia didymobotrya*** (see above).
Eucalyptus maidenii (F. Muell.) MYRTACEAE	Inturusu yera	Leaf, fruit & flower	15	**Used alone**: syrup is made with 5 tablespoons of the powdered dried fruit and 1 liter of water and 3 tablespoons given orally two times a day. Alcohol and milk should be avoided. **In association with *Conyza aegyptia* (leaf), *Tithonia diversifolia* (leaf and flower), *Solanecio mannii* (leaf), *Leucas martinicensis* (whole plant) and *Indigofera arrecta* (root)**: two tablespoons of the filtered decoction made with all plant samples grinded is given orally three

				times a day during 3 days. At the last day the dose is reduced. Alcohol and milk should be avoided.
Tithonia diversifolia (Hensl.) A Gray ASTERACEAE	Cyimbazi	Leaf	11	**Used alone**: A decoction is made with a handful of the leaf and 1 liter of water. The dose is one cup 2 times a day. **In association with *Physalis sp.***: A handful of the two plant samples (leaves) is extracted with 2 liters of water. The decoction obtained is filtered and 3 sips taken orally two times a day. The total dose is between 1 litter and 2 liters of the preparation
Rumex bequaertii De Wild POLYGONACEAE	Nyiramuko	Root	11	**In association with *Zanthoxylum chalybeum* and *Cinchona officinalis***: the leaf, stem bark and root bark of *Z. chalybeum*, the stem bark of *C. officinalis* and the root of *R. bequaertii* are dried and crushed and a decoction is made with a mixture of ½ teaspoon of each plant sample and one liter of water. One third of cup of the hot solution obtained is administered orally three times a day during 3 days. One teaspoon and tablespoon are given respectively for infant and child (at least two year of age). This preparation may be appropriate for severe *Plasmodium falciparum* malaria. **In association with *Capsicum sp. + Nasturtium officinale + Ocimum gratissimum + Tetradenia riparia + Bidens pilosa* and *Cinchona officinalis***: the leaf of each plant and the root of Cinchona are crushed and a decoction is made with some water. A half of cup is orally given 3 times a day during 4 days. Alcohol must be avoided.
Aloe dawei Berg LILIACEAE	Igikakarubamba	Leaf	9	In association with other plants (see other preparations)
Zanthoxylum chalybeum Engl. RUTACEAE	Intareyirungu	Root bark	7	**Used alone**: 1) A juice made with the stem bark of the plant is diluted in 250 ml of water and the solution obtained given orally in single dose. This preparation should be avoided in pregnant women. 2) A decoction is made with the root bark of the same plant.

				In association with *Capparis fascicularis*: from a tablespoon of powdered root bark of each plant a decoction is made with 80 ml of water. The dose is 3 tablespoons three times a day. Total dose is 80 ml. Each plant may be used alone with double dose. See above for other associations.
Mitragyna rubrostipulata (K.schum.) RUBIACEAE	Umuzibaziba	Leaf & stem bark	7	**Used alone**: A decoction is made with a handful of the root bark of the plant with 1 liter of water. One cup of the filtered solution is given orally three times a day. **In association with *Indigofera arrecta* (stem bark) and *Conyza aegyptiaca* (leaf)**: The leaf of *M. rubrostipulata*, *C. aegyptiaca* and the stem bark of *I. arrecta* are grinded. A decoction is made with a handful of the mixture and 500 ml of water. The filtered solution is given orally in single dose divided in three doses regiments per day.
Fuerstia africana T.C.E. Fr. LAMIACEAE	Ubushohera	Leaf & stem	6	**Used alone**: 1) A decoction is made with 5 crushed leaves and 250 ml of water. The dose is one tablespoon two times a day per oral administration. Additionally, 2 drops of the infusion made with some leaves are given in each nostril. Alcohol should be avoided. 2) An infusion is made with a handful of the whole plant and 1 liter of water and a cup is administered orally three times a day. **In combination with *Aloe dawei* and *Tetradenia riparia***: the filtrate (± 250 ml) of the decoction made with crushed leaves and water is given in single dose orally. The dose varies according to the age and the weight of the patient.
Trimeria grandifolia (Warb.) FLACOURTIACEAE	Umusabanyama	Leaf	5	**Used alone**: 1) An infusion is made with a handful of the whole plant and 1 liter of water and tablespoonful given three times a day. 2) A half of a cup of the filtrate decoction is given orally in single dose in the morning on an empty stomach. **In association with *Vepris stolzii***: a decoction made with the leaves of

				the plants is filtered and given as a single dose in morning.
Bidens pilosa L. ASTERACEAE	Inyabarasanyi	Leaf	4.5	**Used alone**: a decoction is made with a handful of the leaf and one liter of water and a cup of the filtrate given orally every 3 hours. In association with other plant (see above)
Rumex abyssinicus Jacq POLYGONACEAE	Umufumba	Root	4.5	**Used alone**: an infusion is made with a handful of the whole plant and 1 liter of banana juice and tablespoonful given three times a day.

The plant specimen was identified as *Microglossa pyrifolia* (LAM.) O.Kunte by specialists of the corresponding family, via Belgium National Botanical Garden (Meise). Similarly, *Fuerstia africana* T.C.E. Fr. ("ubushohera") is wrongly called *Orthosiphon australis*. Table 2.1. illustrates the most cited malaria herbal medicines by Rwandan traditional practitioners and their ethnobotanical information. The results indicate that the family of Asteraceae is much more used to cure malaria. In contrast, a recent study conducted in Volcanoes National Park of Rwanda, revealed that the most significant number of species used to cure malaria belongs to the family of Lamiaceae (Nahayo et al., 2010). This may be explained by the fact that the study areas were different. Indeed, this study focused only on plants from Volcanoes Park whose flora is unique. The leaf was the most used part; decoction, the main mode of preparation used by Rwandan traditional healers in the treatment of malaria whereas oral administration was the common way of administration. The majority of malaria herbal remedies is preferentially administered twice a day: in the morning (rising sun) and in the evening (setting sun) and it seems that midday is not well considering. The only reason given by traditional healers is that bad adherence was observed when a medicine was supposed to be taken three (or more) times a day. Overall, the doses were adjusted according to the ages of the patient. Alcohol was the most cited contraindication followed by milk but, in some cases local banana wine was used as solvent or added to preparations probably to facilitate the extraction of active ingredients or to ameliorate the taste of the preparation. In some cases especially paediatric preparations, honey was added to cover up their bitter taste. Traditional healers explained that alcohol must be avoided because it can destroy the given medicine. A great number of participants

declared that they preparations are so safe to cause any side effect. However, according to the respondents, some preparations from plants like *Mycroglossa pyrifolia* should be taken carefully because they can be toxic. Moreover, traditional healers (Eastern Province of Rwanda) said that herbal medicines used during summer period are more diluted than the ones prepared during the rainy or wet seasons. This means that the concentration of the plant extracts is somehow controlled. Most of the time, traditional practitioners use to control the concentration by checking visually the colour or the viscosity of a preparation. In some cases, we noticed that all details about the preparation are not given. Indeed, it is sometimes difficult for traditional practitioners to explain exactly the quantity of water or the amount of the used plant material but, they can also keep it secret. Based on the present ethnobotanical survey, 13 plants were selected from which 19 samples were collected. Some medicinal plants such as *Terminalia mollis* and *Aristolochia elegans* used to treat but also to prevent malaria were selected even if they are not most cited. The selected plants are *Aristolochia elegans*, *Conyza aegyptiaca* (L.) Aiton (Asteraceae), *Fuerstia africana* T.C.E. Fr. (Lamiaceae), *Markhamia lutea* K.Schum (Bignoniaceae), *Microglossa pyrifolia* (LAM.) O. Kunte Asteraceae, *Mitragyna rubrostipulata* (K.schum.) (Rubiaceae), *Rumex abyssinicus* Jacq (Polygonaceae), *Rumex bequaertii* De Wild (Polygonaceae), *Solanecio mannii* (Hook.f.) Jeffrey **(**Asteraceae), *Terminalia mollis* (Combretaceae), *Tithonia diversifolia* (Hensl.) A Gray (Asteraceae), *Trimeria grandifolia* (Warb.) (Flacourtiaceae), *Zanthoxylum chalybeum* Engl. (Rutaceae). Except *S. mannii*, all plant samples selected were collected in the most endemics area (USAID, 2010) (see Figure 2.2). Details about the selected samples were already reported (Muganga et al., 2010). A copy of specimen for each selected plant was deposited at the National Botanical Garden of Belgium (Meise) to uncertain their authentication. The selected Rwandan medicinal plants may be interesting as source of new antimalarial drugs. However, as the problem of energy is associated with environmental challengers (Hansen et al., 2010), the availability and sustainability of Rwandan medicinal plants as a part of environment is a critical problem. Indeed, some medicinal plants are used as source of construction material or energy in Rwanda where, according to Rwanda Environment Management Authority (REMA), 94 % of the overall energy consumption of the country is derived from biomass (REMA, 2010). Furthermore, medicinal plants and generally forest resources in Rwanda decrease steadily because of rapid population growth, environmental deterioration, and in particular land degradation caused by soil erosion (UNDP/GEF-MSP, 2007).

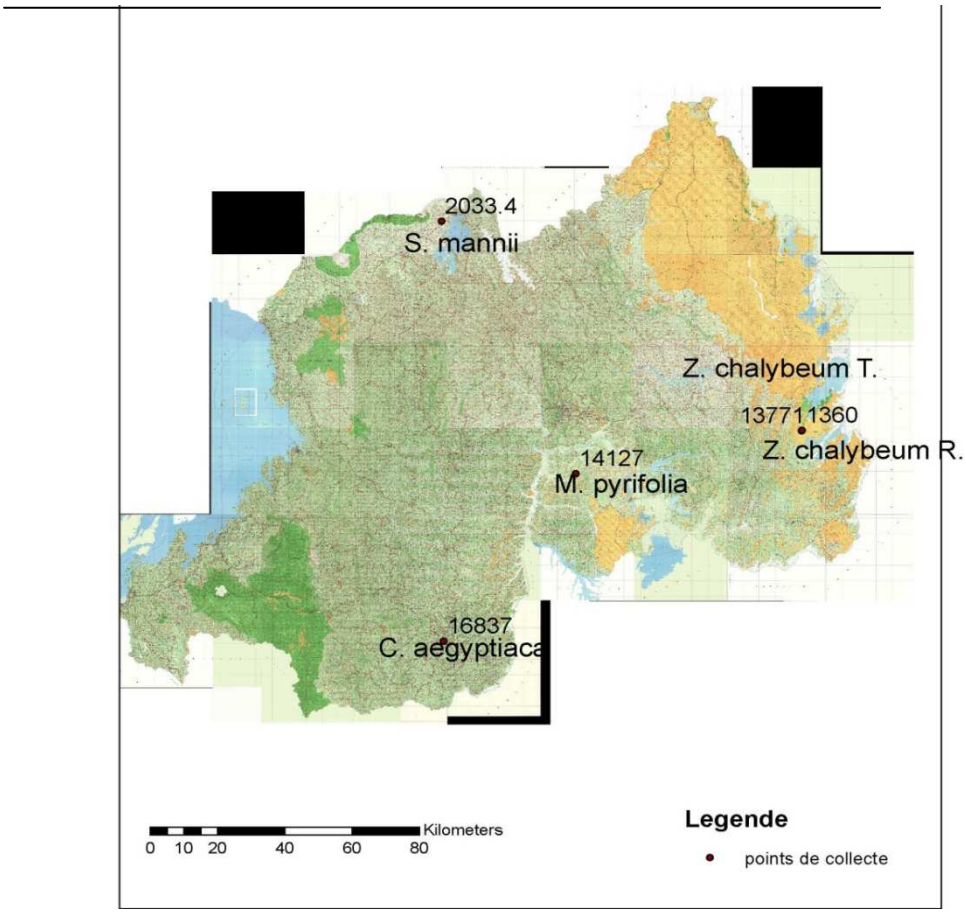

Figure 2.2: Place of samples collection and altitudes. Samples from *Fuerstia africana, Markhamia lutea, Microglossa pyrifolia, Mitragyna rubrostipulata, Rumex abyssinicus, Rumex bequaertii, Solanecio mannii, Tithonia diversifolia* and *Trimeria grandifolia* were collected in the same area as *Conyza aegyptiaca,* whereas *Terminalia mollis'* samples were collected in the same area as *Zanthoxylum chalybeum*

Figure 2.3 *Aristolochia elegans* (from www.jardins-interieurs.com)

Figure 2.4*Conyza aegyptiaca*

Figure 2.5*Fuerestia africana*

Figure 2.6 *Markhamia lutea*

Figure 2.7 *Microglossa pyrifolia*
(from www.zimbabweflora.co.zw)

Figure 2.8 *Mitragyna rubrostipulata*

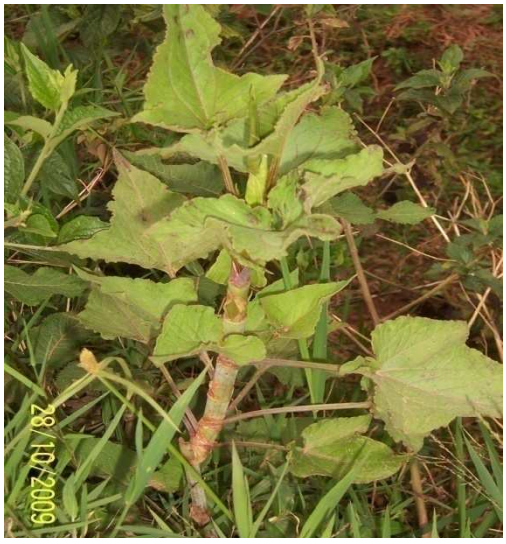

Figure 2.9 ***Rumex abyssinicus***

Figure I2.10 ***Rumex bequaertii***

Figure 2.11 S*olanecio mannii*
(from http://www.flickr.com)

Figure 2.12 ***Tithonia diversifolia***

Figure 2.13 ***Trimeria grandifolia***

Figure 2.14 ***Terminalia mollis***

Figure 2.15 ***Zanthoxylum chalybeum***

Figure 2.16. Traditional healers from Nyamata (Eastern Province, Rwanda)

Figure 2. 17 Traditional healers from Nyamagabe (Southern Province, Rwanda)

Figure 2.18 Traditional healers from Muhanga (Southern Province, Rwanda)

Figure 2.19. Traditional healers from Bugarama (Western Province, Rwanda)

Figure 2.20 Traditional healers from Karongi & Rubavu (Western Province, Rwanda)

Figure 2. 21. Traditional healers from Mutara (Eastern Province, Rwanda)

II.4. CONCLUSION

Our ethnobotanical survey conducted in all provinces of Rwanda showed that local traditional healers still use medicinal plants in the treatment of malaria although local ministry of health and WHO strictly recommend using modern drug, Coartem®, an artemisinin based combination therapy. The survey allowed us to select 13 plants among the most widely used for *in vitro* and *in vivo* antiplasmodial activity evaluation. Rwandan medicinal plants are threatened by rapid population growth and soil erosion and needed to be protected.

II.5. ACKNOWLEDGEMENTS

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CHAPTER III EVALUATION OF ANTIPLASMODIAL ACTIVITY OF RWANDAN MEDICINAL PLANTS USED IN THE TREATMENT OF MALARIA

The ethnobotanical survey described in previous chapter, allowed us to select some Rwandan medicinal plants used in the treatment of malaria. Plant samples were collected in Rwanda, air-dried, grounded under strict hygienic conditions and then transported in Belgium for antiplasmodial activity evaluation. Firstly, plant samples were extracted with methanol, dichloromethane and water and crude extracts submitted for *in vitro* antiplasmodial activity. *Plasmodium falciparum* chloroquine-sensitive and chloroquine-resistant strains were used. Samples which exhibited an interesting antiplasmodial activity were also evaluated for their cytotoxicity. Then selected plant extracts were tested *in vivo* against *Plasmodium belghei* infecting mice.

This chapter is divided in two parts: the first part deals with the *in vitro* antiplasmodial activity of selected plant samples whereas the second one provides the *in vivo* evaluation of the antiplasmodial activity of selected plants extracts.

III.1 *IN VITRO* ANTIPLASMODIAL ACTIVITY OF RWANDAN MEDICINAL PLANTS USED IN THE TREATMENT OF MALARIA

The results from this study are summarised in published article: Raymond Muganga, L. Angenot, M. Tits and M. Frédérich. 2010. Antiplasmodial and Cytotoxic Activities of Rwandan Medicinal Plants used in the Treatment of Malaria**.** Journal of Ethnopharmacology 128: 52-57.

Abstract

Aim of the study

In our study, methanol, dichloromethane and aqueous extracts of thirteen Rwandan medicinal plants used in the treatment of malaria were tested for *in vitro* antiplasmodial activity.

Materials and methods

The growth inhibition of chloroquine-sensitive *Plasmodium falciparum* strain (3D7) was evaluated using the measurement of lactate dehydrogenase activity. The active extracts were

also tested against the chloroquine-resistant *Plasmodium falciparum* strain (W2) and for cytotoxicity assay using human normal foetal lung fibroblasts (WI-38).

Results

The majority of the plants tested showed an antiplasmodial activity and the best results were observed with dichloromethane leaf and flower extracts of *Tithonia diversifolia,* leaf extract of *Microglossa pyrifolia* and root extract of *Rumex abyssinicus,* methanol leaf extract of *Fuerstia africana*, root bark extracts of *Zanthoxylum chalybeum* and methanol bark extract of *Terminalia mollis*. Those extracts were active (IC_{50} < 15 µg/ml) on both chloroquine-sensitive and resistant strains of *Plasmodium falciparum*. *Zanthoxylum chalybeum, Solanecio mannii* and *Terminalia mollis* presented the best selectivity index.

Conclusion

The traditional use of most of the plant evaluated was confirmed by the antiplasmodial test. This study revealed for the first time the antiplasmodial activity of two plants, *Terminalia mollis* and *Rumex abyssinicus.*

III.1.1. Introduction

Malaria is still a major public health problem, especially in tropical and sub-tropical regions. It is estimated that, in 2006, 3.3 billion people were at risk of contracting malaria and that it causes nearly one million deaths each year, mostly of African children aged below 5 years, who are susceptible to this disease. In Sub-Saharan regions, 45 countries were endemic for malaria in 2008 (WHO, 2008). In Rwanda, malaria is one of the leading causes of outpatient attendance and one of the principal causes of morbidity in each province (PNILP, 2005). This is despite the fact that bed nets, artemisinin-based combination therapies (ACT) and indoor spraying have reduced the prevalence of the disease (Fanello et al., 2007). The ACT recommended in Rwanda is artemether-lumefantrine (Coartem®), but the use of this drug is very limited in some rural areas where the population prefer traditional, less expensive preparations (WHO, 2008; Rwanda News Agency, 2009). Artemisinin, isolated from the well-known Chinese medicinal plant *Artemisia annua,* is one of the best compounds used to treat multi-drug resistant strains of *Plasmodium falciparum*. However, artemisinin-resistant malaria parasites were recently detected in Cambodia (Maude et al., 2009). There is therefore an evident need for new anti-malarial drugs, and medicinal plants constitute a reliable source

of these. Many Rwandan medicinal plants are claimed to be active against malaria but, in most cases, there is insufficient explanation or, in fact, any scientific proof of the efficacy of these medicines.

In the present study, based on ethnobotanical data obtained from Rwandan traditional healers and a literature review, thirteen Rwandan medicinal plants used to treat malaria were selected and submitted to *in vitro* evaluation of their antiplasmodial and cytotoxic activities. These plants are listed in Table 1. Samples collected were dried, then extracted with methanol and dichloromethane and the crude extracts obtained were evaluated for *in vitro* antiplasmodial activity. The aqueous crude extracts of the most active plants were also evaluated. Cytotoxic evaluation was only carried out for crude extracts which showed antiplasmodial activity (IC_{50}) < 50 µg/ml.

III.1.2. Material and methods

III.1.2.1. Plant material

Plant samples were collected from the Rwandan regions where malaria is most endemic, East, South and South-West, in November 2007 and in August to October 2008 (see Table 3.1.1). Each species was identified and one voucher specimen deposited in the Rwandan National Herbarium at Butare and another at the National Botanic Garden of Belgium at Meise. All samples collected were air-dried at room temperature with no direct sunlight for 3 days, except the leaf of *Solanecio mannii* which were dried during 5 days. Dried plant samples were then pulverized using an electrical grinder under strict hygienic conditions.

III.1.2.2. Preparation of extracts

Crude methanolic and dichloromethane extracts were obtained by maceration of 5 g of each powdered plant sample three times in 25 ml of solvent, for 30 min under constant shaking at room temperature. For each solvent, a new plant sample was used separately. The extracts were filtered and evaporated to dryness under reduced pressure with a rotatory evaporator. For the aqueous extracts, 100 ml of distilled water were used to extract 2 g of powdered plant material and the mixture obtained was boiled for 1 hour. The solutions obtained were filtered and the filtrate freeze-dried to obtain the dried crude aqueous extracts. All extracts obtained were weighed and their yield calculated.

III.1.2.3. In vitro *antiplasmodial assays*

The culture of Plasmodium falciparum strains was carried out as previously described (Frédérich et al., 2001). All crude extracts were evaluated *in vitro* for their activity against a *Plasmodium falciparum* chloroquine-sensitive strain (3D7) and the most active extracts were also evaluated against a *Plasmodium falciparum* chloroquine-resistant strain (W2). For each crude extract, a series of 8 threefold dilutions (from 200 to 0.09 µg/ml) was prepared, placed in 2 rows of a 96-well microplate and tested in triplicate. Artemisinin (98%, Sigma-Aldrich) and chloroquine diphosphate salt (Sigma-Aldrich) were used as standards, and infected and uninfected erythrocytes were added as positive and negative controls respectively. After 48 hours of incubation at 37ºC, the level of parasitaemia was estimated by measuring lactate dehydrogenase activity, as previously described (Kenmogne et al., 2006). The results were expressed as the mean IC_{50} (the concentration of a drug that reduced the level of parasitaemia to 50%).

III.1.2.4. In vitro *cytotoxic assay*

Cells from the human normal foetal lung fibroblast cell line, WI-38, was cultivated in vitro in DMEM: Dubecco's Modified Eagle's Medium (Lonzo, Belgium), which contains 5% of L-glutamate (Lonzo, Belgium), 5% of penicillin-streptomycin (Lonzo, Belgium) and 10% of heat inactivated foetal bovine serum (Lonzo, Belgium). Then, the cells were incubated at 37 ºC in a humidified atmosphere with 5% CO_2. For each sample, 6 threefold dilutions (from 200 to 0.82 µg/ml) were prepared, placed in 3 rows of a 96-well microplate and tested at least twice. Camptothecin (Sigma) was used as a positive control. After 48 hours' incubation, cell viability was determined by measuring the fibroblast mitochondrial enzyme activity, as previously described (Stevigny et al., 2002). The results were expressed by the mean of IC_{50}s of at least 2 independent assays and the selectivity index (the ratio between the cytotoxic (WI-38 cells) and antiparasitic (3D7 strain) activity) was calculated.

Table 3.1.1: Selected species, their scientific names, parts used, voucher number and place of collection (altitude)

Scientific name of the plant (family), date	Plant part[a]	Voucher number Rwanda	Voucher number Meise Botanical Garden, Belgium	Place of collection (altitude)
Aristolochia elegans MAST (Aristolochiaceae), 2007	Seed	2007V39	BR0000005093953	Huye (1 684 m)
Conyza aegyptiaca (L.) AIT (Asteraceae), 2007	L	2007V38	BR0000005093878	Huye (1 684 m)
Markhamia lutea K.SCHUM (Bignoniaceae), 2008	L	2008R17	BR0000005093979	Nyaruguru (1 753 m)
Microglossa pyrifolia (LAM.) O.KUNTE (Asteraceae), 2007	L	2007R1	BR0000005093977	Bugesera (1 413m)
Mitragyna rubrostipulata (K.SCHUM.) (Rubiaceae), 2008	L&SB	2008R19	BR0000005093595	Nyaruguru (1 648 m)
Fuerstia africana T.C.E. Fries (Lamiaceae), 2008	L&S	-	BR0000005088850	Huye (1 719 m)
Rumex abyssinicus JACQ (Polygonaceae), 2008	R	2008R15	BR0000005093670	Huye (1 670 m)
Rumex bequaertii DE WILD (Polygonaceae), 2008	R	2007R3	BR0000005094059	Huye (1 600 m)
Solanecio mannii (Hook.f.) JEFFREY (Asteraceae), 2008	L	2007R2	BR0000005093472	Musanze (2 033 m)
Terminalia mollis LAWSON (Combretaceae), 2007 and 2008	L, SB & RB	-	BR0000005087167	Ndego (1 592 m)
Tithonia diversifolia (HENSL.) A GRAY (Asteraceae), 2008	F&L	2007R12	BR0000005093793	Huye (1 680 m)
Trimeria grandifolia (HOCHST.) warb. *subsp. tropica* (Burkill) Sleumer (Flacourtiaceae), 2008	L	2008R16	BR0000005093496	Nyaruguru (1 722 m)
Zanthoxylum chalybeum ENGL. (Rutaceae), 2007 and 2008	SB&RB	-	BR0000005087266	Ndego (1 377 m)

[a] F = flower; L = leaves; R=root; RB= root bark; S = stem; SB = stem bark

III.1.3. Results and Discussion

Thirteen plants were selected, 19 samples collected and from these 46 extracts tested for antiplasmodial activity on the 3D7 chloroquine sensitive strain of *Plasmodium falciparum*. According to WHO guidelines and previous results from our team (Pink et al., 2005; Jonville et al., 2008), antiplasmodial activity was classified as follows: highly active at $IC_{50} < 5$ µg/ml, promising at 5-15 µg/ml, moderate at 15-50 µg/ml and inactive at > 50µg/ml.

Most of the plants tested (77%) showed an antiplasmodial activity with $IC_{50} < 50$ µg/ml. Twelve extracts showed a promising level of activity ($IC_{50} < 15$ µg/ml) and seven of them had a very high level of activity ($IC_{50} < 5$ µg/ml) against 3D7 and W2 (*Plasmodium falciparum* chloroquine resistant strain). We found that the dichloromethane extracts were generally more active against *Plasmodium falciparum*, but for some plant samples the methanolic extracts were the more active ones. Nine extracts with a promising level of activity ($IC_{50} < 15$ µg/ml) were assessed for their cytotoxic activity in order to determine the selectivity index (see Table 3.1.2).

Aristolochia elegans, Rumex bequaertii and *Trimeria grandifolia* did not show any antiplasmodial activity *in vitro*, although they are traditionally used to treat malaria (Hakizamungu and Weri, 1988; Rwangabo, 1993). This does not mean that those plants are not active against *Plasmodium falciparum*. Indeed, the *in vitro* antiplasmodial activity was assessed on the asexual erythrocytic stage of *Plasmodium falciparum* and those plants may act on other stages of the parasite. Those plants may also be active *in vivo* where the pharmacokinetics and metabolic process are involved and could therefore make the plant samples active. This is the case with the methanolic leaf extract of *Markhamia lutea*, which had already been reported to be active *in vivo* (Hakizamungu and Weri, 1988) with an important growth inhibition on *Plasmodium berghei* (62.1%) but was found to be inactive *in vitro* ($IC_{50} > 50$ µg/ml). Most of the time, traditional healers use a mixture of different plants so as to increase the activity but also to hide the real active plant. This means that some plant samples, which did not show any activity in our study, might still be active against *Plasmodium falciparum*. It must be noted that, often, traditional healers do not distinguish fever from malaria. Some medicinal plants used in the treatment of malaria, in combination with others, may have antipyretic activity or may reduce joint pain and therefore impact on the patient's recovery. *Aristolochia elegans* did not show any antiplasmodial activity and this plant contains aristolochic acid (Jou et al., 2004), which has already been reported to be

nephrotoxic (Vanherweghem et al., 1993; Nortier et al., 2000). The seed of this plant is used by some traditional healers in the prevention of malaria and this use should be avoided.

III.1.3.1 Mitragyna rubrostipulata

The only active extract from this plant was its dichloromethane stem bark extract, which presented a weak antiplasmodial activity (IC_{50} = 39.9 µg/ml). Further studies are needed to confirm the use of the root and stem bark of the plant in the treatment of malaria by Rwandan traditional healers, as a metabolization step may be necessary to obtain a good activity. The stem bark is also used to treat intestinal worms, especially amoebiasis and it also has antibacterial and antifungal activities (Rwangabo, 1993). Another species of *Mitragyna*, known as *Mitragyna inermis*, has been found to be more active against *Plasmodium falciparum* (Fiot, 2005) and has proved to also have antibacterial activity (Zongo et al., 2009).

III.1.3.2. Markhamia lutea

Dichloromethane leaf extract showed a weak antiplasmodial activity (IC_{50} = 29 µg/ml) but in another recent study (Lacroix et al., 2009), the ethyl acetate extract was found to be two times more active (IC_{50} = 10.2 µg/ml). The methanolic leaf extract of the plant has been reported also to be active *in vivo* (Hakizimungu and Weri, 1988). Our results, together with the results of these studies may confirm the use of the plant in traditional medicine. The plant has other therapeutic properties and has been identified as a potential treatment for viral respiratory infections (Kernan et al., 1998).

III.1.3.3 Conyza aegyptiaca

Conyza aegyptiaca is one of the most used plants to treat malaria in Rwandan traditional medicine. It is also used in the treatment of haematuria (Rwangabo, 1993). In our study, the methanolic leaf extract of the plant showed a moderate antiplasmodial activity (IC_{50} = 22.7 µg/ml) and was slightly more active than the dichloromethane leaf extract (IC_{50} = 36.8 µg/ml). The methanolic leaf extract showed a low cytotoxicity (IC_{50} = 80.9 µg/ml) but presented a low selectivity index (3.6). Further study *in vivo* is needed to confirm the use of the plant in traditional medicine and its absence of toxicity.

Table 3.1.2: *In vitro* antiplasmodial and cytotoxic activity and selectivity index of the selected samples

Species	Plant part[a]	Extract	Yield	3D7 IC_{50} (µg/ml)[c]	W2 IC_{50} (µg/ml)[c,d,e]	WI-38 IC_{50} (µg/ml)[d, e]	SI[d, e]
Aristolochia elegans	Seed	MeOH	5.4	> 50	Nd	nd	nd
		CH_2Cl_2	7.6	> 50			
Conyza aegyptiaca	L	MeOH	5.4	22.7 ± 4.2	24.66 ± 2.4	81.9 ± 1.6	3.6
		CH_2Cl_2	7.6	36.8 ± 6.0			
		H_2O	31.7	> 50			
Markhamia lutea	L	MeOH	10.7	> 50	Nd	nd	nd
		CH_2Cl_2	1.7	29.0 ± 0.8			
Microglossa pyrifolia	L	MeOH	18.7	**4.2 ± 1.9**			
		CH_2Cl_2	7.4	**1.5 ± 0.1**	**2.4 ± 0.1**	4.7 ± 0.8	3.2
		H_2O	25.2	**14.3 ± 2.1**			
Mitragyna rubrostipulata	SB	MeOH	16.1	> 50	Nd	nd	nd
		CH_2Cl_2	0.9	39.9 ± 2.8			
	L	MeOH	29.9	> 50	nd		
		CH_2Cl_2	2.4	> 50			
Fuerstia africana	L&S	MeOH	13.3	**6.9 ± 2.3**	**4.1 ± 1.6**	13.0 ± 2.3	1.9
		CH_2Cl_2	2.8	40.2			
		H_2O	5.3	> 50			
Rumex abyssinicus	R	MeOH	16.2	> 50			
		CH_2Cl_2	7.4	**4.3 ± 2.0**	**3.1 ± 1.2**	13.3 ± 0.9	3.1
		H_2O	45.4	> 50			
Rumex bequaertii	R	MeOH	19.1	> 50	Nd	nd	nd
		CH_2Cl_2	0.3	> 50			
Solanecio mannii	L	MeOH	10.6	21.6 ± 3.4	26.2 ± 0.1		
		CH_2Cl_2	4.2	18.2 ± 6.0	**12.7 ± 2.8**	122.3 ± 3.8	6.7
		H_2O	40.7	> 50			
Terminalia mollis	L	MeOH	10.6	> 50	Nd		nd
		CH_2Cl_2	4.2	> 50			
	SB	MeOH	16.9	> 50			
		CH_2Cl_2	7.4	> 50			
	RB	MeOH	26.3	**11.7 ± 0.9**	18.9 ± 1.6	77.2 ± 8.9	6.6
		CH_2Cl_2	0.4	> 50			
		H_2O	34.23	33.5 ± 0.3			
Tithonia diversifolia	F	MeOH	14.9	**8.1 ± 3.3**	**6.5 ± 1.9**		
		CH_2Cl_2	48.0	**1.1 ± 0.3**	**1.0 ± 0.3**	5.3 ± 1.3	4.7
		H_2O	28.6	24.5 ± 3.9			
	L	MeOH	5.7	**1.2 ± 0.4**	**1.5 ± 0.4**		
		CH_2Cl_2	49.1	**0.6 ± 0.1**	**0.7 ± 0.2**	2.5 ± 0.2	4.2
		H_2O	42.1	15.6 ± 1.8			
Trimeria grandifolia	L	MeOH	23.0	> 50	nd	nd	nd
		CH_2Cl_2	4.3	> 50			
Zanthoxylum chalybeum	SB	MeOH	12.3	42.5 ± 0.4			
		CH_2Cl_2	4.4	41.5 ± 0.9			
	RB	MeOH	16.8	**4.2 ± 2.7**	**1.9 ± 0.5**	40.0 ± 8.5	9.5
		CH_2Cl_2	3.7	**6.2 ± 0.6**			
		H_2O	13.4	> 50			
	RB[b]	MeOH	15.2	38.34 ± 2.0			
Artemisinin				0.0063 ± 0.0009	0.0024 ± 0.0005	nd	nd
Chloroquine				0.0016 ± 0.003	0.329 ± 0.110	nd	nd
Camptothecin						0.0197±0.025	nd

[a] F, flower; L, leaves; R, root; RB, root bark; S, stem; SB, stem bark, [b] root bark collected in August; [c] IC_{50} values shown in bold express promising antiplasmodial activity; [d] For chloroquine, n = 11; [e]nd, not determined.

III.1.3.4. Solanecio mannii

This plant, also called *Senecio mannii*, is used by Rwandan traditional healers to treat malaria but also fever, burns, abscesses, leprosy, anthrax and poisoning (Hakizamungu and Weri, 1988). The dichlomethane leaf extract of the plant showed promising antiplasmodial activity (IC_{50} = 18.2 and 12.9 µg/ml respectively on 3D7 and W2) with a quite low level of cytotoxicity (IC_{50} = 122.3 µg/ml) and a significant selectivity index of 6.7. Our results were able to confirm the use of the plant in Rwanda traditional medicine. This plant is then a potential source of a new anti-malarial drug.

III.1.3.5. Terminalia mollis

The methanolic and aqueous root bark extract of the plant are active against *Plasmodium falciparum* with IC_{50} values of 11.7 and 33.5 respectively. This justifies the traditional use of the plant in the treatment of malaria, as water is used as the solvent in the traditional preparation. Subsequently, the cytotoxicity level of the plant was quite low (IC_{50} = 77.2 µg/ml) and its selectivity index was significant (6.6). However, further investigation *in vivo* is needed to confirm its safety. The plant also has antifungal, antibacterial and antiviral activities (Maregesi et al., 2008). A recent study (Maregesi et al., 2009) reported nevertheless that 80% methanol root extract has a very weak activity (IC_{50} = 125 – 150 µg/ml) against *Plasmodium falciparum*. The difference in the results of both studies may be explained by the fact that the plant samples were collected in two different regions and the *Plasmodium* strains used were different. The antiplasmodial activity is already known in other species of Combretaceae; the species *Terminalia bentzoe* has already been reported to have a promising effect on *Plasmodium falciparum* strains (Jonville et al., 2008) and *Terminalia bellirica* on the 3D7 strain (Valsaraj et al., 1997).

III.1.3.6. Fuerstia africana

The whole plant without its root is used to treat malaria and fever in Rwandan traditional medicine and the leaf is used to treat gonorrhoea (Rwangabo, 1993). The methanolic leaf and stem extract of the plant showed a very high antiplasmodial activity towards both strains of *Plasmodium falciparum*, 3D7 and W2 (IC_{50} = 6.9 and 4.1 µg/ml respectively) but the plant was quite cytotoxic (IC_{50} = 13.0 µg/ml) and therefore the selectivity index was low (1.9). The same antiplasmodial activity was already reported (Muthaura et al., 2007) but selectivity

index were quite different. The high selectivity indexes found in the paper from Muthaura and co-authors could be explained by the fact that they calculated the selectivity indexes by using CI_{50} for Plasmodium and CC_{50} for cells (and not CI_{50} in the two cases, as usual). One compound, known as Ferruginol, has been previously isolated from this plant and this substance presented a strong antimalarial activity, with an IC_{50} of 1.95 µg/ml, but also a cytotoxic activity. Therefore, ferruginol is not a desirable antimalarial candidate (Koch et al., 2006).

III.1.3.7. Rumex abyssinicus

The root of the plant is traditionally used to treat malaria, gonorrhoea, constipation, poisoning, hepatitis, constipation and sciatic neuralgia, and the leaf is used to treat coughs and gastric ulcers (Rwangabo, 1993). The dichloromethane root extract showed a very high antiplasmodial activity (IC_{50} = 4.3 and 3.1 µg/ml on 3D7 and W2 respectively). To date, there has not been any scientific proof of the antiplasmodial activity of this plant. The cytotoxicity level of the same extract was not negligible (IC_{50} = 13.3 g/ml) but there was a low to moderate selectivity index (3.1). The cytotoxicity of this plant could nevertheless compromise its medicinal use and more clarification regarding this toxicity is needed.

III.1.3.8. Microglossa pyrifolia

The leaf of the plant is used traditionally against malaria, and for fever, pain relief, intestinal worms, rheumatism, diarrhoea, gonorrhoea etc. in Rwanda and in many other African countries (Rwangabo, 1993; Neuwinger, 1996). Aqueous, methanolic and dichloromethane leaf extracts of the plant presented a promising and very high effect on 3D7 strain, with IC_{50} values of 14.2, 4.2 and 1.5 µg/ml respectively. A previous study had shown that the lipophilic extract of the plant was active against malaria with an IC_{50} = 10.5 and 13.1 µg/ml, respectively on the chloroquine-sensitive strain PoW and the chloroquine-resistant clone Dd2. Two diterpenes, E-Phytol and 6E-geranylgeraniol-19-oic acid, which have also been found to be detectable in aqueous extract, have been found to be responsible for the antiprotozoal activity of the plant (Köhler et al., 2002). The level of antiplasmodial activity obtained in our study was higher than the one already reported. This slight difference could be explained by the fact that we used different strains of *Plasmodium falciparum* on the one hand and, on other hand, by the fact that the plant samples used came from different countries: Rwanda and Ghana. Although the plant has a good effect on *Plasmodium*

falciparum, its cytotoxicity level is relatively high (IC_{50} = 4.7 µg/ml) and the selectivity index moderate (3.2). The cytotoxicity and hepatotoxicity of the plant is already described in the literature (Zirihi et al., 2005; Mukazayire et al., 2009).

III.1.3.9. Tithonia diversifolia

The best antiplasmodial activities were found in leaf and flower, especially when extracted with dichloromethane (1.0 and 0.7 µg/ml). The methanolic leaf and flower extract presented an interesting level of activity (1.2 and 8.1µg/ml) and a moderate level of activity was found with aqueous extracts (15.6 and 24.5 µg/ml respectively for leaf and flower extract). An artemisinic acid analogue was isolated from the mature stem of the plant and may contribute to the antiplasmodial effect (Bordoloi et al., 1996). The aerial parts of the plant also contain Tagitinin C, a lactone sesquitene with a very promising antiplasmodial activity, but this component is also cytotoxic (Goffin et al., 2002). Recently, the toxicity of the aerial parts has also been shown in rats and this compromises the use of the plant in the treatment of malaria (Elufioye et al., 2009).

III.1.3.10. Zanthoxylum chalybeum

The plant is commonly used in traditional medicine of Eastern African countries (Tabuti, 2008; Gessler et al., 1995). Different extracts of the plant were analysed and the best results were obtained with the methanolic root bark extract [IC_{50} = 4.2 µg/ml (3D7) and 1.9 µg/ml (W2)]. *In vitro* antiplasmodial activity of the plant has been previously reported (Gessler et al. 1994; Runkunga et al., 2009). Our results were quite similar to other IC_{50} values already reported for Kenyan and Tanzanian samples, except in the case of aqueous extracts (Gessler et al., 1995; Rukunga et al., 2009). The aqueous root bark extract in the present study showed a negligible activity towards 3D7, but previous studies have reported that this extract was really active with IC_{50} < 6 µg/ml (Gessler et al., 1994; Rukunga et al., 2009). This noticeable difference may be explained by the fact that the sample used in this study was not fresh. Indeed, plant samples used in this study were collected in Rwanda and then analysed in Belgium some months later. We found that the plant sample collected in August, during the summer period, was less active (IC_{50} = 38.3 g/ml) than the one taken in November during the rainy season, suggesting that the rainy season is appropriate for the synthesis of the active ingredients of our interest. It is known that the time of collection and the locality of plant material may play a major role in its beneficial properties (Capasso, 1985; Gessler, 1994). In

addition, the same methanolic extract presented the best selectivity index from all plant samples analysed (9.5). The level of cytotoxicity of the methanolic root bark extract shown in this study (IC_{50}= 40.0 µg/ml) is similar to that already reported (Kamuhabwa et al., 2000). This plant has a very promising antiplasmodial activity and a good selectivity index, and it could be very interesting in further antimalarial studies if the problem of differences in activity between batches is resolved.

III.1.4. Conclusion

The majority of the plants analysed in this study presented an antiplasmodial activity, which could justify their use in Rwandan traditional medicine. This preliminary study confirmed the interesting antiplasmodial activities of some plants already used and studied in other countries and revealed for the first time the antiplasmodial activity of *Terminalia mollis and Rumex abyssinicus*. Some plant samples, such as *Fuerstia africana*, *Rumex abyssinicus* and *Microglossa pyrifolia*, showed a very promising antiplasmodial activity against two plasmodial strains, but they were also cytotoxic and further investigations are therefore needed to clarify which compound is responsible for one or both activities. Some extracts were more active against the chloroquine resistant strain than against the chloroquine sensitive one (i.e. *Zanthoxylum chalybeum* and *Fuerstia africana*). This could be an indication of a specific mode of action of these extracts against *P. falciparum.* This study indicated particularly that three plants, *Terminalia mollis*, *Solanecio mannii* and *Zanthoxylum chalybeum*, have an important antiplasmodial activity and significant selectivity index, making them good candidates for further pharmacological study.

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III.2 *IN VIVO* ANTIPLASMODIAL ACTIVITY OF SOME RWANDAN PLANTS USED IN THE TREATMENT OF MALARIA

The following sub chapter deals with *in vivo* antiplasmodial assay conducted on selected plant extracts, based on the results from *in vitro* antiplasmodial and cytotoxic assays. The results from this subchapter are given in the following article under submission. Some results were taken from a poster presented in the Trends in Natural Product Research: a PSE Young Scientists Meeting (Crete, June 2011) (see annexe 4).

Abstract

Introduction

It is imperative to have new antimalarial drugs because of a real increase of parasite's resistance to current drugs. Medicinal plants constitute an interesting source of new drugs but, some plant extracts active *in vitro* may be active or not *in vivo*. Previous study reported the *in vitro* antiplasmodial activity of some Rwandan plant extracts, hence the need for them to be also evaluated *in vivo*.

Aim of the study

The aim of our study was to evaluate *in vivo* the antiplasmodial activity and the safety of the selected Rwandan medicinal plants used in the treatment of malaria with a view to developing them into improved traditional medicines.

Methods

Plant extracts were selected according to their selectivity index and the *in vivo* antiplasmodial activity of aqueous, methanolic and dichloromethane extracts was evaluated using the classical 4 days suppressive test on *Plasmodium berghei* infecting mice. The activity of the plant extracts was estimated as the percentage of parasitemia inhibition and the possible animal deaths were monitored.

Results

Four plants were selected namely *Fuerstia africana*, *Rumex abyssinicus*, *Terminalia mollis* and *Zanthoxylum chalybeum*. The highest activity was observed with the methanolic extract of *Fuerstia africana* (> 70%) on day 4 and 7 post-treatment after intra-peritoneal injection and on day 7 using oral administration. After oral administration, the best parasitemia inhibition was observed on day 4 post infection with the dichloromethane extract of *Rumex abyssinicu*s (49 %) followed by the aqueous extract of *Terminalia mollis* (44 %) and *Zanthoxylum chalybeum* (37 %). However, *Zanthoxylum chalybeum* and *Rumex abyssinicus*

extracts presented a high toxicity after intra-peritoneal injection with 0 % mouse survival on day 1 post-infection whereas *Fuerstia africana* was safer with 40 % mouse survival on day 20 post-infection.

Conclusion

Fuerstia africana has a very promising antiplasmodial activity *in vivo*. Although most of plants tested showed an antiplasmodial activity some of them may be toxic. On one hand, the use of those plants at low dose orally could be safe but a chronic toxicity is still possible. On the other hand, compounds responsible for the beneficial activity of those plants may not be equally responsible for their toxicity. Therefore, further investigations will be required to clarify which compound is responsible for each activity.

III.2.1 . Introduction

Malaria remains a major public health problem especially in Sub-tropical regions. Worldwide, about 3.3 billion people were exposed to malaria and the highest risk is in sub-Saharan Africa regions where approximately 81% of cases and 91% of deaths occur mostly in children under five years of age and pregnant women (WHO, 2011). The rise and spread of *Plasmodium falciparum* malaria resistance to chloroquine and sulfadoxine-pyrimethamine (Ginsburg et al., 2011) as well as the resistance of falciparum malaria vector to pyrethroids, insecticides used to prevent malaria in endemic regions, is a serious challenge especially in developing countries such as Rwanda (WHO, 2011). Medicinal plants constitute an interesting source of new drugs and worldwide there is now a real interest in antiplasmodial plants (Rasoanaivo et al., 2011). Many plant extracts have early exhibited a promising antiplasmodial activity *in vitro* and may be more or not active *in vivo*. In our previous report some medicinal plants used in Rwanda to treat malaria showed an interesting antiplasmodial activity *in vitro* (Muganga et al., 2010*)*. However, before those plants could be recommended in the treatment of malaria, their activity and safety *in vivo* should be evaluated. Therefore, based on their antiplasmodial and cytotoxic activity, four plants, *Fuerstia africana*, *Rumex abyssinicus, Terminalia mollis* and *Zanthoxylum chalybeum* were selected for *in vivo* antiplasmodial assay. Those plants are used traditionally in many countries and the antiplasmodial activity of *Fuerstia, Terminalia* and *Zanthoxylum* species is already well known (Jonville et al., 2008; Koch et al., 2006; Pinmai et al., 2010; Rukunga et al., 2009). This work reports the *in vivo* antiplasmodial activity of the selected plants on *Plasmodium berghei* in mice.

III.2.2. Materials and methods

III.2.2.1.Preparation of plant extracts

Crude methanolic, aqueous and dichloromethane plant extracts were prepared as early described (Muganga et al., 2010) and then dissolved in polyethylene glycol (PEG), in normal saline (NaCl 0.9%) or in a mixture of 7% tween 80 and 3% ethanol according to their solubility. The last vehicle was used for all plant samples used intra-peritoneally.

III.2.2.2.In vivo antiplasmodial activity

Permission and approval were obtained for the present study by the University of Liège Ethical Committee for using animals (Case file number 721). All mice used were SPF (Specific Pathogen Free) females approximately 4-5 weeks old (18-20 g) free from *Eperythrozoon coccoides* and *Haemobartonella muris* and were obtained from Charles River (France). The parasite used to infect mice is *Plasmodium berghei*, NK173 strain. *In vivo* antiplasmodial assays were performed based on the classical 4-day suppressive test as previously reported (Frédérich et al., 2004; Jonville et al., 2008). Briefly, female Swiss mice (5 mice /group) were infested by *Plasmodium berghei* NK173 four hours before treatment. Treatment doses (200 and 300 mg/kg of plant extract) were given intra-peritoneally and orally once daily from day 0 to day 3. On day 4 and 7, thin mouse tail blood smears were prepared and stained with Giemsa. Parasitaemia was determined counting at least 500 erythrocytes under microscope. Chloroquine at 4 mg/kg doses and physiological serum (To) or PEG were used as positive control and negative control respectively. The percentage of parasitaemia inhibition (activity) was determined measuring the difference between the mean number of parasites in the control group (100%) and those of the experimental group on day 4 and 7 using the formula below (Fidock et al., 2004):

$$\% \text{ inhibition} = 100 - \left\{\frac{\text{mean parasitaemia treated}}{\text{mean parasitaemia control}} \times 100\right\}$$

III.2.3. Results and discussion

In our previous report (Muganga et al., 2010) some plant extracts exhibited a high antiplasmodial activity *in vitro* with IC_{50} < 15 µg/ml. The cytotoxic activity of those plant samples was then evaluated and selectivity index determined. Based on the selectivity index four plant extracts, methanol leaf and stem extract of *Fuerstia africana*, methanolic root bark extract of *Zanthoxylum chalybeum* and *Terminalia mollis* and the dichloromethane root extract of *Rumex abyssinicus* were selected. Firstly, the antiplasmodial assay *in vivo* was done with tree plant extracts, the methanolic leaf and stem extract of *Fuerstia africana* and the methanolic

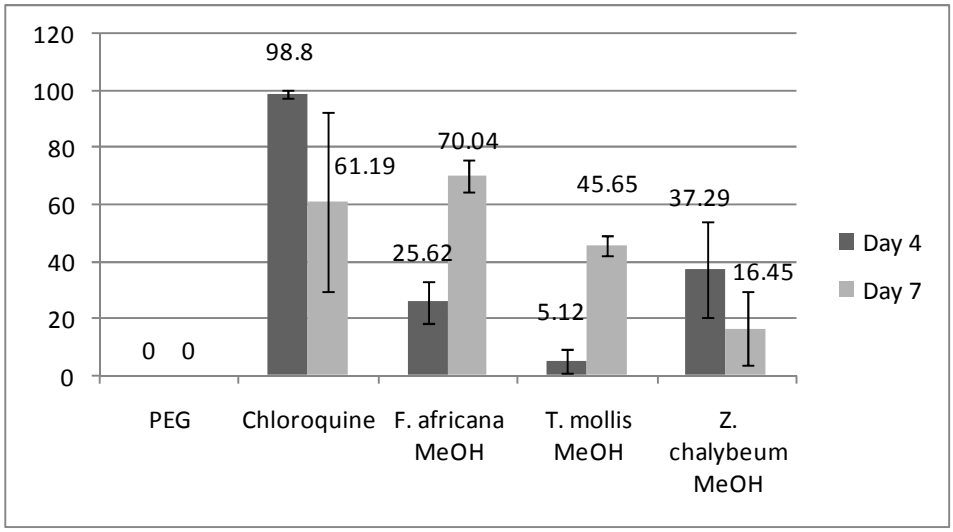

Figure 3.2.1 Results of *in vivo* assay on parasitaemia inhibition in mice infected by *Plasmodium berghei*. PEG (Polyethylene glycol) was used as negative control and chloroquine 4 mg/kg used as positive control. Mice (n = 5) received orally 300 mg/kg of methanolic extract of *Fuerstia africana* (leaf and stem), *Terminalia mollis* (root bark) and *Zanthoxylum chalybeum* (root bark) separately during four days. The percentage of parasitaemia inhibition indicated at the top of each column was calculated for each plant sample versus the parasitaemia in the control group taken as 100 %.

root bark extract of *Terminalia mollis* and *Zanthoxylum chalybeum* using oral administration. On day 4 post-infection the methanolic extract of *Zanthoxylum chalybeum* exhibited the best parasitaemia inhibition (37 %) followed by the methanolic extract of *Fuerstia africana* (25 %). On day 7, the highest parasitaemia inhibition was obtained with the methanolic extract of *Fuerstia africana* (70 %) followed by the methanolic extract of *Terminalia mollis* (45 %) (see Figure 1). As methanol extracts of *Z. chalybeum* and *T. mollis* did not exhibit a high activity

in vivo and traditional healers use water as solvent in their preparation, aqueous extracts of *Terminalia mollis* and *Zanthoxylum chalybeum*, active also *in vitro*, were also assessed for their antiplasmodial activity *in vivo* in the same conditions as the dichloromethane root extract of *Rumex abyssinicus*. On day 4 the activity of aqueous and methanolic extract of *Zanthoxylum chalybeum* was quite similar (34 and 37 %) whereas the aqueous extract of *Terminalia mollis* was more active (44 %) then the methanolic one (5 %) (see Figure 3.2.2). This may be explained on one hand by the solubility of active ingredients in both solvents and on the other hand by the fact that we used different batches. Unfortunately, we did not determine the activity of plant samples on day 7 as all mice from negative control

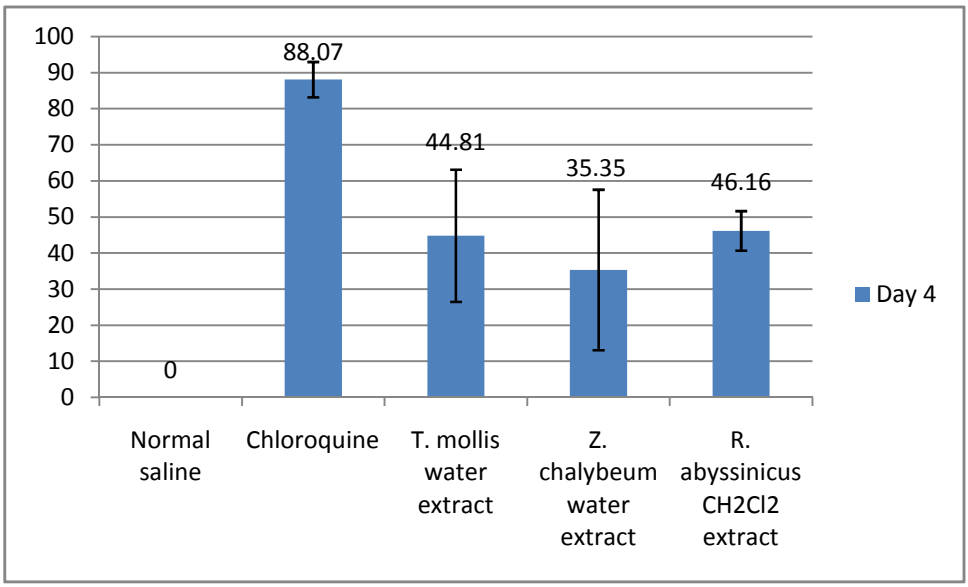

Figure 3.2.2 Results of *in vivo* assays on parasitaemia inhibition in mice infected by *Plasmodium berghei*. Normal saline (NaCl 0.9%) was used as negative control and chloroquine 4 mg/kg used as positive control. Mice in group of five received orally 300 mg/kg of aqueous root bark extract of *Terminalia mollis* and *Zanthoxulum chalybeum* as well as a dichloromethane root extract of *Rumex abyssinicus* separately during four days. The percentage of parasitaemia inhibition indicated at the top of each column was calculated for each plant sample versus the parasitaemia in the control group taken as 100 %

group had died before. In some cases standard deviation calculated for the activity of plant extract in one given group (five mice) was relatively high. This may be due to immune response which differs from one mouse to another as in this study we use wild mice. The fact that the antiplasmodial activity of *Fuerstia africana* and *Terminalia mollis* on day 7 was greater than the activity obtained on day 4 may result on the low elimination rate of active

ingredients from those plants or on the immune response. Furthermore, it is known that saponins stimulate lymphocyte proliferation (Adão et al., 2012; Jianshuang et al., 2011) and their presence in *Terminalia mollis* extract given to infected mice may help. Methanolic root extract of *Rumex abyssinicus* showed a good antiplasmodial activity on day 4 post-infection (46.16%). Methanolic root bark extracts of *Terminalia mollis* and *Zanthoxylum chalybeum* as well as the dichloromethane root extract of *Rumex abyssinicus* have a good antiplasmodial activity *in vitro* (IC_{50} < 15 µg/ml) (Muganga et al., 2010). However, those extracts are less active *in vivo* suggesting that the oral absorption of active ingredients from those plants may be poor. Therefore, we tried to measure the antiplasmodial activity of the two plant extracts using intra-peritoneal administration. The *in vivo* antiplasmodial assay using intra-peritoneal route was carried out for four plant extracts, namely methanolic extract of *Fuerstia africana*, *Terminalia mollis* and *Zanthoxylum chalybeum* as well as the dichloromethane root extract of *Rumex abyssinicus*. Only *Fuerstia africana* extract gave good results with parasitaemia inhibition of 74 % and 71 % on day 4 and 7 respectively whereas, mice which received *Zanthoxylum chalybeum* and *Rumex abyssinicus* extract (separately) did not survive more than one day post-treatment (see Table 1 below). Previous study reported the antiplasmodial and cytotoxic activity of ferruginol, the active ingredient from *Fuerstia africana* (Koch et al., 2006). However, the plant presented a good mouse survival time indicating that either ferruginol toxicity in mouse is weak, or the level of the toxic compound is very low in the plant. Other constituents of the plant may also modulate its activity. All mice which received the methanolic root bark extract of *Zanthoxylum chalybeum* died within 5-10 minutes after intra-peritoneal injection indicating the high toxicity of the extract in mice. The toxicity of this plant may result from its quaternary alkaloids (Neuwinger, 1996) in particular candicine which was early reported to have prominent curariform, stimulating nicotinic and paralyzing nicotinic actions (Takeshi et al., 1974). Furthermore, just before sudden death, mice revealed convulsions and paralysis and those symptoms were already observed in mice after intra-peritoneal injection of candicine (Hori et al., 1980). All mice treated with *Rumex abyssinicus* extract died one day post-treatment. This plant exhibited already cytotoxicity toward human normal foetal lung fibroblast cell (WI-38) but the compounds responsible for this toxicity are unknown so far. The majority of mice treated with *Terminalia mollis* extract died on day 2 post-infection and only one mouse survived until day 20 post-treatment; suggesting that the plant extract is also toxic in mice. The toxicity of *Terminalia species* was previously reported and may be attributed to saponins (Bulus et al., 2007) or to hydrolysable tannins (Mbwambo

et al., 2007). We tried then to check if hydrolysable tannins present in *Terminalia mollis* such as punicalagin and its derivatives are responsible for this toxicity. For this purpose, a methanolic extract free of hydrolysable tannins was made using a size exclusion column (sephadex) and then administered intra-peritoneally to mice. The majority of mice (60%) treated with this preparation free of hydrolysable tannins died within 6 days after infection, meaning that other constituent may intervene in the toxicity of the plant. Moreover, a condensed tannin called terminalin (MW= 603) isolated from *Terminalia oblongata* was reported to be toxic (Oelrichs et al., 1994) and this toxic compound may be also present in other *Terminalia species* such as *Terminalia mollis*. Unfortunately, we were not be able to verify the presence of this toxic condensed tannin in our preparation as the compound is not commercialised. The toxicity of the plant was also previously attributed to its saponins contents (Bulus et al., 2007).

Table 3.2.1. Viability of the mice infected by *Plasmodium berghei* after plant extracts treatment per oral and intra-peritoneal administration

Plant	Extract tested	Mouse survival time (day) (n=5)	
		Per oral	**Intraperitonially**
Fuerstia africana (L&S)	Methanol extract	11.8 ± 6.7	17.6 ± 1.2
Terminalia mollis (RB)	Methanol extract	7.8 ± 0.4	6
	Aqueous extract	8.2 ± 6.1	-
	Aqueous extract free of tannins	-	9.2 ± 3.9
Zanthoxylum chalybeum (RB)	Methanol extract	14.2 ± 5.98	0
	Aqueous extract	10.6 ± 5.3	-
Rumex abyssinicus (R)	Dichloromethane extract	7.4 ± 6.5	1

L, leaf; S, stem; R, root; B, bark. RB, root bark

as they possess deleterious haemolysing effect on circulating red blood cells (Lacaille-Dubois et al., 1996). However, haemolysis test done on aqueous and methanolic extract of *Terminalia mollis* at a concentration of 200 µg/ml revealed no haemolysis effect from the extracts (results not shown) suggesting that at therapeutic dose there no toxicity of the plant from its saponins. After oral administration of 300 mg/kg methanolic root bark extract of *Terminalia mollis* per day during 3 days, no acute toxicity was observed in uninfected mice.

This means that malaria caused by *Plasmodium berghei* in mice influences negatively mouse survival time.

III.2.4. Conclusion

From all plant samples tested *in vivo*, *Fuerstia africana* has the best antiplasmodial activity and good survival time after oral and intra-peritoneal administration. It would be interesting to carry out further investigations on this plant. Even if extracts from *Terminalia mollis*, *Zanthoxylum chalybeum* and *Rumex abyssinicus* exhibited antiplasmodial activity *in vivo*, we must pay attention on the use of those plants in traditional medicine especially when they are continuously used or used at high doses. Further toxicological studies are then recommended to guaranty the safety of those plants. Nevertheless, active ingredients from those plants may not be toxic and the isolation and determination of the active compound are under investigation.

III.2.5. Acknowledgment

The authors wish to thank ULg-GIGA Unit for facilitating to perform the *in vivo* assays on mice. This study was sponsored by the CUD (Coopération Universitaire au Développement) and by the Belgian National Fund for Scientific Research (FNRS).

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PART TWO

PART TWO

Like the first part, the second comportment of this work is divided in two chapters:

- Chapter IV which reports the identification of the active ingredient(s) responsible for the observed antiplasmodial activity of *T. mollis*, other antiparasitic activities and the possible anti-inflammatory activity of the plant;
- Chapter V deals with the isolation and the identification of the main antimalarial constituent(s) of *Z. chalybeum*, the effect of the crude extracts of this plant on other parasites and the evaluation of its anti-inflammatory.

CHAPTER IV ANTIMALARIAL COMPOUND(S) FROM *TERMINALIA MOLLIS* AND THE EFFECT OF THE PLANT ON OTHER PARASITES AND ON INFLAMMATION

Based on the results from previous chapters, two plants namely *T. mollis* and *Z. chalybeum*, were selected for further phytochemical and pharmacological studies. This chapter provides the results from the investigation performed on the root bark of *T. mollis*. This chapter is divided in three parts. Firstly, the active ingredient responsible for the antiplasmodial activity of the plant was isolated and determined. Secondly, *T. mollis* root bark extracts were assessed for their possible effect on other parasites like trypanosoma, leishmania and amoeba. Thirdly, the extracts were evaluated for their anti-inflammatory activity *in vitro*.
This chapter is presented as an article which will be submitted in the near future.

In vitro antiplasmodial, antileishmanial, antitrypanosomal, antiamoebic and anti-inflammatory activity of *Terminalia mollis* root bark

Abstract

Aim of the study: In this study, the antiplasmodial, antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activities of *Terminalia mollis* root crude extract were investigated *in vitro*. The total polyphenol contents were determined and the active ingredient responsible for the antiplasmodial activity of the plant identified.

Material and method: The antiplasmodial activity was tested on chloroquine-sensitive and mefloquine-resistant *Plasmodium falciparum* strains (3D7 and F32). The antitrypanosomal and antileishmanial activities were evaluated *in vitro* respectively on *Trypanosoma brucei brucei* (strain 427) (Tbb) and promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) (Lmm). The antiamoebic activity was investigated using an *Entamoeba histolytica* strain from a patient with acute amoebic dysentery. The total antioxidant activity was carried out using 2, 2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation decolorization assay. The anti-inflammatory activity of the plant was determined by evaluating the effect of crude extracts on reactive oxygen species (ROS) production and on myeloperoxidase (MPO) release by phorbol 12-myristate 13-acetate (PMA)-stimulated equine neutrophils and on purified equine MPO activity using lucigenin-enhanced chemiluminescence (CL) and Specific Immuniological Extraction Followed by Enzymic Detection (SIEFED). Total polyphenol and tannin content were determined as described by European Pharmacopeia 7.0.

Results: Crude methanolic and aqueous extracts of *Terminalia mollis* root bark showed a very promising antiplasmodial activity (IC_{50} 3.76 and 5.59 µg/ml respectively) and ellagic acid was identified as the active constituent (IC_{50} = 0.175 µg/ml). The methanolic, aqueous crude extract and aqueous crude extract free of tannins exhibited good growth inhibition on Tbb (IC_{50} 3.72, 6.05 and 4.45 µg/ml respectively) but were inactive against Lmm (IC_{50} > 100 µg/ml). The ethanolic crude extract of the plant displayed a moderate antiamoebic activity (MIC ≤ 250 µg/ml). A very interesting anti-inflammatory activity was observed with 50% hydroethanolic, aqueous crude extracts and aqueous crude extract free of tannins (IC_{50} < 5 µg/ml for ABTS, CL, and SIEFED assay).

Conclusion: This study revelled for the first time the antitrypanosomal, antiamoebic and anti-inflammatory activity of *Terminalia mollis* as well as the presence of ellagic acid in aglycone form which plays a main role in the aforementioned properties particularly in the antiplasmodial activity. More researches on this plant are needed and its eventual toxicity *in vivo* is under investigation.

IV.1. INTRODUCTION

Many studies have focused on the importance of medicinal plants in the treatment of various diseases. The management and control of malaria, trypanosomiasis, leishmaniasis and

amoebiasis is still among the major health problems especially in developing countries (WHO, 2007; WHO, 2008; WHOb, 2010).

Terminalia mollis M.A. Lawson (Combretaceae) is one of Rwandan medicinal plants used to treat malaria and gastrointestinal diseases. The plant is elsewhere used to treat diarrhoea, gonorrhoea and may be beneficial for the management and the control of HIV diseases (Kisangau et al., 2007; Maregesi et al., 2010). Its antifungal and antibacterial activities were all ready indicated (Baba-Moussa et al., 1999; Maregesi et al., 2008).

Several plant preparations are used to treat various gastrointestinal disorders in Rwanda and most of time local traditional healers do not distinguish different intestinal parasites. It is already known that some plants may be active against various parasites (Asres et al., 2001; Fernandez et al., 2010; Morais et al., 2012). This means that *Terminalia mollis* based preparation used by traditional healers to treat malaria and gastrointestinal disorders (not specified) may also treat amoebiasis and eventually be active against other parasitosis such as leishmaniasis, trypanosomiasis. Furthermore, intestinal amoebiasis is often responsible for diarrhoea (Tona et al., 1998) on which, according to traditional healers, *Terminalia mollis* is active (Liu et al., 2009).

The antiplasmodial activity of the plant was previously reported (Muganga et al., 2010) but active constituents responsible for this activity need to be identified.

Previous phytochemical study of *T. mollis* reported the presence of polyphenolic compounds in stem bark (Liu et al., 2009) and the plant exhibited a good antioxidant activity (Masoko et al., 2007). Polyphenolic are known to have many therapeutic properties such as antitumor, antiviral and anti-inflammatory (Kurokawa et al., 2001; Owen et al., 2000; Paixão et al., 2007). 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 (Rosdahl et al., 2008) and then to prevent various diseases.

Many methods were already developed to evaluate the antioxidant and anti-inflammatory property of various samples. In this study, prior to evaluation of anti-inflammatory activity, the total antiradical capacity of different root bark crude extracts were evaluated using the

well known ABTS assay (Miller et al., 1996; Re et al., 1999). This assay measures radical scavenging capacity of the extract by reducing the pre-formed radical cation $ABTS^{\bullet+}$ generated by oxidation of ABTS with sodium persulfate. Even if reactive oxygen species (ROS) are produced by normal cellular process and help for immune function, their excessive production can also cause various diseases such as inflammation (Conforti et al., 2008). This may happen when the quantity of ROS exceeds the normal physiological level which is controlled by the antioxidant defence systems in the body (Masoko et al., 2007). On one hand, we investigated the anti-inflammatory property of plant extracts by evaluating their capacity to reduce ROS produced by stimulated equine neutrophils using lucigenin-enhanced chemiluminescence (CL) and their modulatory activity on myeloperoxidase (MPO) by SIEFED on the other hand. MPO is a hemic peroxidase present in the granules of the neutrophils involved in the inflammatory response (Kohnen et al., 2007; Van Antwerpen, 2006). Indeed, MPO play a major role in acute and chronic inflammation (Deby-Dupont et al., 1999). SIEFED is new orginal method developed to study the direct interaction of some molecules with the enzyme MPO (Franck et al., 2006).

The present study reports firstly the identification of the active ingredient responsible for the antiplasmodial activity of *T. mollis* and then the *in vitro* antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activities of the plant.

IV.2. MATERIAL AND METHODS

IV.2.1. Plant material

Root bark samples of *T. mollis* were collected from Eastern Province of Rwanda in October 2010. After authentication a voucher number was given (BR0000005087167). The voucher species of the plant was deposited at the Rwandan National Herbarium (Butare) and another at the National Botanic Garden of Belgium (Meise). The samples were treated as previously reported (Muganga et al., 2010). Commercial Green tea was obtained from Tilman® Pharmaceutical plant (Liège Pharmaceutical plant, Belgium) and Rwandan green tea purchased from Rwandan Mountain Tea Rubaya-Nyabihu Tea Factories.

IV.2.2. Bio-guided fractionation

In our previous report, the highest antiplasmodial activity of the plant was found with methanol extract (Muganga et al., 2010), which was then fractionated to isolate active constituents. Crude methanolic root bark extract was obtained by macerating 500 g of the plant material with 6 L of methanol for 72 hours at room temperature. Extracts were then evaporated to dryness under reduced pressure. An aliquot of the methanolic extract was solubilised in a mixture of methanol and water (8:2) and then consecutively extracted with hexane, ethyl acetate and water. Fractions were tested for antimalarial activity and the most active fraction was fractionated by preparative high performance liquid chromatography (Armen Instrument AP 100) (size exclusion (Fractogel® TSK HW-40 (s) (Merck, Germany) and SEPHADEX® (Sigma-Aldrich, Belgium) to isolate pure compounds which were also tested. HPLC (Agilent Technologies 1200 series, California) analysis was performed using acetonitrile – pure water as mobile phase, with gradient mode and a Hypersil ODS C18 column (Alltech). Methanol was used to separate compound on fractogel while ethanol and pure water were used as eluents for SEPHADEX. Pure compounds were further submitted to NMR (Bruker Avance 500 MHz DRX 500 pectrometer) and mass spectroscopy (Micromass ESI-Q-TOF II instrument) to determine their chemical structures.

IV.2.3. Antiplasmodial activity

Crude methanolic, aqueous extracts and different fractions of the plant were prepared as previously reported (Muganga et al., 2010) and submitted to *in vitro* antiplasmodial test against two *Plasmodium falciparum* strains, 3D7 (chloroquine-sensitive) and F32 (mefloquine-resistant). The *Plasmodium falciparum* mefloquine-resistant strain F32 was chosen because it is coming from Tanzania, a country close to Rwanda where the plant is used to treat malaria. The culture of the parasite was carried out as already described (Frédérich et al., 2001) and the antiplasmodial activity assay was performed according to the method previously reported (Muganga et al., 2010). Artemisinin (98 %) and chloroquine (Sigma-Aldrich) were used as reference, all samples were tested in triplicate and the results expressed as mean ± standard deviation (SD).

IV.2.4. *In vitro* haemolysis with plant extracts

Methanolic and aqueous root bark extract of *T. mollis* at a final concentration of 200 and 100 µg/ml in DMSO (1%) were tested with 10 % freshly heparinised human blood (A+) in

phosphate buffer (PBS). To 10 µl of plant extract 190 µl of 10 % human blood was added and the mixture incubated for 1 hr under constant and slow shaking at room temperature. After incubation, solutions were centrifuged for 5 min at 2000 rpm in Eppendorf centrifuge 5417 R (Hamburg, Germany). The supernatant was removed and the absorbance (A) of liberated haemoglobin measured at 550 nm with Spectrophotometer Perkin Elmer Wallac Victor^2 (USA). DMSO (20 %) (Sigma-Aldrich, Belgium), Triton -100 (20 %) and PBS (Lonza, Belgium) were used respectively as solvent, positive (100 % haemolysis) and negative control. Each sample was analysed in triplicate and mean ± S.E. was calculated. The percentage of haemolysis was calculated as follows:

% haemolysis: [(A product – A solvent) / (A triton – A PBS)] x 100

IV.2.5. In vitro *antitrypanosomal and antileishmanial* assay

Samples free of tannins were prepared accordingly to European pharmacopeia 7.0 (2.8.14). Briefly, plant material was powdered to a size of 250 µm; a known amount was placed in 150 ml of distilled water and boiled in water-bath at 100^{o}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 shaked for 1 hr at room temperature. After filtration, the filtrate obtained was freeze-dried to give a crude aqueous extract free of tannins. All dilutions and extraction operations were protected from light.

The blood-stream forms of *Trypanosoma brucei brucei* (Tbb) strain 427 were cultured in HMI9 medium which contains 10% heat-inactivated foetal bovine serum and incubated at 37 $^{\circ}$C in a humidified atmosphere with 5% CO_2 as already described (Hirumi et al., 1994).

The strain MHOM/BZ/84/BEL46 of *Leishmania mexicana mexicana* (Lmm) in prostigote form was cultivated *in vitro* in a semi-defined medium (SDM-79) (Brun et al., 1994; Brun et al., 1979), supplemented with 15% heat-inactivated foetal bovine serum and incubated at 28°C.

The *in vitro* antitrypanosomal and antileishmanial activity of plant extracts was evaluated as previously described (Hoet et al., 2004). Three extracts were tested, methanol, aqueous and aqueous crude extract free of tannins and ellagic acid. Suramine and Amphotericine B were used as positive controls for antitrypanosomal and for antileishmanial activity respectively. Plant material samples and positive controls were prepared as described (Bero et al., 2011)

and tested in duplicate. The results were expressed as the mean IC_{50} (the concentration of a product that can reduce the level of parasitaemia to 50%).

IV.2.6. *In vitro* antiamoebic test

Crude hydro-ethanolic extract was obtained by maceration of 40 g of the powdered plant material with 400 ml of 70% ethanol for 24 hours at room temperature for 30 minutes under constant shaking. The mixture was filtered and the filtrate evaporated to dryness under reduced pressure.

In this study, the *Entamoeba histolytica* strain used is a laboratory strain isolated from patient with acute amoebic dysentery and was kindly offered by Professor G. Muhirwa from the Department of Medical Biology, University Teaching Hospital at Butare (Rwanda). *Entamoeba histolytica* was cultured in sterile test tube containing 5 ml of Balamuth medium (egg yolk based medium) (Anastopoulos et al., 1950). The infected medium was incubated at 37^{o}C and parasitic growth controlled one week before the test. Amoxicillin was added to avoid contamination and pH maintained at 7 – 7.5 with diluted NaOH. Neubauer's cell was used to determine the daily parasitaemia and only cultures presenting suitable growth of motile forms were selected for the test. Metronidazole (Axopharma, Belgium) was used as standard while infected and uninfected medium played the role of positive and negative controls respectively. The number of amoeba in un-contaminated tubes was estimated to be approximately 2.5 millions/ml. To investigate antiamoebic activity, plant extract in the final concentrations range of 0.007 – 2 mg/ml was added to a series of test tubes containing the contaminated medium. All tubes were filled up with sterile cotton, stirred and incubated at 37^{o}C for 72 hours. Each test was performed in duplicate.

IV.2.7. Anti-inflammatory activity

IV.2.7.1. Measurement of antioxidant capacity

Crude hydro-ethanolic extract was obtained by maceration of 2 g of the powdered plant material with 3 x 25 ml of 50% ethanol for 30 minutes under constant shaking at room temperature. The mixture was filtered and the filtrate evaporated to dryness under reduced pressure. Samples free of tannins were prepared as described in section 2.5.

The total antiradical capacity of crude extracts was determined using 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) radical cation decolourisation (Re et al.,

1999). An amount of ABTS was oxidised overnight in dark and at room temperature by sodium persulfate to generate $ABTS^{\cdot+}$. $ABTS^{\cdot+}$ was diluted 200 times with methanol to obtain the absorbance of 0.800 ± 0.1 at 740 nm. Three crude extracts, hydro-ethanolic, aqueous and aqueous crude extract free of tannins, were prepared at different concentrations in water and in DMSO. Gallic acid (Sigma-Aldrich, Belgium), ellagic acid (Alfa Aesar, Germany) and a mixture of punicalagin A (30.7%) & B (65.6) (Chromadex, California) were used as standards and methanol as 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 added to 1980 µl of diluted $ABTS^{\cdot+}$. The mixture was left to stand for 90 minutes at room temperature in dark room and then the absorbance read and recorded. The percentage decrease of absorbance was calculated, assuming that the absorbance of the solvent (pure water or DMSO) is equal to100% and IC_{50} estimated.

IV.2.7.2. Isolation of equine neutrophils

Horse blood was drawn from the jugular vein of healthy horses fed and bred in identical conditions (Faculty of Veterinary Medicine, University of Liège, Belgium) using EDTA as anticoagulant. Neutrophils were isolated using a discontinuous percol density gradient as already reported (Pycock et al., 1987). For each batch of neutrophils, 60 ml of blood from one horse was used. The neutrophils were carefully collected, washed with two volumes of physiological saline solution and resuspended in 20 mM phosphate buffered saline (PBS) (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.

IV.2.7.3. 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 et al. (Benbarek et al., 1996). Briefly, neutrophils suspensions were distributed in the wells of a 96-well-microtiter plate (10^6 neutrophils per well) (white combiplate 8, Fisher Scientific) and incubated for 10 min at 37°C with PBS-solutions of the extracts (*T. mollis* and green tea) at final concentrations of 0.4; 2; 4; 10; 20 and 40 µg/ml. After 10 min incubation, 25 µl of $CaCl_2$ (Merck, VWRI, Leuven, Belgium), 2 µl of 5 µM lucigenin and 25 µl of 16 µM PMA (Sigma, Bornem, Belgium) were added. The chemiluminescence of the neutrophils was immediately monitored for 30 min (Fluoroscan Ascent, Fischer Scientific) and expressed as the integral value of the

total CL emission. 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. Stimulated neutrophils incubated with PBS containing 1% of the vehicle (DMSO or pure water) instead of plant extracts was used as control and taken as 100% of CL response. The percentages of inhibition for all samples were calculated in relation to the control.

IV. 2.7.4. Viability test

This test was carried out to verify if the decrease of chemiluminescence response is not due to the toxicity of the samples analysed. For that we used the Trypan bleu exclusion test (Tenant, 1964). 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. Unstimulated neutrophils and DMSO or water, in place of sample were used as control.

IV.2.7.5. Measurement of active MPO by SIEFED method

The SIEFED method allows to specifically detecting the active equine neutrophil MPO (Franck et al., 2006). This method includes three steps, the first one consists of the extraction of MPO from a solution or a biological sample by specific immobilised antibodies followed by a washing step to eliminate all other compounds of the sample (proteins, modulating and interfering substances) that do not bind to the antibodies. The third step deals with the *in situ* detection of the nitration-peroxidasic activity of MPO using 10 µM H_2O_2 (Merck, VWRI, Leuven, Belgium) and nitrite anions (Sigma, Bornem, Belgium) as reaction enhancer and the fluorogenic substrate Amplex® Red (Molecular Probes, Invitrogen, Merelbeke, Belgium).
The MPO solution (50 ng/ml) was prepared from purified equine MPO diluted in PBS at pH 7.4 with 5 % Bovin Serum Albumin (BSA) (Bornem, Belgium) and 0.1% Tween 20 (Merck, VWRI, Leuven, Belgium). The extracts and references were incubated for 10 min with MPO at a final concentration of 25 ng/ml. After incubation, the solutions were placed in the well of SIEFED microtiter plate already coated with rabbit polyclonal antibodies (3 µg/ml) against equine MPO and then incubated again for 2 h at 37°C in darkness. This immunoextraction step, during which MPO is specifically captured by the antibodies, was followed by 4 washing steps with PBS/Tween solution. The peroxidase activity of MPO was measured

after adding 100 µl of a 40 µM Ampex® Red solution freshly prepared in phosphate buffer (50 µM) containing H_2O_2 (10 µM), and NO_2^- (10 mM). MPO in diluted buffer with 1% DMSO or pure water was used as control and taken as 100 % MPO activity. After incubation, fluorescence was determined with Fluoroscan Ascent plate reader at excitation (λ = 544 nm) and emission (λ = 590 nm). The percentages of inhibition were calculated for all samples in relation to the control.

IV.2.8. Determination of total polyphenol and tannin content

Total polyphenol was determined for *T. mollis* root bark extracts and for green teas according to the method recommended by European Pharmacopeae 7.0 (2.8.14) (EDQM, 2011). Plant powder (± 1 g) was treated as described above in section 2.5. (samples free of tannins). From the first filtrate obtained, 5.0 ml was taken and diluted to 25.0 ml with distilled water. To 2.0 ml of the resulting solution we added 1.0 ml of phosphomolybdotungstic reagent, 10.0 ml of distilled water and we completed the mixture to 25.0 ml with sodium carbonate (290.0 mg of $NaCO_3$.10 H_2O in 1.0 L distilled water). After 30 min incubation, the absorbance (A_1) was read at 760 nm with spectrophotometer UVICON 922 using distilled water as blank. Pyrogallol (Baker, Netherlands) was used as standard, for that about 50.0 mg of the product was immediately dissolved in distilled water and 2 ml of the obtained solution treated as for the plant extract. The absorbance of the pyrogallol solution was recorded as A_3. Total polyphenols was calculated according to the formulae: the percentage of total polyphenols = $[(62.5 \times A_1) \times m_2] / (A_3 \times m_1)$ where m_1 is the mass of the sample to be analysed and m_2 the mass of pyrogallol in grams.

To determine tannin content, samples were treated as already described above in section 2.2. An aliquot from the second filtrate, obtained after adding hide powder, was treated following the same procedure as for the determination of total polyphenols and the absorbance recorded as A_2. The percentage content of tannins = $[62.5 \times (A_1 - A_2) \times m_2] / A_3 \times m_1$.

IV2.9. Quantification of ellagic acid, punicalagin A & B and gallic acid in *T.mollis* root bark

The level of ellagic acid, punicalagins and gallic acid was determined using HPLC-UV/DAD analysis. Acetonitrile and 0.05 % trifluoacetic acid were used as mobile in gradient mode and ODS C18 column HYPERSIL 250/4.6 mm, (5 µm) as stationary phase.

IV.2.10. Statistical analysis

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

IV.3. RESULTS AND DISCUSSION

IV.3.1. Bio-guided fractionation

All *T. mollis* root bark extracts tested showed an antiplasmodial activity and the best activity was found with methanolic extract (TMM) on both *Plasmodium falciparum* strains (3D7 and F32). Even aqueous crude extract of the plant (TMH) exhibited a high antiplasmodial activity (IC_{50} = 4.66 µg/ml) against 3D7 (Table 4.1), supporting the use of this plant in traditional medicine. However, the previous study reported a weak activity of the aqueous extract of this plant (Muganga et al., 2010). This difference may be explained by the fact that the sample used in the present study was fresh compared to that one used in the previous study. Furthermore, significant difference in terms of activity was observed between samples collected during summer period and those collected during rainy season and it was already observed that the time of collection and the locality of plant material may play a major role in its beneficial properties (Capasso, 1985; Gessler et al., 1994; Muganga et al., 2010). After liquid-liquid partition, ethyl acetate fraction (TMEA) was more active (IC_{50} = 2.10 µg/ml) than the aqueous one (IC_{50} = 4.66 µg/ml) and the insoluble fraction (IC_{50} = 39.71 µg/ml) while the hexane fraction was inactive ($IC_{50} > 50$ µg/ml). The fractionation of ethyl acetate fraction yields different fractions from which compound TMEA3 was isolated (from fraction 87 – 88) and presented a very high activity, IC_{50}= 0.175 µg/ml (see Table 1). TMEA2 presented also a very important activity (IC_{50}= 0.75 µg/ml) and its further analysis revealed that it was a semi pure fraction containing additional TMEA3.

The spectral data of all constituents isolated were compared with their corresponding references. Compound T1A was identified as an ellagic acid derivative (his structure is under

elucidation) and was inactive against *P. falciparum* strain 3D7. These results support previous report in which the important role of free hydroxyl groups in the antiplasmodial activity of ellagic acid was observed (Simões-Pires et al., 2009; Sturm et al., 2009). Compound TMEA3 was determined as ellagic acid and was responsible for the activity of the plant. Ellagic acid is a polyphenolic compound commonly found in different plant tissues (leaves, fruits, stalks, stem and root bark). The *in vitro* antiplasmodial activity of ellagic acid is already known (Banzouzi et al., 2002; Soh et al., 2009; Verotta et al., 2001) and our results are in accordance with those already found (IC_{50} between 90 and 175 ng/ml).

Moreover, ellagic presented synergy activity with other antimalarial drugs like chloroquine, atovaquone, mefloquine and artesunate and slight antagonism with artemisinin (Soh et al., 2009). The mechanism of action of ellagic acid on *Plasmodium falciparum* is not very well known. Some researchers suggested that, as this compound has ability to make a π-π complex (Dell'Agli et al., 2003), it may act at the mature trophozoites and young schizont stage of the erythrocytic life cycle by inhibition of β-hematin formation in the parasite like aminoquinoline antimalarial agents such as chloroquine (Egan et al., 1999). Although ellagic acid is very active against malaria, its bioavailability is low. Therefore, the modification of this compound is needed to improve its activity after oral administration. Indeed, ellagic acid has poor absorption (less than 1 %) and is rapidly excreted after oral administration (Hamad et al., 2009; Seeram et al., 2004; Stoner et al., 2005).

Table 4.1: *In vitro* antiplasmodial activity and percentage of haemolysis with *T. mollis* root bark extracts and isolated compounds. Data are expressed as mean ± SD, n ≥ 3

Plant sample	Yield	3D7 (IC_{50} µg/ml)	F32 (IC_{50} µg/ml)	% Haemolysis	
				100 µg/ml	200 µg/ml
TMM	27.22	3.84 ± 1.23	3.42 ± 0.77	0.05 ± 0.03	0.11 ± 0. 4
TMH	36.3	4.66 ± 0.89	12.3 ± 1.18	0.08 ± 0.06	0.03 ± 0.02
TMFT	Nd	25.77 ± 4.89	Nd	Nd	Nd
FxTMnH	1	>50	Nd	Nd	Nd
FxTMEA	45.66	2.1 ± 0.48	Nd	Nd	Nd
FxTMH	38.14	19.72 ± 4.86	Nd	Nd	Nd
Fx Ins.	11.7	39.71 ± 2.18	Nd	Nd	Nd
T1A	Nd	>50	Nd	Nd	Nd
TMEA2	Nd	0.75 ± 0.059	Nd	Nd	Nd
TMEA3 (ellagic acid)	Nd	0.17 ± 0.17	0.140 ± 0.028	Nd	Nd
TMEA4	Nd	> 50	> 50	Nd	Nd
Artemisinin		0.005 ± 0.00021	0.003 ± 0.0003	Nd	Nd
Chloroquine		0.0017 ± 0.004	Nd	Nd	Nd

TMM : crude methanolic root bark extract of *T. mollis*
TMH : crude aqueous root bark extract of *T. mollis*
TMFT : crude aqueous root bark extract of *T. mollis* free of tannins
FxTMnH : n hexane fraction of *T. mollis*
FxTMEA : ethyl acetate fraction of *T. mollis*
$FxTMH_2O$: aqueous fraction of *T. mollis*
Fx Ins. : fraction insoluble in water
T1A : fraction 1
TMEA2 : fraction 2
TMEA3 : fraction 3
TMEA4 : fraction 4

Ellagic acid possesses other beneficial properties like anticancer, antimutagenic (Aggarwal et al., 2006; Kasimsetty et al., 2010; Kaur et al., 1997; Loarca-Pina et al., 1998), antioxidant, anti-inflammatory (Devipriya et al., 2007; Priyadarsini et al., 2002; Rogerio et al., 2008; Tasaki et al., 2008), antiviral (Ruibal, 2003) and antibacterial activity (Reddy et al., 2007). The fact that this compound presents antiplasmodial, antioxidant and anti-inflammatory activities may be an advantage, especially in case of severe malaria (Dell'Agli et al., 2010; Watt et al., 2002). Ellagic acid has also cardioprotective activity (Lakovleva et al., 1998), exerts antiulcer activity (Beserra et al., 2011) and hepatoprotective activity (Singh et al., 1999). Moreover, the fact that a part of *Plasmodium falciparum* life cycle occurs in the liver, the hepatoprotective activity of ellagic acid is a real advantage.

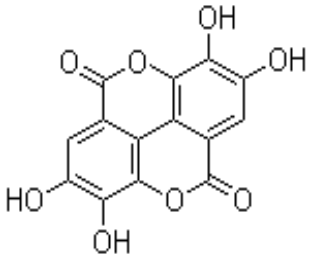

Figure 4.1 Ellagic acid

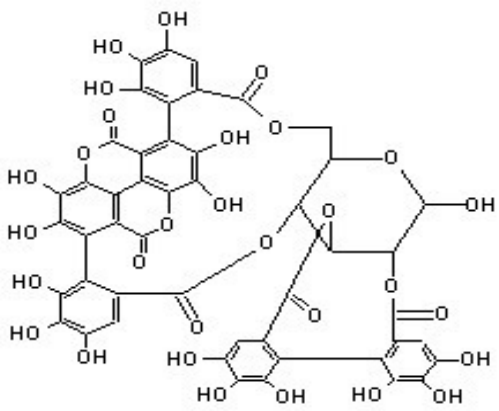

Figure 4.2 Punicalagins

In this study, ellagic acid was found in *T. mollis* root bark in aglycone form (free form). Most of time the ellagic acid is present in conjugated form with a glycoside moiety and its free form in the nature is uncommon (Clifford et al., 2000).

Fraction TMEA4 was identified as a mixture of punicalagin A & B (anomeric isomers) by comparison of their spectral data with those of a commercial sample. Punicalagin, like ellagic acid, is a hydrolysable polyphenolic compound (Wilson et al., 1900) commonly called ellagitanin and found in plants where it plays the physiological role of protection against microbial decay (Haslam, 1996). Various biological activities from punicalagin were reported: antioxidant, anti-inflammatory, anti-genotoxic, antiviral (Chen et al., 2000; Haidari et al., 2009; Kulkarni et al., 2007) and hepatoprotective (Lin et al., 2001). However, there has been a great deal of discussion about the toxicity of this compound as it may provoke liver necrosis and nephrotoxicity (Cerda et al., 2003; Doig et al., 1990; Filippich et al., 1991; Oelrichs et al., 1994). Further studies are needed to prove the safety of punicalagin in human. The fraction TMEA4 did not present any antiplasmodial activity on both *Plasmodium falciparum* strains. In contrast, previous studies reported the antiplasmodial activity of punicalagin but did not specify which one (punicalagin A or B) was the active constituent (Reddy et al., 2007; Shuaibu et al., 2008). Asres et al. (2001) reported that punicalagin was active against 3D7 (IC_{50} = 27.73 µg/ml). This punicalagin activity was very low for a pure compound and should be qualified as negligible compared to that one found with other pure compounds like ellagic acid. Furthermore, in our study, the crude aqueous extract free of tannin (TMFT) was also active suggesting that the active ingredient(s) is/are not totally adsorbed by hide powder. HPLC analysis of this TMFT extract demonstrated that ellagic acid

was still present whereas punicalagin and its derivatives were almost absent. Gallic acid and some condensed tannins such catechin, gallocatechin, and epigallocatechin were also identified in the plant (data not shown) but were reported to have lower antiplasmodial activity, (IC_{50} > 25 µg/ml) (Ramanandraibe et al., 2008) then the one found with ellagic acid. Consequently, ellagic acid is the main antiplasmodial constituent and those other compounds present in the plant may reinforce its antiplasmodial activity.

No haemolysis was observed on erythrocytes with the highest concentrations tested for antiplasmodial activity (100 and 200 µg/ml). This means that, at the concentrations presenting antiplasmodial activity, there is no toxicity of the plant extracts towards erythrocytes.

The toxicity of aqueous extract of *T. mollis* in rats was already reported (Bulus et al., 2007) and the safety of this plant in human must be determined to guaranty its traditional use.

IV.3.2. The effect of T. mollis root bark extract on other parasites

IV. 3.2.1.In vitro antitrypanosomal and antileishmanial activity

Methanolic and aqueous extracts of *T. mollis* showed an interesting antitrypanosomal activity and the highest activity was observed with the methanolic extract (IC_{50} = 3.725 µg/ml), but they were both inactive toward leishmania (IC_{50} > 100 µg/ml) (Table 4.2). The antitrypanosomal activity is already known in *Terminalia species* (Abiodun et al., 2011; Shuaibu et al., 2008). In this study, ellagic acid presented a good activity (IC_{50} = 1.92 µg/ml) against trypanosome but this activity was not significantly different from that obtained from the crude extract; suggesting that the plant possesses others active compounds. Indeed, punicalagin and gallic acid, also present in the plant were reported to have trypanocidal activity (Asres et al., 2001; Hoet et al., 2004; Koide et al., 1998) meaning that those compounds may act as antitrypanosomal by synergism.

Table 4.2: *In vitro* antitrypanosomal and antileishmanial activities of *T. mollis* root bark extracts

Plant extract	Antitrypanosomal activity Tbb (IC_{50}, µg/ml ± SD)	Antileishmanial activity Lmm (IC_{50}, µg/ml ± SD)
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
Suramine	0.11 ± 0.02	Nd
Amphotericine B	Nd	0.1 ± 0.01

TMM : crude methanolic root bark extract of *T. mollis*

TMH : crude aqueous root bark extract of *T. mollis*

TMFT : crude aqueous root bark extract of *T. mollis* free of tannins

TLC analysis of fractions obtained after fractogel confirmed the presence of some terpenoid saponins in this plant (data not shown) which may play as well a role of active constituents (Bulus et al., 2008). However, saponins may occur as minor components in many members of the Combretaceae whereas tannins are often major constituents (Hegnauer, 1989). Furthermore, another study (Asres et al., 2001) reported that saponins isolated from another combretaceae species, *Combretum molle*, present low activity against trypanosoma (IC_{50} > 30 µg/ml). The determination of the active constituents and evaluation of the antitrypanosomal activity of *T. mollis in vivo* are then recommended.

As already mentioned above, *T. mollis* root bark extracts were not active against leishmania. However, ellagic acid and punicalagin, two ellagitannins present in this plant, have weak antileishmanial activity. Indeed, in the present study, ellagic acid was moderately active (IC_{50} = 18.43 µg/ml) and punicalagin was previously reported to have also a moderate antileshmanial activity (IC_{50} > 10 µg/ml) (Asres et al., 2001). In contrast, gallic acid was reported to have a good leishmanicidal activity (Kolodziej et al., 2001). Based on our results, *T. mollis* is not recommended to treat leishmania. According to a Rwandan traditional healer, *T. mollis* based preparation is used to treat "gastrointestinal disorders". As we know, most of time traditional healers do not have enough information about the diseases they are claiming

to treat. This means that the term "gastrointestinal disorders" may describe for them various diseases such as trypanosomia or another parasitosis sensitive to *T. mollis* extract.

IV.3.2.2. In vitro antiamoebic activity

Balamuth medium (an egg yolk based medium) was used because it is a simple medium, cheap and able to maintain parasite life up to 8 days but we opted to change the culture medium every 3 days. Hydroethanolic extract of *T. mollis* root bark presented a moderate activity (MIC ≤ 250 µg/ml) against vegetative forms of *Entamoeba histolytica* obtained from a patient while metronidazole, used as standard, was active (MIC ≤ 31.125 µg/ml). Previous studies used also a laboratory strain isolated from patient with acute amoebic dysentery to evaluate the antiamoebic activity. The antiamoebic activity of metronidazole found in this work was low compared to that one reported in previous studies (Calzada et al., 2003; McGaw et al., 2000; Sohni et al., 1995) suggesting the possible resistance to metronidazole of the used *Entamoeba histolytica* strain. However, the resistance of *Entamoeba histolytica* to metronidazole was already observed (Aguirre-Cruz et al., 1990) and comparable low antiamoebic activity of metronidazole (MIC = 10 µg/ml) was previously reported (Sohni et al., 1995). Therefore, further study using a standardized *Entamoeba histolytica* strain is needed to confirm the eventual resistance. The amoebicidal property of gallic acid and ellagic acid is known (Alanís et al., 2003; Derda et al., 2009) and ellagic acid may play a major role in the antiamoebic activity of *T. mollis*. Unfortunately, we did not be able to evaluate the antiamoebic activity of ellagic acid. HPLC analysis of *T. mollis* root bark extract shows that ellagic acid and punicalagins derivatives are the major constituents and ellagic acid is a metabolite of punicalagin (Aguilera-Carbó et al., 2008). This means that an important amount of ellagic acid in free form is available in situ (intestine) after oral administration and will then react actively against vegetative forms of amoeba. Moreover, Alanís et al. (2003) demonstrated that epigallocathechin has an interesting amebicidal activitity (IC_{50} 1.9 µg/ml) comparable to emetine (Alanís et al., 2003). Previous phytochemical study of the stem bark of the plant indicated the presence of epigallocathechin among others condensed tannins (Liu et al., 2009). According to a traditional healer from eastern province of Rwanda, the stem bark of the plant is used to treat gastrointestinal disorders in young children. The antiamoebic activity of *T. mollis* found in the present study confirms its use in traditional medicine, but more investigations are needed including the toxicity of the plant. The *in vivo* determination

of the activity of *T. mollis* against this parasite is highly recommended as well as the determination of active constituents.

IV.3.3. *In vitro* anti-inflammatory activity of T. mollis root bark extracts

T. mollis root bark extracts and green tea aqueous extract, used as reference, displayed an interesting total antioxidant activity on the ABTS test (IC_{50} < 10 µg/ml). Comparable radical scavenger activity was observed for *T. mollis* root bark and green tea. Overall, the percentage of ABTS radical activity decreased in a dose dependent manner for all samples tested. A significant inhibition ($p < 0.05$) was observed for all plant extracts from concentration of 2 µg/ml and form 0.5 and 1 µg/ml for gallic acid, punicalagins and ellagic acid respectively. The antioxidant activities of plant extracts using pure water and DMSO as solvents were not significantly different ($p > 0.05$) (data not shown). However, we preferred to use pure water as solvent with the exception of hydroethanolic extract and references which were more soluble in DMSO. Pure water was chosen as solvent because it is used to prepare traditional remedies. Green tea is known to have a good antioxidant activity and was used in this test for better evaluation of our plant extracts. Nevertheless, gallic acid famously known for its high radical scavenger activity (Hagerman et al., 1998) was used as reference and compared to two polyphenolic compounds, ellagic acid and punicalagin, present also in *T. mollis*. Rwandan and commercial green teas presented similar antioxidant activity and only tea from Rwanda was used in further analysis. The best results were found with 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) (Figure 4.3). 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 (Figure 4.4). The antioxidant activity of ellagic acid and punicalagin was previously reported (Reddy et al., 2007). *T. mollis* crude aqueous extract free of tannins showed also a promising antioxidant activity because the plant contains not only ellagitannins which are adsorbed on hide powder, but also ellagic acid and some condensed tannins such as catechin, epicatechin, gallocatechin

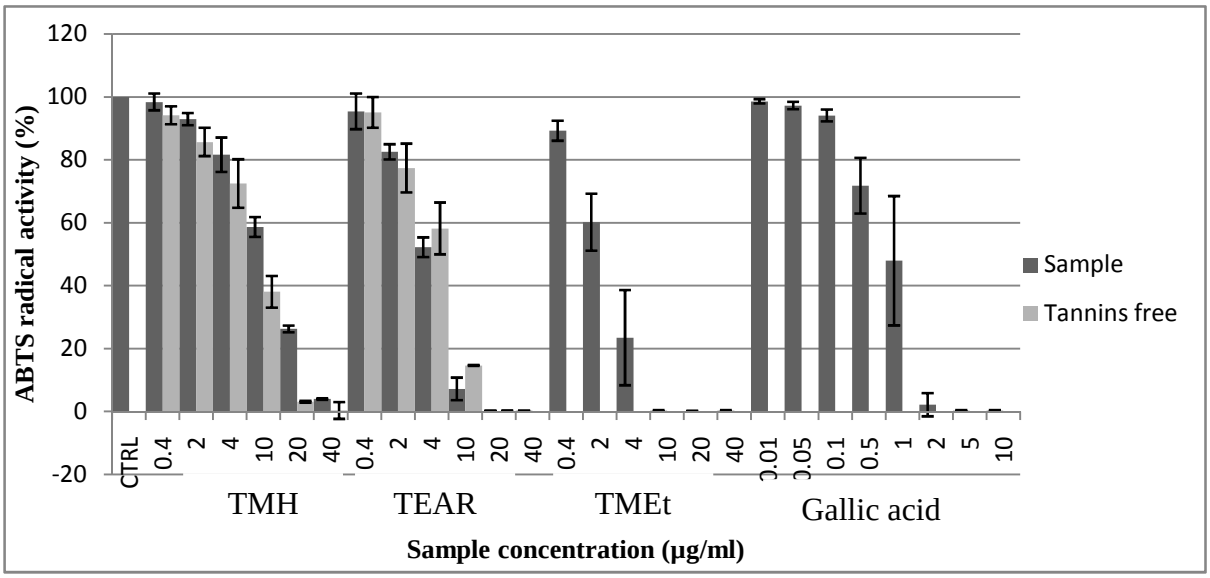

Figure 4.3 Effect of plant extracts and gallic acid on the ABTS cation radical aassay. Samples were tested at final concentration of 0.4, 2, 4, 10, 20 and 40 µg/ml for plant extracts and 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml for gallic acid. The percentage of inhibition was calculated for each sample concentration versus the control DMSO taken as 100 % (mean ± SD, n ≥ 3).

CTRL: control (pure water or DMSO)
TMH: *T. mollis* crude aqueous extract
TEAR: Crude aqueous extract of green tea from Rwanda
TMEt: 50% hydroethanolic extract of *T. mollis*

and epigallocatechin (Liu et al., 2009) as well as gallic acid and derivatives. *T. mollis* also contains also some terpenoids saponins which may intervene in radical scavenging activity. Previous study reported the antioxidant activity of triterpenoid saponins from another species of Terminalia, *Terminalia ivorensis* (Ponou et al., 2010). The hydroethanolic extract of *T. mollis* at a concentration of 10 µg/ml exhibited total inhibition of ABTS radical activity. This very high activity may be explained by the fact that some antioxidant compounds present in the plant like gallic acid and ellagic acid are more soluble in organic solvent. The antioxidant capacity of this plant may also be beneficial in the treatment of other diseases because various pathology conditions are associated with oxidative stress (Ponou et al., 2010). The results from this test show the capacity of our plant extracts to scavenge free radicals and the results from CL and SIEFED assays gave further information about the anti-inflammatory activity of the extracts.

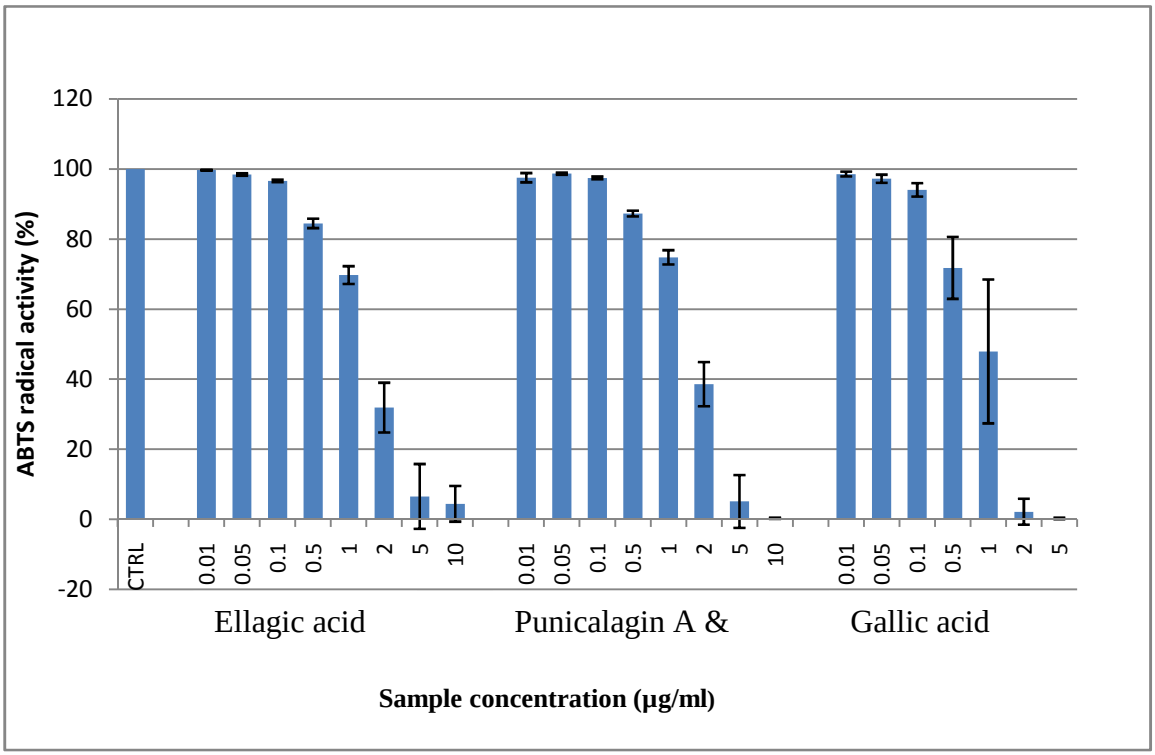

Figure 4.4 Comparison of the effect of pure compounds found in *T. mollis* on ABTS radical activity using DMSO as solvent. Samples were tested at a final concentration of 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml. The percentage of inhibition indicated at the top of the column was calculated for each sample concentration versus the control DMSO taken as 100 % (mean ± SD, n = 3).

CL assay allows us evaluating the effect of plant extract and references on the total ROS produced by equine neutrophils stimulated with PMA. The aqueous extracts of *T. mollis* and references reduced ROS production in concentration-dependent manner (Figure 4.5). 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 higher than the one of corresponding extracts with tannins but no significantly ($p < 0.05$). 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). Considering the molecular weight of the components, punicalagin is the most potent and its high activity results on the presence of 16 phenolic hydroxyls per molecule.

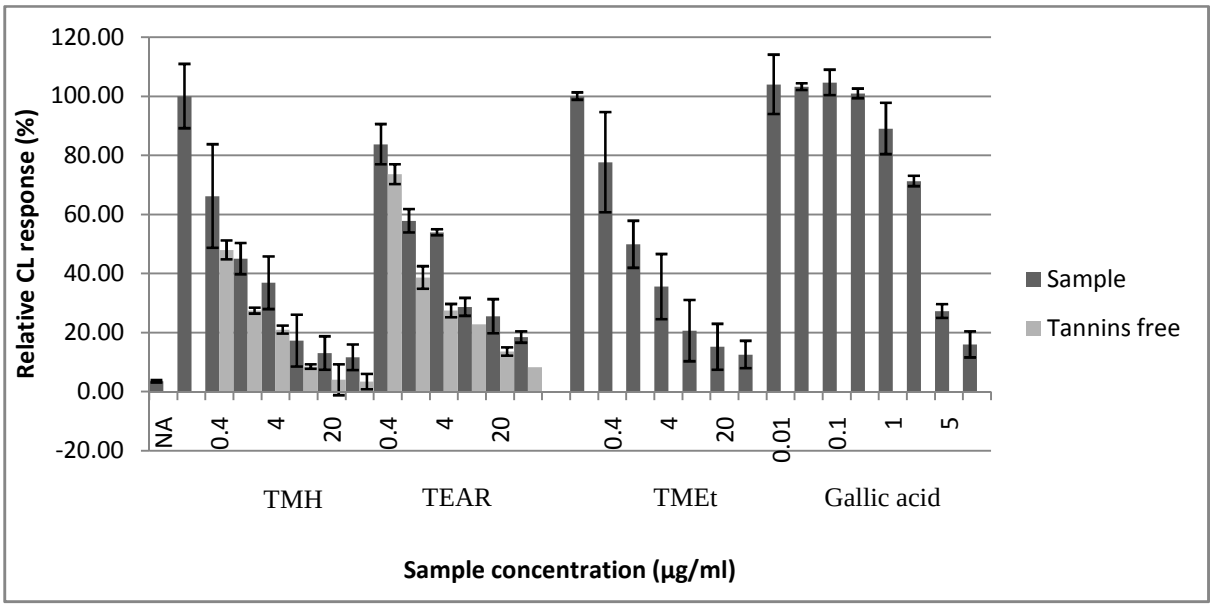

Figure 4.5 Inhibitory effect of *T. mollis*, green tea extracts and gallic acid on the chemiluminescence response produced by PMA-stimulated equine neutrophils. Aqueous extracts of *T. mollis* and green tea were dissolved in pure water, hydroethanolic extract and gallic acid dissolved in DMSO and tested at final concentrations of 0.4, 2, 4, 10, 20 and 40 µg/ml for plant extracts and 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 ± S,D, n ≥ 6 and experiments were performed using different batches of neutrophils.

NA : not activated cells
CTRL DMSO: DMSO + stimulated cells
CTRL W : Pure water + stimulated cells
TMH: *T. mollis* crude aqueous extract
TEAR: Crude aqueous extract of green tea from Rwanda
TMEt: 50% hydroethanolic extract of *T. mollis*

Plant extracts and pure compounds exhibited inhibition of MPO in dose-depending manner as for CL assay. Significant inhibition of MPO was observed for all concentrations tested for plant extracts. 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 4.3). Significant inhibition was observed from 5 µg/ml for ellagic acid, from 2.5 µg/ml for punicalagin and from 0.5 µg/ml for gallic acid. Similarly punicalagin was again the most active whereas ellagic acid was less active compared to other pure compounds tested. According to the results of this study, punicalagin and ellagic acid may have pro-oxidant activity at low concentrations (≤ 0.1 for punicalagin and ≤ 1 µg/ml for ellagic acid).

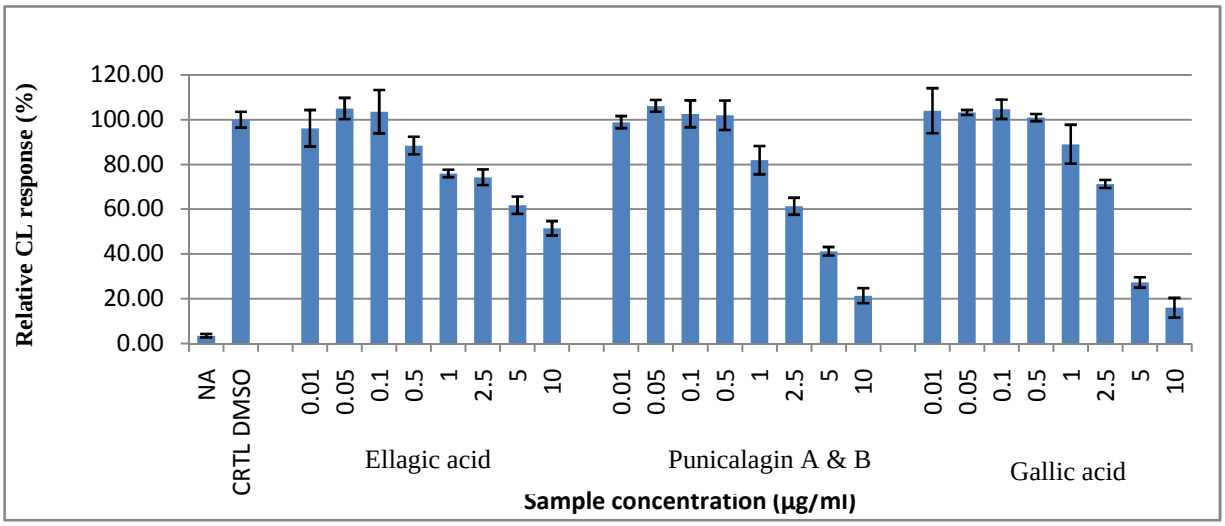

Figure 4.6 Inhibitory effect of ellagic acid, punicalagin and gallic acid on the chemiluminescence response produced by PMA-stimulated equine neutrophils. Pure compounds were dissolved in DMSO and tested at final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml. The percentage of inhibition was calculated versus the control DMSO taken as 100%. All data were expressed as means ± SD, n= 6 and experiments were performed using different batches of neutrophils.

NA: not activated cells
CTRL DMSO: DMSO + stimulated cells

Similar results were also found for ellagic acid (Soh et al., 2009) but further study is needed as standard deviation values found at those concentrations were relatively high (Figure 4.6). It is possible to found the two opposite activity from one compound. Indeed, previous study demonstrated that curcumin, another antioxidant compound, may act as pro-oxidant and antioxidant (Kang et al., 2005; Sandur et al., 2007).

The anti-inflammatory activity of gallic acid is already known (Kroes et al., 1992) as well as the anti-inflammatory property of ellagic acid and punicalagin (Adams et al., 2006; Bae et al., 2010; Chao et al., 2010; Corbett et al., 2010; Rogerio et al., 2008). As already mentioned above, the root bark of *T. mollis* contains gallic acid, ellagic acid, punicalagins and their derivatives which may be mainly responsible for the anti-inflammatory activity of the plant. Indeed, those compounds, especially punicalagin and gallic acid, presented strong radical scavenging and modulatory activities of on the oxidant activity of stimulated neutrophils and myeloperoxidase.

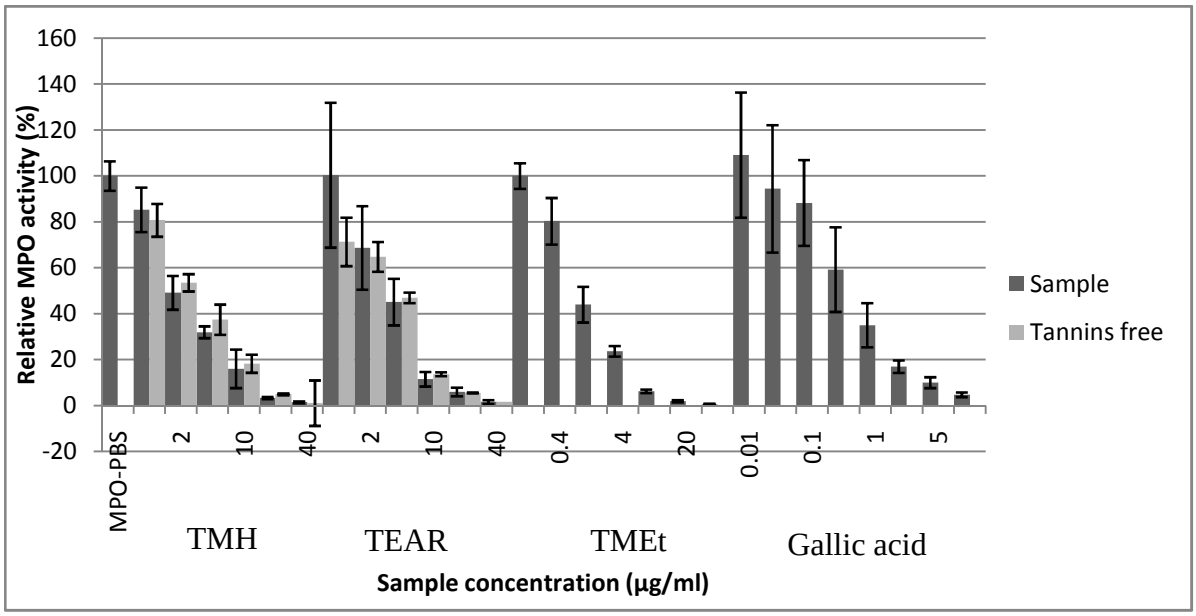

Figure 4.7 Effect of plant extracts and gallic acid on MPO activity measured by SIEFED. Samples were analysed at final concentrations of 0.4, 2, 4, 10, 20 and 40 µg/ml. The percentage of inhibition was calculated for each sample concentration versus MPO-PBS or MPO-DMSO taken as 100% (mean ± SD, n ≥ 6).

MPO- DMSO: Control for gallic acid
MPO - PBS: Control for plant extracts
TMH: *T. mollis* crude aqueous extract
TEAR: Crude aqueous extract of green tea from Rwanda
TMEt: 50% hydroethanolic extract of *T. mollis*

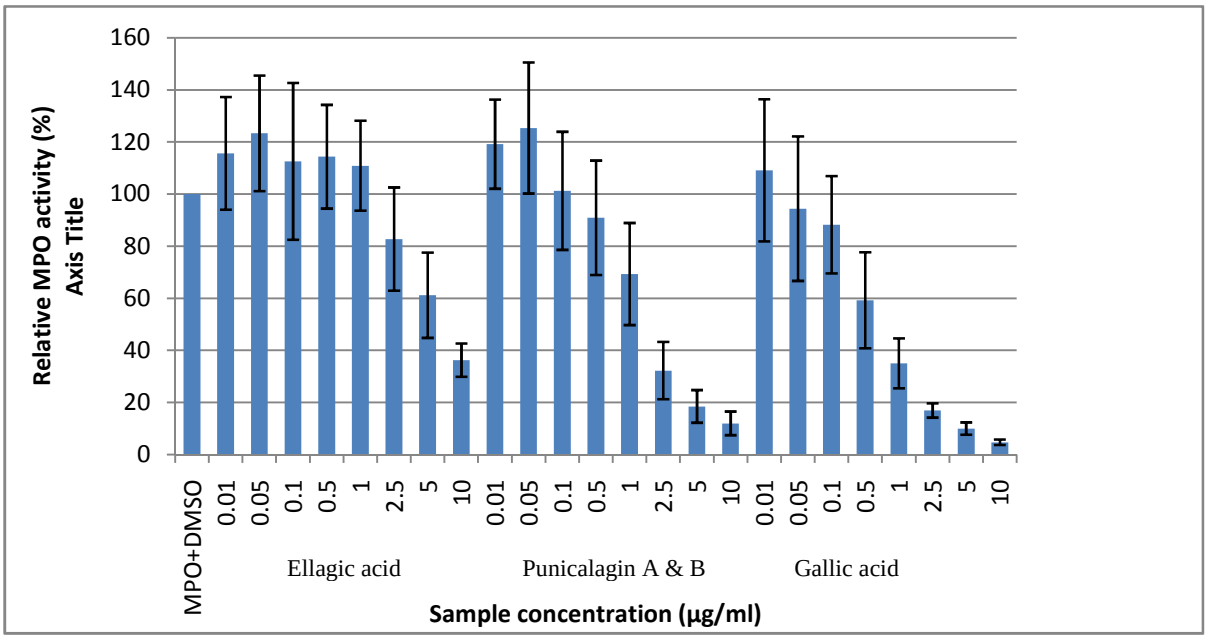

Figure 4.8 Effect of ellagic acid, punicalagin and gallic acid on MPO activity measured by SIEFED. Samples were analysed at final concentrations of 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml. The percentage of inhibition was calculated for each sample concentration versus MPO + DMSO taken as 100% (mean ± SD, n = 6). MPO+ DMSO: Control

Table 4.3 Summary of IC_{50} (µg/ml) and R2 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 (IC50), n ≥ 3 for ABTS assay and n ≥ 6 for CL and SIEFED. CL experiments were performed using different batches of equine neutrophils.

	IC_{50} in µg/ml ± SD (R^2)					
	ABTS test		**Chemiluminescence**		**SIEFED technique**	
	Sample	**Tannins free**	**Sample**	**Tannins free**	**Sample**	**Tannins free**
TMH	4.23 ± 0.21 (0.979)	6.76 ± 0.69 (0.972)	1.45 ± 0.26 (0.962)	0.38 ± 0.04 (0.96)	1.99 ± 0.59 (0.9562)	2.11 ± 0.65 (0.9266)
TEAR	4.01 ± 0.02 (0.992)	4.38 ± 0.48 (0.978)	3.79 ± 0.38 (0.963)	1.32 ± 0.11 (0.957)	3.06 ± 1.57 (0.820)	4.13 ± 0.77 (0.831)
TMEt	2.47 ± 0.32	nd	1.75 ± 0.43 (0.957)	nd	1.51 ± 0.41	nd
E.A.	1.56 ± 0.14 (0.986)	-	10.51 ± 1.28 (0.896)	-	6.65 ± 2.14 (0.926)	-
G.A.	0.89 ± 0.16 (0.974)	-	3.5 ± 0.09 (0.897)	-	0.62 ± 0.37 (0.928)	-
PUN.	1.77 ± 0.13 (0.985)	-	3.71 ± 0.09 (0.97)	-	1.74 ± 0.82 (0.870)	-

TMH : Crude aqueous extract of *T. mollis*
TEAT : Crude aqueous extract of green tea from Rwanda
TMEt : 50% hydroethanolic extract of *T. mollis*
E.A. : ellagic acid
G.A. : gallic acid
PUN. : punicalagins

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 no cytotoxicity towards neutrophils.

IV.3.4. Determination of total polyphenol and tannin content and the level of punicalagins, ellagic acid, gallic acid and derivatives in plant samples

Both *T. mollis* root bark and the green tea from Rwanda are rich in polyphenols and tannins. They contain quite similar level of total polyphenol and no noticeable difference between their tannins and non-tannins content (Table 4.4). Similarly, *T. mollis* and green tea from Rwanda presented comparable anti-inflammatory activity suggesting that polyphenol play a key role in the anti-inflammatory capacity of those plants. This results support previous works on the importance of polyphenol in the anti-inflammatory activity of various samples (Gil et al., 2000; Tsumbu et al., 2012).

HPLC-UV/DAD analysis using commercial pure compounds allowed us to quantify the amount of punicalagin A & B, ellagic acid, gallic acid and their respective derivatives in methanolic root bark extract of *T. mollis*, (r^2= 0.99934, 0.99924, 0.99964 and 0.99995 for punicalagins, ellagic acid and gallic acid respectively (annexe 1). The highest level was obtained with punicalagins (3.16% and 2.31% for punicalagin A and B respectively), followed by ellagic acid (2.0 %) whereas gallic acid level appears to be low (0.08%). The amount of punicalagins and derivatives in plant was estimated to be around 11.35 % based on punicalagin A (Table 4.4). Consequently, we may say that the high level of punicalagins and their derivatives play a major role in the anti-inflammatory capacity of the plant. However, samples free of tannins (like punicalagins and derivatives) exhibited also a remarkable anti-inflammatory activity, suggesting the synergistic action of the other *T. mollis* constituents such as gallic, ellagic acid and their derivatives. It should be noticed that other aspects of inflammation process should be also considered as the present study explored some of them.

The antidiarrhoea of tannins is already known (Nazarov, 1958) and the fact that this plant contains a big amount of tannins may confirm its use in traditional medicine to treat diarrhoea.

Many researchers agreed about the role of pro-inflammatory cytokines in malarial disease (Clark et al., 2006). Ellagic acid, the major antiplasmodial constituent of *T .mollis* may also

act on the release of pro-inflammatory cytokines. Unfortunately we did not explore this point of view. Inflammation is a complex biological response and only some causes were considered. Further studies are recommended to clarify it.

Table 4.4 Quantitative determination of total polyphenol and tannin content, level of punicalagins, ellagic acid, gallic acid and derivatives in *T. mollis* root bark and green tea from Rwanda. The percentages of total polyphenol and tannin content were calculated according to European Pharmacopeae 7.0 whereas the amount (%) of pure constituents was estimated using HPLC-UV/DAD

	***T. mollis* root bark**	**Rwandan green tea**
Total polyphenol	14.03 ± 1.06	14.33 ± 0.75
Tannins	10.59 ± 1.38	8.75 ± 0.42
Non-tannins	3.43 ± 0.32	5.58 ± 1.16
Gallic acid	0.08	0.08
Gallic acid & derivatives	1.85	1.09
Ellagic acid	1.42	Nd
Ellagic acid and derivatives	3.33	Nd
Punicalagin A	3.16	Nd
Punicalagin B	2.31	Nd
Punicalagins & derivatives	11.35	Nd

IV.4. CONCLUSION

The root bark of *T. mollis* has a very promising antiplasmodial activity and ellagic acid is the main active constituent. However, the efficacy of the plant in the treatment of malaria per oral administration may be low because of poor bioavailability of ellagic acid. The evaluation of *T. mollis* antiplasmodial activity *in vivo* is under investigation.

The very high antitrypanosomal activity of *T. mollis* leads this plant to be a good candidate to development of a standardised plant extract in the treatment of trypanosomiasis. Punicalagins, Ellagic acid, gallic acid and derivatives may be the major active constituents.

T. mollis is not recommended for the treatment of leshmaniasis even if it contains ellagic acid active against this parasitosis. Our primary study on the antiamoebic activity revels that *T. mollis* has antiamoebic activity and further study is needed to confirm its traditional use.

The plant possesses a very interesting anti-inflammatory activity mainly due to its high polyphenolic content particularly punicalagin and related compounds. This plant rich in

polyphenol may have other beneficial properties. This study revealed for the first time the antitrypanosomal, anti-inflammatory and antiamoebic activity of *T. mollis* as well as the presence of ellagic acid in an aglycone form as the compound responsible for the antiplasmodial activity of the plant.

IV.5. ACKNOWLEDGEMENTS

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IV.6. SUPPORTING INFORMATION

Details about HPLC analysis of the plant (method and chromatogram) and ^{1}H NMR spectra of ellagic acid are given as supporting information (Annexe 1).

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CHAPTER V ANTIMALARIAL COMPOUND(S) FROM *ZANTHOXYLUM CHALYBEUM* AND THE EFFECT OF THE PLANT ON OTHER PARASITES AND ON INFLAMMATION

The results reported in previous chapters illustrated the interesting antiplasmodial activity of the root bark of *Zanthoxylum chalybeum*. We were then interested in the isolation and the determination of the compounds responsible for the antiplasmodial activity of the plant. Bio-guided fractionation was performed on methanolic root bark extract of the plant, the most active extract from the plant. Compounds were isolated chromatographically, and their chemical structure determined by using NMR and MS. Since the main active antimalarial compounds known, the plant extract was then evaluated *in vitro* for its antiparasitic activity and its possible effect on inflammation which is associated to infectious diseases such as malaria and other studied parasitosis. The anti-inflammatory activity was evaluated only on the aqueous extract of the plant for good comparison with the results previously reported on the same extract (Muller-Jakic et al., 1993; Matu et al., 2003). Moreover, water is the solvent mainly used by traditional healers to prepare their remedies. This chapter is based on the following article under submission:

In vitro **antiplasmodial, antileishmanial, antitrypanosomal, antiamoebic and anti-inflammatory activity of *Zanthoxylum chalybeum* root bark**

Abstract

The aim of the study

The aim of this study was to determine the active substances responsible for the antiplasmodial activity of *Zanthoxylum chalybeum*, to evaluate the effect of the plant on other parasites like trypanosome, leishamania and amoeba and to determine its anti-inflammatory activity.

Methods

The antiplasmodial activity was tested on chloroquine-sensitive and mefloquino- resistante *Plasmodium falciparum* strains (3D7 and F32). Antitrypanosomal and antileishmanial activity was evaluated *in vitro* respectively on *Trypanosoma brucei brucei* (strain 427) (Tbb) and promastigotes of *Leishmania mexicana mexicana* (MHOM/BZ/84/BEL46) (Lmm). The antiamoebic activity was assessed using an *Entamoeba histolytica* strain from a patient with

acute amoebic dysentery. Total antiradical activity was estimated using 2, 2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation decolorization assay. The anti-inflammatory activity of the plant was determined by evaluating the effect of crude extract on the production of reactive oxygen species (ROS) by stimulated neutrophils and on purified equine myeloperoxidase (MPO) activity. Total polyphenol and tannin content were determined as described by European Pharmacopeia 7.0.

Results

Crude methanolic root bark extract showed a very promising antiplasmodial activity (IC_{50} 6.18 µg/ml respectively) and nitidine was identified as the main active constituent (IC_{50} = 0.175 µg/ml). The methanolic crude extract of the plant exhibited a remarkable growth inhibition on Lmm (IC_{50} 1.82 µg/ml) and moderate activity against Tbb (IC_{50} 19.38 µg/ml). The ethanolic crude extract of the plant presented a weak antiamoebic activity (MIC ≤ 312.5 µg/ml). Aqueous crude extract presented a high antioxidant activity and interesting capacity to inhibit total ROS produced by stimulated neutrophils (IC_{50} < 5 µg/ml for ABTS and CL), but its effect on myeloperoxidase was low (IC_{50} 67.69 µg/ml).

Conclusion

This study revealed the active constituent responsible for the antiplasmodial activity of *Z. chalybeum* which is potentially a good candidate for further investigations into natural antileishmanial field. The plant has an interesting anti-inflammatory activity and may act on NADPH oxydase or on superoxide anion.

V.1. INTRODUCTION

Although a lot of effort has gone into making the control of malaria a success, the disease remains one of the most health challenges particularly in developing countries where, according to WHO, 81% of cases reported worldwide occurs (WHOb, 2011). In Rwanda, as in many African countries, the majority of people prefer the usage of medicinal plants to cure various diseases (Elujoba et al., 2005) including malaria. Several studies reported the promising antiplasmodial activity of some medicinal plants used in the treatment of malaria (Jonville et al., 2008; Kantamreddi et al., 2012; Muthaura et al., 2007; Rukunga et al., 2009) but few is finally known about their active constituents. In our previous report (Muganga et al., 2010) some Rwandan plants, among which we can cite *Zanthoxylum chalybeum*, were of possible value for the development of new antimalarial drugs. *Zanthoxylum chalybeum*

(Rutaceae) locally called "intareyirungu", is one of Rwandese plants used traditionally to treat malaria. It is widely distributed in other countries such as Ethiopia, Somalia, Kenya, Uganda, Tanzania, Burundi, Zambia particularly in dry bushland and woody grassland (Neuwinger, 1996). Concerned about the possible valorisation of this plant in the treatment of malaria, the determination of the active ingredient responsible for the antiplasmodial activity constitutes an important step. Moreover, as some *Zanthoxylum species* have been reported to possess antiparasitic properties in addition to antiplasmodial activity (Akhtar et al., 2000; Ferreira et al., 2011; Ferreira et al., 2007), *Zanthoxylum chalybeum* was also assessed against other parasites like trypanosoma, leishmania, and amoeba. Trypanosomiasis, leishmaniasis and amoebiasis are tropical neglected diseases which are directly associated with poverty and then affect mainly developing countries. However, the global emigration phenomenon may modify the geographical distribution of those diseases worldwide (WHOa, 2011; Ximénez et al., 2011). Since there is little incentive to pharmaceutical industries to develop new medicines against neglected diseases, medicinal plants offer an opportunity to provide new effective products that are readily available and affordable, and *Zanthoxylum chalybeum* could be a good candidate. Malaria, trypanosomiasis, leishmaniasis and amoebiasis are infectious diseases and they are accompanied by inflammation response (Clark et al., 2006). Inflammation is an immune response to protect tissues from irritants, or pathogens. However, inflammation can sometimes be inappropriately set of or poorly regulated, and leads to various disorders even death (Kumar et al., 2009; Revillard, 2001; Serhan et al., 2010). The control of inflammatory is then important in the management of various illnesses such as infectious diseases. Some alkaloids constituents of *Zanthoxylum chalybeum* such as protoberberines and benzophenanthridine have been reported to possess anti-inflammatory activity (Muller-Jakic et al., 1993). Previous researches demonstrated the interesting anti-inflammatory activity of the plant using cyclooxygenase (Matu et al., 2003) and 5-lipooxygenase assay (Muller-Jakic et al., 1993). As inflammation is a complex reaction, it is interesting to determine the effect of the plant on other aspects of inflammation process. One of the main active agents in inflammation is the polymorphonuclear neutrophil (Firestein et al., 2008; Serhan et al., 2010). When polymorphonuclear neutrophils are stimulated, they produce reactive oxygen species (ROS) and release myeloperoxidase (MPO)**,** an enzyme involved in the destruction of pathogens and actively implicated in acute and chronic inflammations (Deby-Dupont et al., 1999; Kohnen et al., 2007; Van Antwerpen, 2006)**.** The anti-inflammatory capacity of a plant extract can then be assessed by measuring its

potentiality to reduce ROS produced by stimulated equine neutrophils. Additionally, the anti-inflammatory activity can also be determined by evaluating the capacity of the plant extract to modulate the activity of myeloperoxidase. One of the most reliable methods that can be used to measure the activity of MPO is a technique called SIEFED (Specific Immuniological Extraction Followed by Enzymic Detection). SIEFED is a new orginal method developed to detect specifically the activity of MPO (without interference with the products and the enzyme activity) and to study the direct interaction of some substances with the enzyme (Franck et al., 2006). The anti-oxidant property can be predicted by estimating the total antiradicalar activity of a sample by 2, 2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) radical cation decolorization assay (ABTS assay). ABTS allows measuring the radical scavenging capacity of the sample by reducing the pre-formed radical $ABTS^{\bullet+}$ generated by oxidation of ABTS with sodium persulfate (Miller et al., 1996; Re et al., 1999). In this paper, we are reporting the antiplasmodial, antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activity of *Zanthoxylum chalybeum*, as well as the determination of the active ingredient responsible for its antiplasmodial activity.

V.2. MATERIAL AND METHODS

V.2.1 Plant material

The root bark samples of *Zanthoxylum chalybeum* were collected at Ndego, Kayonza (Eastern Province of Rwanda) in October 2010. Samples were authenticated, a voucher specimen representing the species was deposited at the Rwandan National Herbarium (Butare) and another at the National Botanic Garden of Belgium (Meise) (BR0000005087266). Rwandan green tea sample was purchased from Rwandan Mountain Tea Rubaya-Nyabihu Tea Factories and all samples were treated as previously reported (Muganga et al., 2010).

V.2.2. Bio-guided fractionation

Powdered root bark of *Zanthoxylum chalybeum* (500 g) was extracted with 6 L of a mixture of methanol and acetic acid (1%) for 72 hours at room temperature. The solution obtained was concentrated to about 500 ml and pure water was added to precipitate pigments. After filtration the solution was then extracted with dichloromethane to get two phases. Aqueous phase (ZA1) was basified by sodium carbonate and then extracted with dichloromethane.

Three fractions were obtained: aqueous fraction (ZA2), organic fraction (ZD2) and an insoluble fraction (ZP) which were submitted to *in vitro* antiplasmodial test. The most active fraction was fractionated using preparative high performance liquid chromatography (HPLC) with acetonitrile and trifluoroacetic acid 0.05 % as mobile phase in gradient mode and a Pursuit Diphenyl column (Varian) as stationary phase. Isolated pure compounds were tested *in vitro* and their chemical structure determined using NMR (Bruker Avance 500 MHz DRX 500 pectrometer) and mass spectrometry (Micromass ESI-Q-TOF II instrument). The aqueous fraction was acidified and quaternary alkaloids precipitated overnight with Mayer's reagent. On the next day the precipitate was cleaned with cold water and then eluted with a mixture of acetone-water-ethanol (6:1:2) on ion exchange resin column (Amberlite®) which will give alkaloids in their chloride form. The alkaloids' fraction obtained was further fractionated using preparative HPLC and isolated pure quaternary alkaloids were identified as described above.

V.2.3. Antiplasmodial activity

Different fractions and pure compounds obtained from the bio-guided fractionation were tested for their antiplasmodial activity toward 3D7 a chloroquine-sensitive *Plasmodium falciparum* strain. Culture of the parasite and the antiplasmodial assay were carried out as previously reported (Frédérich et al., 2001; Muganga et al., 2010). The first active pure compound was also tested against F32, a mefloquine-resistant *Plasmodium falciparum* strain. This strain was chosen because it was found in Tanzania, neighbouring country of Rwanda. All samples were analysed in triplicate and results expressed as mean standard deviation. Chloroquine and artemisine (98%) (Sigma-Aldrich) were used as standards.

V.2.4. *In vitro* haemolysis assay

The *in vitro* haemolysis assay was carried using 10 % freshly heparinised human blood (A+) in phosphate buffer saline (PBS) and the methanolic root bark extract of *Z. chalybeum* was tested at a final concentration of 200 and 100 µg/ml in DMSO (final concentration = 1%). An aliquot of 10 µl of plant extract was dissolved in 190 µl of 10 % human blood and the mixture incubated for 1 hr under constant and slow shaking at room temperature. Solutions were then centrifuged for 5 min at 2000 rpm in Eppendorf centrifuge 5417 R tubes (Hamburg, Germany). After centrifugation, 150 µl of the supernatant was collected and the absorbance (A) of liberated haemoglobin measured at 550 nm with a spectrophotometer

Perkin Elmer Wallac Victor^2 (USA). Triton -100 (20 %) (Lonza, Belgium), DMSO (1%) (Sigma-Aldrich), and PBS (Lonza, Belgium) were used respectively as positive (100 % haemolysis) and negative controls. Each sample was analysed in triplicate and mean ± S.E. was calculated. The percentage of haemolysis was calculated as follows:

% haemolysis: [(A product – A DMSO) / (A triton – A PBS)] x 100

V.2.5. *In vitro* antitrypanosomal and antileishmanial assay

Parasites were treated as previously reported (Bero et al., 2011). HMI9 medium containing 10% heat-inactivated foetal bovine serum was used to culture the blood-stream forms of *Trypanosoma brucei brucei* (Tbb) strain 427 which were then incubated at 37 °C in a humidified atmosphere with 5% CO_2 as already described (Hirumi et al., 1994). Promostigotes of *Leishmania mexicana mexicana* (Lmm) (MHOM/BZ/84/BEL46) were cultivated *in vitro* in a semi-defined medium (SDM-79) (Brun et al., 1994; Brun et al., 1979), supplemented with 15% heat-inactivated foetal bovine serum and incubated at 28°C.

The *in vitro* antitrypanosomal and antileishmanial activity of methanol root bark extract of *Zanthoxylum chalybeum* was evaluated as previously described (Hoet et al., 2004). Suramine and Amphotericine B were used as positive controls for antitrypanosomal and for antileishmanial activity respectively. Plant material samples and positive controls were prepared as previously described (Bero et al., 2011) and tested in duplicate. The results were expressed as the mean IC_{50} (the concentration of a product that can reduce the level of parasitaemia to 50%).

V.2.6. *In vitro* antiamoebic activity assay

The strain *Entamoeba histolytica* used in this study was isolated from patient with acute amoebic dysentery and was kindly offered by Professor G. Muhirwa from the Department of Medical Biology, University Teaching Hospital at Butare (Rwanda). Parasites were cultured in Balamuth medium (a egg yolk based medium) in sterile test tubes (5 ml) (Anastopoulos et al., 1950) and then incubated at 37°C. Daily parasitaemia was determined using Neubauer's cell and parasitic growth controlled one week before the test. Amoxicillin was added to avoid contamination and pH maintained at 7 – 7.5 with diluted NaOH. Only cultures presenting a suitable growth of motile forms were selected for the test. Metronidazole (Axopharma, Belgium), infected and uninfected medium were used as standard, positive and negative

controls respectively. Crude hydro-ethanolic root bark extract of *Zanthoxylum chalybeum* was obtained by maceration of 40 g of the powdered plant material with 400 ml of 70% ethanol for 24 hours at room temperature. The antiamoebic activity of the plant extract was tested as follows: plant extract in the final concentrations range of 0.007 – 2 mg/ml was added to a series of test tubes containing the contaminated medium. The initial parasitaemia was estimated at 2.8 millions parasites /ml. All tubes were filled up with sterile cotton, stirred and incubated at 37^{o}C for 72 hours. Each test was performed in duplicate.

V.2.7. Anti-inflammatory activity

V.2.7.1. Measurement of antioxidant capacity

The antioxidant or total antiradical capacity of *Zanthoxylum chalybeum* was estimated using its aqueous extract. The aqueous extract was prepared by extracting 1 g of powdered plant material with 100 ml of distilled water heated on water-bath during 30 min. The solution obtained was filtered and the filtrate freeze-dried to get crude extract. The antioxidant activity of the plant extract was evaluated using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) radical cation decolourisation assay (ABTS assay) (Re et al., 1999). The blue-green ABTS radical cation chromophore ($ABTS^{\cdot+}$) was obtained by oxidising overnight in the dark and at room temperature an amount of ABTS by sodium persulfate. The resulting coloured compound was diluted 200 times with methanol to obtain an absorbance of 0.800 ± 0.1 at 740 nm. Green tea and gallic acid (Sigma-Aldrich) known for their antioxidant capacities were used as references, methanol as blank sample and pure water or DMSO used as solvent. An aliquot of 20 µl of reference (at different concentrations) and plant extracts adjusted to 0.4, 2, 4, 10, 20 and 40 µg/mL final concentration, were added to 1980 µl of diluted $ABTS^{\cdot+}$. The mixture was left to stand for 90 minutes at room temperature in the dark and then the absorbance was read and recorded. The percentage of decrease of absorbance was calculated and the IC_{50} estimated assuming that the absorbance of the solvent (pure water for plant extracts and DMSO for gallic acid) is equal to100%.

V.2.7.2. Isolation of equine neutrophils

Horse blood was drawn from the jugular vein of healthy horses fed and bred in identical conditions (Faculty of Veterinary Medicine, University of Liège, Belgium) using EDTA as anticoagulant. Neutrophils were isolated using a discontinuous percol density gradient as previously described (Pycock et al., 1987). Each batch of neutrophils was carefully collected

from 60 ml of one horse blood, washed with two volumes of physiological saline solution and then resuspended in 20 mM phosphate buffered saline (PBS) (at pH 7.4 with 137 mM NaCl and 2.7 mM KCl). Cells were used within 4 hours and each experiment was repeated at least twice with different batches of neutrophils.

V.2.7.3. Measurement of the total ROS produced by neutrophils activated with PMA (CL assay)

The amount of ROS produced by activated neutrophils was measured by lucigenin-enhanced chemiluminescence (CL) under adapted the method from Benbarek et al. (Benbarek et al., 1996) as described by Tsumbu et al. (Tsumbu et al., 2012). Briefly, neutrophils suspensions were placed in the wells of a 96-well-microtiter plate (10^6 neutrophils per well) (white combiplate 8, Fisher Scientific) and incubated for 10 min at 37°C with PBS-solutions of the extracts (*Z. chalybeum* and green tea used as reference) at final concentrations of 0.4; 2; 4; 10; 20 and 40 µg/ml in pure water. After an incubation of 10 min, 25 µl of $CaCl_2$ (Merck, VWRI, Leuven, Belgium), 2 µl of 5 µM lucigenin and 25 µl of 16 µM PMA (Sigma, Bornem, Belgium) were added to neutrophils suspensions. The chemiluminescence of the neutrophils was immediately monitored for 30 min (Fluoroscan Ascent, Fischer Scientific) and expressed as the integral value of the total CL emission. Gallic acid, initially dissolved in DMSO, was used as reference at final concentration of 0.01; 0.05; 0.1; 0.5; 1; 2 and 4 µg/ml. Stimulated neutroplils incubated with PBS containing 1% of the used solvent (DMSO or pure water) in place 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.

V.2.7.4. Viability test

This test allows assessing the safety of sample toward neutrophils during CL assay. It helps then to verify if the decrease of chemiluminescence response is not due to the toxicity of the samples analysed. For this purpose, we used the trypan bleu exclusion test (Tenant, 1964). Plant sample at final concentration of 20 and 40 µg/ml was incubated for 40 min with unstimulated neutrophils (10^6 cells) in PBS. After incubation, the percentage of viability was microscopically estimated for each sample. Unstimulated neutrophils and water, instead of sample, were used as control.

V.2.7.5. Measurement of active MPO by SIEFED method

The SIEFED method allows to specifically detecting the activity of equine neutrophil MPO (Franck et al., 2006). This method includes three steps, the first one deals with the immunoextraction of MPO from a solution or a biological sample by specific immobilised antibodies followed by a washing step so as to eliminate all other compounds of the sample such as proteins, modulating and interfering substances that do not bind to the antibodies. The third step consists of the *in situ* detection of the peroxidase activity of MPO by using 10 µM H_2O_2 (Merck, VWRI, Leuven, Belgium), Amplex® Red (fluorogenic substrate) (Molecular Probes, Invitrogen, Merelbeke, Belgium) and nitrite anions (Sigma, Bornem, Belgium) as substrate, electron donor and enhancer of the reaction respectively. By using this method, when a studied compound increases or diminishes the fluorescence response, its enhancing or inhibiting activity may be attributed to a direct interaction with the enzyme catalytic centre (Franck et al., 2006).

The MPO solution (50 ng/ml) was prepared from purified equine MPO diluted in PBS at pH 7.4 with 5 % Bovine Serum Albumin (BSA) (Bornem, Belgium) and 0.1% Tween 20 (Merck, VWRI, Leuven, Belgium). Samples were incubated for 10 min with MPO at final concentration of 25 ng/ml. Solutions were then placed in the well of SIEFED microtiter plate coated with rabbit polyclonal antibodies (3 µg/ml) against equine MPO and incubated again for 2 h at 37°C in darkness. After the specific capture of MPO by the antibodies, the solution with potential interfering compounds was removed by four washings with PBS/Tween solution. The peroxidase activity of MPO was measured after adding 100 µl of a 40 µM Ampex® Red solution freshly prepared in phosphate buffer (50 µM) containing H_2O_2 (10 µM), and NO_2^- (10 mM). MPO in diluted buffer with 1% DMSO or pure water was used as control and taken as 100 % MPO activity. After incubation, fluorescence was determined with Fluoroscan Ascent plate reader using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The percentages of inhibition were calculated for all samples in relation to the control.

V.2.8. Determination of total polyphenol and tannin content

Total polyphenol and tannin content was determined for *Z. chalybeum* and green tea according to the method recommended by European Pharmacopeaie 7th edition (2.8.14) (EDQM, 2011). A known amount of plant powder (fineness of 250 µm) was dissolved in 150 ml of distilled water and boiled in water-bath at 100^{o}C for 30 min. The mixture was cooled, completed to 250 ml with pure water and then filtrated. From the obtained filtrate 5.0 ml were taken and diluted to 25.0 ml with distilled water. To 2.0 ml of the solution 1.0 ml of phosphomolybdotungstic reagent and 10.0 ml of distilled water were added. The solution was completed to 25.0 ml with sodium carbonate (290.0 mg of $NaCO_3$.10 H_2O in 1.0 L distilled water). After 30 min of incubation, the absorbance (A_1) was read at 760 nm with a spectrophotometer UVICON 922 using distilled water as blank. About 50.0 mg of Pyrogallol (Baker, Netherlands) was used as standard, was immediately dissolved in distilled water and 2 ml of the obtained solution was treated as described above for the plant extracted. The absorbance of the pyrogallol solution was recorded as A_3. Total polyphenols were calculated according to the formulae: the percentage of total polyphenols = $[(62.5 \times A_1) \times m_2] / (A_3 \times m_1)$ where m_1 is the mass of the sample to be analysed and m_2 the mass of pyrogallol in grams.

To determine tannin content, crude aqueous extract was obtained as for the determination of total polyphenols (see above). To a portion of the filtrate, hide powder was added, the mixture shaked for 1 hr at room temperature and then filtered. From the filtrate obtained, 2 ml were taken, treated following the same procedure as for the determination of total polyphenols and the absorbance recorded as A_2. The percentage content of tannins = $[62.5 \times (A_1 - A_2) \times m_2] / A_3 \times m_1$.

V.2.9. Statistical analysis

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

V.3. RESULTS AND DISCUSSION

V.3.1. Bio-guided fractionation, antiplasmodial activity of fractions and isolated compounds

Our previous work conducted on *Z. chalybeum* showed that the methanolic root bark extract of the plant has a very promising antiplasmodial activity (IC_{50} = 4.2 µg/ml) (Muganga et al., 2010). The bio-guided fractionation performed targeted alkaloids because this plant is rich in alkaloids which may intervene in its various therapeutic properties (Neuwinger, 1996). After alkalinisation and extraction of the aqueous fraction (ZA1) by dichloromethane, the best antiplasmodial activity was obtained with the organic fraction ZD2 (IC_{50} = 4.8 ± 0.26 µg/ml). A bio-guided preparative HPLC of the organic fraction ZD2 yielded different fractions among which fraction 7 (F7), a yellow needle-like crystal, was the most active with IC_{50} = 77.74 and 27 ng/ml respectively on 3D7 and F32 strain. Based on NMR and mass spectrometry spectra the active compound was identified as nitidine (see Figure 1). The yield of nitidine isolated from the root bark of *Z. chalybeum* was estimated to be 0.002%. Previous study reported that the yield of nitidine from the root of *Z. chalybeum* was about 0.0123% (Kato et al., 1996) suggesting that the whole root of the plant contains more nitidine. This explains also why the root of the plant free of bark was also active against *Plasmodium falciparum* (IC_{50}= 7.31 µg/ml) (data not published). Nitidine is a quaternary alkaloid already known in *Z. chalybeum* and generally in Rutaceae family (Liang et al., 2006; Nakanishi et al., 1998; Talontsi et al., 2011). It was isolated for the first time in *Zanthoxylum nitidium* (Arthur et al., 1959) and is commonly responsible for the antiplasmodial activity of many anti-malaria remedies (Bouquet et al., 2012; Gakunju et al., 1995; Jullian et al., 2006; Nyangulu et al., 2005). Considered as a potential anticancer drug, nitidine was reported to have other therapeutic properties such as antileukemic (Chen et al., 2012; Nakanishi et al., 1998; Tillequin, 2007), antimicrobial (Del Poeta et al., 1999) anti-inflammatory and analgesic (Hu et al., 2006) and anti-HIV (Tan et al., 1991). In our study, the high antiplasmodial activity of nitidine on chloroquine-sensitive *Plasmodium falciparum* strains is in accordance with what was already reported (Bouquet et al., 2012; Gakunju et al., 1995; Nyangulu et al., 2005). The slight differences between the IC_{50} values may be explained by the fact that different methods were used. Other alkaloids such as methyl canadine, chelerythine and tembetarin were also isolated from the plant but their activity was much lower (IC_{50} > 1 µg/ml) compared to nitidine's one. Consequently, nitidine could be considered as the main antiplasmodial

ingredient of *Zanthoxylum chalybeum* and the other alkaloids like chelerythine and methyl canadine already known in the plant (Neuwinger, 1996) may intervene by synergism in this activity. Nitidine was already reported to be the active ingredient of another *Zanthoxylum species, Zanthoxylum gilletii* (Zirihi et al., 2009). The mechanism of action of nitidine may be due to its ability to inhibit topoisomerase in the parasite (Gakunju et al., 1995). However, it was recently reported that nitidine may possesses chloroquine-like mechanism of action because of its capacities to bind to haem and to inhibit β-haematin formation (Bouquet et al., 2012). More investigations on this compounds are still of interest to exploit its remarkable antiplasmodial activity.

The plant extract at the highest concentration tested for antiplasmodial activity *in vitro* (100 and 200 µg/ml) did not induce any haemolysis in erythrocytes, suggesting that methanolic root bark extract of *Z. chalybeum* at therapeutic concentration *in vitro* has no toxicity towards erythrocytes. Consequently, the plant extract is able to specifically attack the parasite.

Table 5.1: *In vitro* antiplasmodial activity and percentage of haemolysis with *Z. chalybeum* root bark extracts and isolated compounds. Data are expressed as mean ± SD, n ≥ 3

Plant sample	3D7 (IC_{50} µg/ml)	F32 (IC_{50} µg/ml)	% Haemolysis	
			100 µg/ml	200 µg/ml
MeOH crude extract	6.18 ± 1.23	-	0.17 ± 0.15	0.39 ± 0.04
ZD2	4.8 ± 0.26	-	-	-
ZA2	14.37 ± 5.49	-	-	-
ZP (precipitate)	22.53 ± 2.37	-	-	-
Methyl canadine	2.01 ± 0.9	-	-	-
Chelerythine	1.35 ± 0.7	-	-	-
NITIDINE	0.0775 ± 0.0062	0.021 ± 0.01	-	-
Artemisinin	0.005 ± 0.00021	0.003 ± 0.0003	-	-
Chloroquine	0.0017 ± 0.004	Nd	-	-

ZD2 : Second dichloromethane fraction of *Z. chalybeum*
ZA2 : second aqueous fraction of *Z. chalybeum*
ZP : precipitate obtained after liquid-liquid fractionation of ZD2

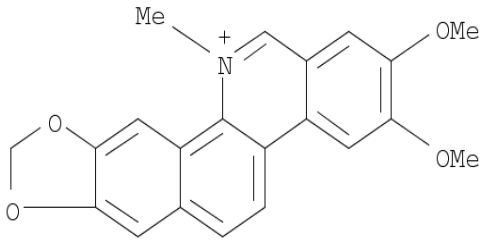

Figure 5.1: Chemical structure of nitidine

V.3.2. Activity of the plant on other parasites

V.3.2.1. Antitrypanosomal and antileishmanial activity

Methanol root bark extract of *Zanthoxylum chalybeum* exhibited a very significant antileishmanial activity (IC_{50} = 1.82 µg/ml). The antileishmanial activity in Rutaceae family is known (Fournet et al., 1994) and may be due mainly to

their benzophenanthridine alkaloids like chelerythine, dihydrochelerythrine, nitidine and dihydronitidine. Indeed, chelerythine and dihydrochelerythine were reported to have a very promising leishmanicidal activity (IC_{50} = 6 and 69 ng/ml respectively) whereas nitidine and dihydronitidine IC_{50} value was 480 ng/ml (Fuchino et al., 2010). Therefore, *Z. chalybeum* could be a good candidate in further antileishmanial research particularly in external use as the active constituents are toxic (Ulrichová et al., 1996). The same plant sample was also active against Trypanosoma (IC_{50} = 19.38 µg/ml) in accordance with a previous study (Nibret et al., 2010). The antitrypanosomal activity of *Z. chalybeum* is not surprising as Rutaceae species have been found to be active against trypomastigote form of *Trypanosoma cruzi* (Mafezoli et al., 2000) and on *Trypanosomal brucei rhodesiense*. Some lignans such as cubebin (Freiburghaus et al., 1996) isolated from *Zanthoxylum naranjillo* showed potent antitrypanosomal activity (Bastos et al., 1999) and may be the active ingredient of the plant. Previous study reported that nitidine is inactive *in vivo* against *Trypanosoma cruzi* and *T. brucei rhodesiense* (Kinnamon et al., 1998) probably because of the poor absorption of this quaternary alkaloid. In contrast to our results, only a low trypanocidal activity was observed from the seed and stem of *Z. chalybeum* (Olila et al., 2001). This may be explained by the fact that different plant samples were used in both studies meaning that the root of the plant may exhibit trypanocidal activity whereas the seed and the stem are inactive.

Table 5.2: In vitro antitrypanosomal and antileishmanial activities of methanol root bark extracts of *Z. chalybeum*

Sample	Antitrypanosomal activity Tbb (IC_{50}, µg/ml ± SD)	Antileishmanial activity Lmm (IC_{50}, µg/ml ± SD)
Z. chalybeum (MeOH)	19.38 ± 0.25	1.82 ± 0.94
Suramine	0.11 ± 0.02	Nd
Amphotericine B	Nd	0.1 ± 0.01

V.3.2.2. Antiamoebic activity

The hydro-ethanolic extraction gave a good yield (35.12%). The antiamoebic activity of the crude hydro-ethanolic root bark extract of *Z. chalybeum* was assessed using Balamuth medium (an egg yolk based medium) which was changed every three days. Crude hydroethanolic extract of *Z. chalybeum* root bark presented weak activity against vegetative forms of *Entamoeba histolytica* with MIC value ≥ 312.5 µg/ml. Surprisingly, metronidazole, used as standard, showed also a very low activity (MIC ≤ 156.25 µg/ml) compared to values obtained in previously published experiments (Calzada et al., 2003; McGaw et al., 2000; Tona et al., 1998). This may be explained by the possible resistance of the used *Entamoeba histolytica* strain to metronidazole. However, the resistance of *Entamoeba histolytica* to metronidazole was already observed (Aguirre-Cruz et al., 1990) and low antiamoebic activity of metronidazole (MIC = 10 µg/ml) was previously reported (Sohni et al., 1995). *Entamoeba histolytica* strain used in this study was obtained from a patient living in southern province of Rwanda but no information about metronidazole resistance was available. However, considering the low availability of appropriate drugs, common self-medication and poor drug compliance in developing countries such as Rwanda, the metronidazole resistance is very possible. Therefore, further study using a standardized *Entamoeba histolytica* strain is needed to confirm the possible metronidazole resistance. According to a traditional healer from eastern province of Rwanda, tea from the leaf of the plant is used to treat intestinal disorders. Unfortunately, we did not be able to know which intestinal disorder is targeted or to evaluate the activity of this plant using an *Entamoeba histolytica* strain from this area. Previous researchers reported high amoebicidal and weak giaridicidal activity from the leaf of *Zanthoxylum liebmannianun* (Arrieta et al., 2001). More investigations are then recommended to certify the possible antiamoebic activity of this plant.

V.3.3. Anti-inflammatory activity

Water is generally mostly used to prepare traditional remedies, then pure water was chosen as solvent for plant. Green tea is known to have a good antioxidant activity and was used in this test for better evaluation of our plant extracts.

ABTS radical cation decolourisation assay revealed that the root bark of *Z. chalybeum* has a very promising radical scavenger activity (IC_{50} = 4.27 µg/ml) which is comparable to the activity of the green tea (IC_{50} = 4.01). IC_{50} value of the reference (gallic acid) was 0.89 µg/ml (see Table 3). Overall, the percentage of ABTS radical activity decreased in a dose dependent manner for all samples tested. A significant inhibition ($p < 0.05$) was observed for all samples from concentration of 0.5 and 2 µg/ml for gallic acid and plant extracts respectively (see Figure 1). HPLC analysis revealed that the plant contains caffeic acid derivatives (results not shown) which may account for its antioxidant activity. The antioxidant capacity of this plant may also be beneficial in the treatment of other diseases because various pathology conditions are associated with oxidative stress (Ponou et al., 2010). The results from ABTS test indicate the effect of the root bark of our plant on the reactivity of ABTS cation and the results from CL and SIEFED assays gave further information about the possible effect of the plant on total ROS produced by stimulated neutrophils and on the activity of myeloperoxidase, an enzyme largely implicated in the inflammation process.

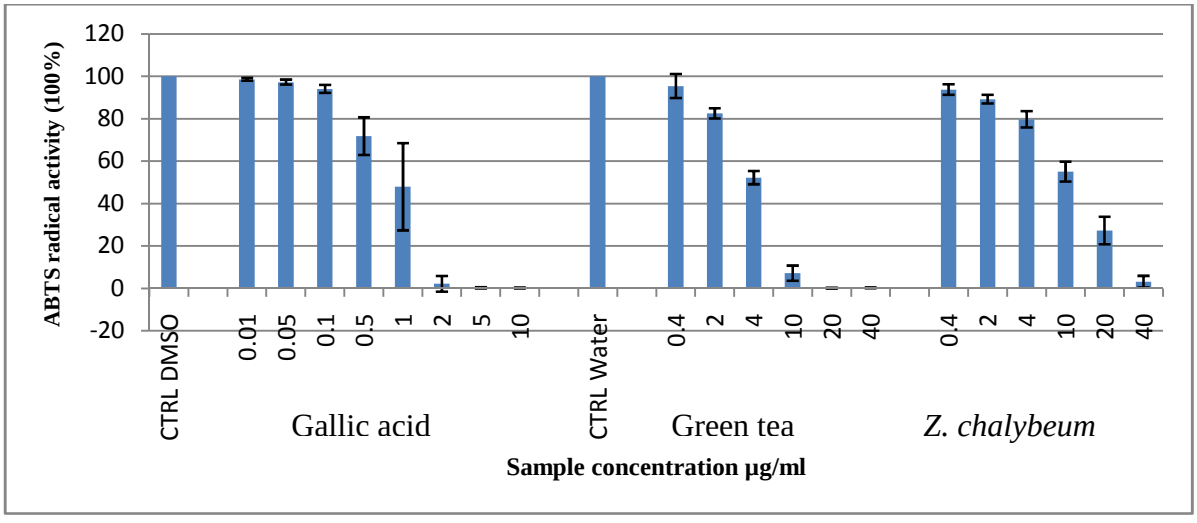

Figure 5.2: Effect of *Z. chalybeum*, green tea aqueous extracts and gallic acid on the ABTS cation radical assay. Samples were tested at final concentration of 0.4, 2, 4, 10, 20 and 40 µg/ml for plant extracts and 0.01, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml for gallic acid. The percentage of inhibition was calculated for each sample concentration versus the control pure water or DMSO taken as 100 % (mean ± SD, n ≥ 3).

Results from chemiluminescence assay show that the plant extract has an important capacity to inhibit the production of ROS produced by stimulated equine neutrophils. The aqueous extracts of *Z. chalybeum* reduced ROS production in concentration-dependent manner (Figure 3). The plant extract exhibited significant inhibition ($p < 0.05$) at all concentrations tested (from 0.4 to 40 µg/ml) whereas for green tea aqueous extract significant inhibition was observed from the concentration of 2 µg/ml. As found for antiradical activity, the activity of the plant extract and green tea was similar with IC_{50} value of 3.78 and 3.79 µg/ml respectively for *Z. chalybeum* and green tea, whereas IC_{50} value of gallic acid, used as reference, was 3.5 µg/ml. Results form trypan bleu exclusion test showed that the cell viability was ≥ 94 % for both PBS (taken as control) and plant extracts at the final concentration of 200 µg/ml indicating no cytotoxicity towards neutrophils. The observed high activity of plant sample with CL and ABTS assay was comparable, clearly indicating the remarkable capacity of the plant to scavenger free radicals in general and particularly ROS produced by stimulated neutrophils.

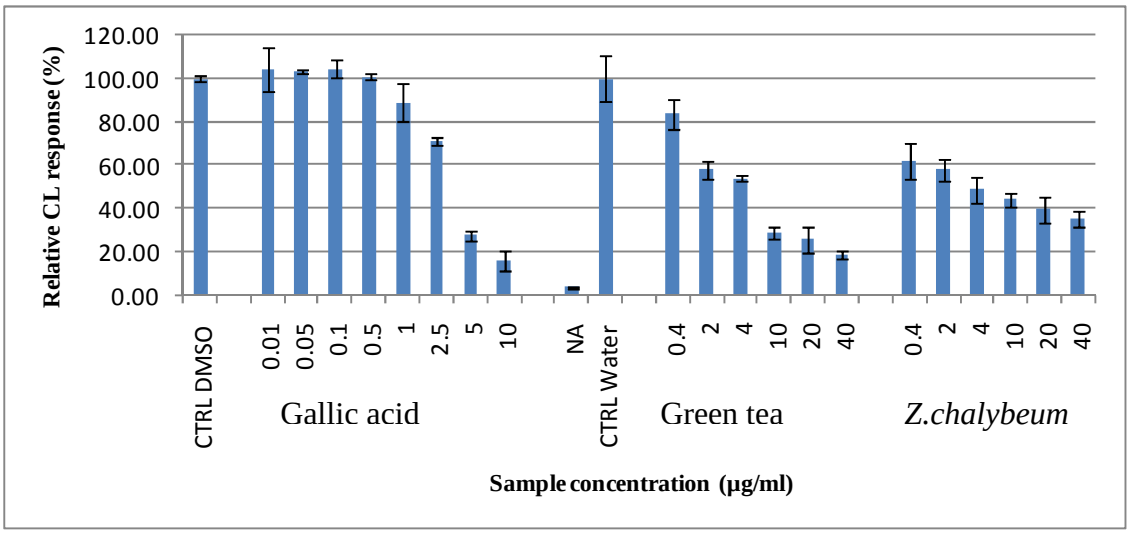

Figure 5.3: Inhibitory effect of *Z. chalybeum*, green tea extracts and gallic acid on the chemiluminescence response produced by PMA-stimulated equine neutrophils. Aqueous extracts of *Z. chalybeum* and green tea were dissolved in pure water, gallic acid dissolved in DMSO and tested at final concentrations of 0.4, 2, 4, 10, 20 and 40 µg/ml for plant extracts and 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% value. All data were expressed as means ± S,D, n ≥ 6 and experiments were performed using different batches of neutrophils.

The results from SIEFED assay gave more information about the possible interaction of the plant extract with MPO, an enzyme involved in the inflammation process. The effect of

aqueous extract of *Z. chalybeum* (root bark) on myeloperoxidase was very low compared to that obtained from green tea or gallic acid. (IC_{50} values = 67.69; 3.06 and 0.62 µg/ml respectively for the plant, green tea and the reference). Even if the plant extract exhibited inhibition of MPO in dose-depending manner, significant inhibition ($p < 0.05$) was observed only from concentration of 10 µg/ml whereas green tea and gallic acid presented significant inhibition at all concentrations tested. Previous study reported the anti-inflammatory activity of *Z. chalybeum* which may result from the capacity of the plant to inhibit cyclooxygenase (Matu et al., 2003) and its benzophenanthridine alkaloids such as nitidine, may play a key role in this activity (Hu et al., 2006; Muller-Jakic et al., 1993). Muller et al. found that root bark extract of the plant reduces more 5-lipooxygenase than cyclooxygenase, enzymes highly involved in biosynthesis of inflammatory leukotrienes and prostaglandins respectively (Muller-Jakic et al., 1993). Indeed, the reported percentage inhibition of cyclooxygenase by the aqueous root bark extract of plant (73.7 %) was obtained at high concentration (500 µg/ml) suggesting that the ability of the crude plant extract to interact with MPO and cyclooxygenase is very weak. Therefore, at this level, it could be argued that the plant exhibits its anti-inflammatory activity by inhibiting mainly 5-lipooxygenase. However, the results from CL assay illustrated well the high capacity of the plant extract to reduce ROS produced by stimulated neutrophils, suggesting that either the plant interact with NADPH oxydase, enzyme responsible for the production of superoxide onion ($O_2^{\bullet -}$), or the plant extract interacts with superoxide anion from which H_2O_2, the substrate of myeloperoxidase is produced. Moreover, *Z. chalybeum* may be able to interact with other enzymes such as NO synthase, or on other aspects of inflammation but unfortunately, we were not able to cover all mediators of inflammation. It should also be noticed that pure compounds isolated from this plant may exhibit more activity than the crude extract. Indeed, crude extract contains many ingredients that can interact with each other. Further studies on other inflammatory aspects are of interest, especially on pro-inflammation cytokines, clearly implicated in infectious diseases such malaria (Clark et al., 2006).

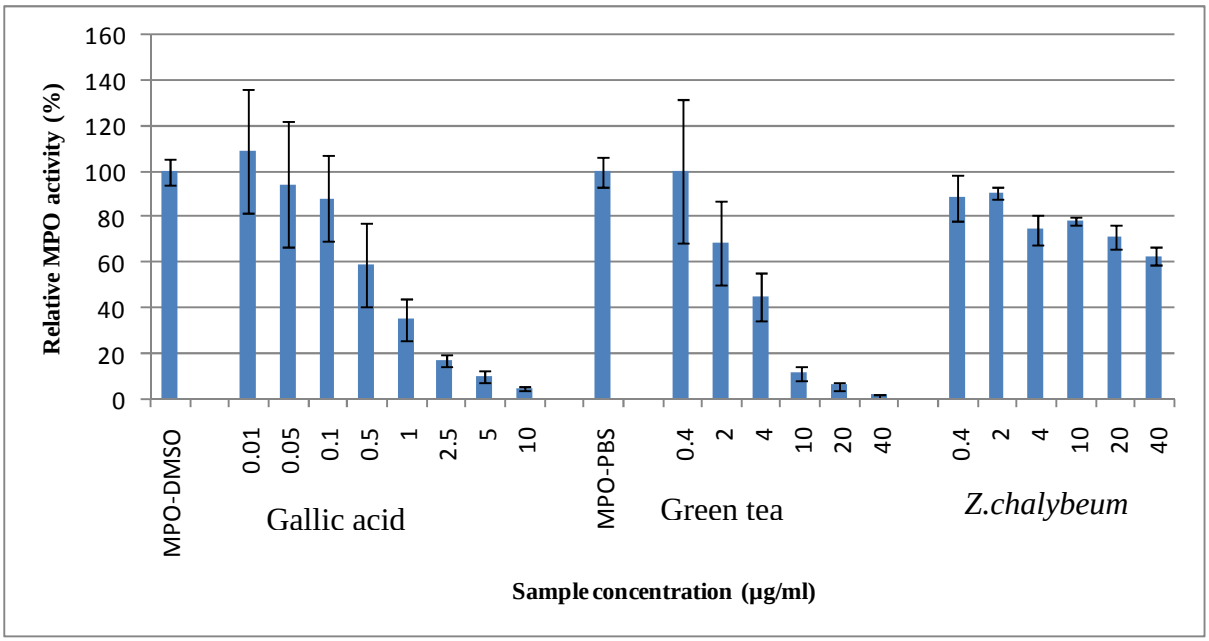

Figure 5.4: **Effect of plant extracts and gallic acid on MPO activity measured by SIEFED. Samples were analysed at final concentrations of 0.4, 2, 4, 10, 20 and 40 µg/ml for plant extracts and 05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml for gallic acid. The percentage of inhibition was calculated for each sample concentration versus MPO-PBS or MPO-DMSO taken as the 100% value (mean ± SD, n ≥ 6).**

As nitidine possesses both antiplasmodial and anti-inflammatory activity, it may be very interesting for the treatment of infection diseases such as malaria.

Table 5.3: Summary of IC_{50} (µg/ml) and R2 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 and n ≥ 6 for CL and SIEFED. CL experiments were performed using different batches of equine neutrophils.

Sample	IC_{50} in µg/ml ± SD (R^2)		
	ABTS test	**Chemiluminescence**	**SIEFED technique**
Z. chalybeum	4.27 ± 0.07 (0.997)	3.78 ± 0.74 (0.812)	67.69 ± 5.87 (0.606)
Green tea	4.01 ± 0.02 (0.992)	3.79 ± 0.38 (0.963)	3.06 ± 1.57 (0.820)
Gallic acid	0.89 ± 0.16 (0.974)	3.5 ± 0.09 (0.897)	0.62 ± 0.37 (0.928)

V.3.4. Determination of total polyphenol and tannin content

The total level of polyphenols in *Z. chalybeum* was estimated to be 5.61 ± 0.31% whereas the percentage of total tannins content in the plant was 1.18 ± 0.30. The analysis of the root bark of the plant using HPLC-diode array showed that the presence of caffeic acid derivatives (results not shown) may mainly contribute to its polyphenols content. *Z. chalybeum* contains

polyphenols and some alkaloids such us nitidine known for their antioxidant and anti-inflammatory activity (Hu et al., 2006; Tsumbu et al., 2012; Zirihi et al., 2009). Those compounds may intervene in the anti-inflammatory activity of the plant by a possible synergism.

V.4. CONCLUSION

The antiplasmodial activity of *Z. chalybeum* is mainly due to nitidine, but other alkaloids such as chelerythine and methyl canadine may contribute to this activity in a synergistic manner. This study reported for the first time the marked antileishmanial activity of the plant. The root bark of *Z. chalybeum* has an interesting anti-inflammatory activity, moderate antitrypanosomal and weak antiamoebic activity. It seems that the mechanism of action of the plant extract in its anti-inflammatory activity does not target MPO activity. However, the plant may interact with other enzymes implicated in the production of ROS such as NO synthase or NADPH oxidase, or with superoxide anion.

V.5. ACKNOWLEDGMENTS

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V.6. SUPPORTING INFORMATION

Scheme of the bio-guided fractionation of *Z. chalybeum*, details about HPLC analysis of the plant (method and chromatograms), ^{1}H NMR spectra of nitidine are given as supporting information (Annexe 2 & 3).

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CHAPTER VI. DISCUSSION

This chapter deals with the general discussion of the results obtained in the present work and it is divided in three parts:

- Antiplasmodial activity which was the main objective of our study;
- Effect of *Terminalia mollis* and *Zanthoxylum chalybeum* on other parasites;
- Anti-inflammatory activity of *Terminalia mollis* and *Zanthoxylum chalybeum*.

VI. 1. ANTIPLASMODIAL ACTIVITY

VI. 1.1. Ethnobotanical survey

The evaluation of the antiplasmodial activity of plant samples was preceded by an ethnobotanical survey conducted in Rwanda on traditional medicinal plants used locally to treat malaria. Despite the reservations expressed by traditional practitioners, relevant ethnobotanical data were obtained which allowed us to select some plants for further scientific evaluation. The selection of plants samples was based not only on the ethnopharmacological information obtained, but also on the literature. Then, some medicinal plants among the most cited, were not selected since their antiplasmodial activity was already known by the scientific community. However, some plants whose antiplasmodial activity was scientifically proved but, their active ingredients are still unknown were also selected. This case concerns for instance *Zanthoxylum chalybeum* and *Markamia lutea*. Medicinal plants such as *Terminalia mollis* and *Aristolochia elegans* were selected due to the fact that they have been used also to prevent malaria. In some cases we were obliged to collect different parts of the selected plants since the part of the plant usually used was not revealed or varies from one respondent to another. This happened with *Z. chalybeum and T. mollis*.

VI.1.2. Screening of plant samples for antiplasmodial activity

The *in vitro* antiplasmodial activity of selected plants samples was determined on methanol and dichloromethane extracts. Aqueous extracts were only evaluated for plant samples whose methanolic extract presented an interesting activity. The antiplasmodial activity of each sample was classified as high, important, moderate or weak and negligible based on IC_{50} values (Jonville et al., 2008; Pink et al., 2005). As results, a great number of samples analysed were active against *Plasmodium falciparum* including 16 % with high activity

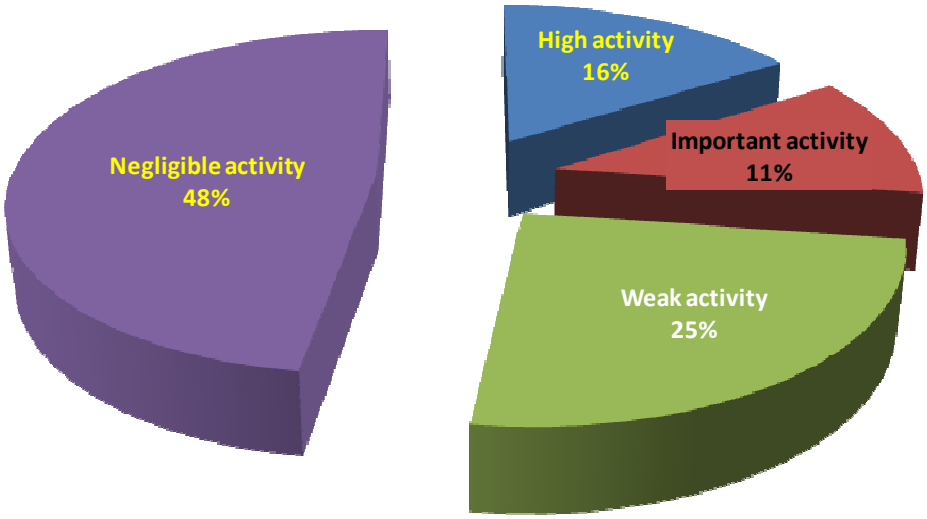

Figure 6.1 The *in vitro* antiplasmodial activity of 46 plant samples analysed

(IC_{50} < 5 µg/ml), confirming the use of those plants in traditional medicine (see Figure 6.1). Overall, the methanolic extracts exhibited the highest activity and were more active than the aqueous ones. However, aqueous extracts of samples from *Z. chalybeum* and *T. mollis* showed comparable activity (IC_{50} < 10 µg/ml). Nevertheless, plant samples which indicated low or negligible activity *in vitro*, may be active *in vivo* or on another *Plasmodium falciparum* stage, such as human liver stage (not assessed in this work). The *in vitro* assay used in the present study was based on human blood stage of *Plasmodium falciparum* and is appropriate for the purpose of this study (Frédérich, 2006). Furthermore, possible non-polar active ingredients from those plant samples were in little quantity or were absent in the aqueous extracts. The *in vitro* assay allowed us to select plants based on their IC_{50} values. Their possible cytotoxic activity was evaluated to determine their ability to selectively target the parasite. Even if this test was performed on crude extracts which contain various substances, it helped us to detect the presence of any cytotoxic compound in the plant samples before investing their *in vivo* antiplasmodial activity. Nevertheless, it doesn't mean that all plant samples with low selectivity index will be toxic *in vivo*. Indeed, toxic compound from plant samples may have low absorption or high elimination or could be transformed in less toxic metabolites.

Four plants were selected for *in vivo* antiplasmodial activity evaluation. Methanol and aqueous extracts were tested using both oral and intra-peritoneal route, except *Rumex*

abyssinicus for which only a dichloromethane extract was tested. Methanol and aqueous extracts of this plant did showed any interesting activity *in vitro*. Oral administration was firstly used to test the activity of the plant samples as it is the administration route commonly used by traditional healers in the treatment of malaria. Similarly, aqueous extracts were analysed to mimic the mode of preparation mainly used in traditional medicine (data not yet published). The methanolic extract of *Fuerstia africana* gave the best activity with a percentage of parasitaemia inhibition > 70 % after oral and intra-peritoneal administration. Unfortunately, we did not do any further investigation on this plant since the active ingredient was cytotoxic (Koch et al., 2006). This may explain the observed low selectivity index of the plant sample (1.9) (Muganga et al., 2010). Mouse viability was very low with *Z. chalybeum*, *R. abyssinicus* and *T. mollis* extracts intra-peritonealy administered; clearly illustrating the toxicity of the plant samples. It should be noticed that the results from *in vivo* antiplasmodial assay using animal model cannot be transposed directly in humans. Indeed, on one hand, even if the metabolism system of mouse (used in this study) is comparable to human being one, they are not totally the same. On the other hand, Plasmodium strain used in this *in vivo* assay (*Plasmodium berghei*) is genetically different from *Plasmodium falciparum*, which affects human and then the two strains present different sensitivities. Even if the methanolic extracts of *Z. chalybeum* and *T. mollis* did not exhibit any good antiplasmodial activity using intra-peritoneal administration; they were active with oral administration. Therefore, a bioguided fractionation was carried out on the two plants to identify the active ingredients.

VI.1.3. Bio-guided fractionation

The bio-guided fractionation was based on the most active crude extracts *in vitro*. Consequently, crude methanolic root bark extracts of *Z. chalybeum* and *T. mollis* were used.

The fractionation of *Z. chalybeum* was done using different chromatographic techniques and allowed us to isolate nitidine as the main antiplasmodial ingredient of the plant (IC_{50} ± 77 ng/ml). Indeed, a compound is potentially interesting as antiparasitic if it has IC_{50} < 1 µg/ml or < 1 µM/ml (Frédérich, 2006; Pink et al., 2005). Other alkaloids such as chelerythine and methyl canadine present in this plant, were also active on the parasite but presented IC_{50} > 1 µg/ml. Nitidine was already found in various *Zanthoxylum species* (Bouquet et al., 2012; Gakunju et al., 1995; Rajkumar et al., 2010; Talontsi et al., 2011) and reported as the active ingredient of other plants but, to our knowledge, this is the first time that this molecule is reported to be the main antiplasmodial compound of *Z. chalybeum*. Despite the very

promising activity of nitidine, as a quaternary salt, this compound has low bioavailability per oral administration. Nevertheless, this may be improved by structure modification of the molecule. However, possible absorption of nitidine was reported (Gakunju et al., 1995), and other substances from the plant may ameliorate its absorption; illustrating the observed antiplasmodial activity *in vivo* and the use of the plant in traditional medicine. There has been growing interest in synthesising nitidine especially for its potent anticancer and antileukemic activities (Ishii et al., 1985; Messmer et al., 1972; Nakanishi et al., 1998) but it may also be an interesting candidate in the development of new antimalarial drugs if the problem of absorption is resolved. Recently, Bouquet et al. demonstrated that Nitidine, known to be cytotoxic (Gakunju et al., 1995), did no present any sign of acute toxicity after intra-peritoneal injection (Bouquet et al., 2012). Nitidine offers a remarkable advantage as it presents a very high and similar antiplasmodial activity on different *Plasmodium falciparum* strains (mefloquino-resistance strain, chloroquine-sensitive and chloroquine-resistant).

The fractionation of *T. mollis* allowed us to isolate ellagic acid as the active substance responsible for the antiplasmodial activity of the plant. Ellagic acid is a polyphenol commonly found in fruits and thanks to its various beneficial properties particularly antioxidant, anticancer. Therefore, it has been used as dietary complement (Ascacio-Valdés et al., 2011; Leonardo et al., 2011). Additionally, ellagic acid has an interesting antiplasmodial activity already reported (Dell'Agli et al., 2003; Verotta et al., 2001) and plays a major role in the antiplasmodial activity of some other plants like *Syzygium cumini* (Simões-Pires et al., 2009) and *Punica granatum* (Dell'Agli et al., 2010). HPLC analysis of *T. mollis* revealed that the plant contains an important amount of ellagic acid and ellagitannins, mainly punicalagines and ellagic acid derivatives. This is not surprising as the genus Terminalia is known to possess high total polyphenol content (Lin et al., 1990). Ellagic acid derivatives, punicalagin and its derivatives do not present an interesting antiplasmodial activity *in vitro* but may release free ellagic acid as metabolite *in vivo* and may then contribute to the activity of the plant (Aguilera-Carbó et al., 2008). However, the low bioavailability of ellagic acid per oral administration compromises its very promising antiplasmodial activity (Seeram et al., 2004). Some researchers attempted structure modifications of the ellagic acid to enhance its absorption and solubility. It seems that free hydroxyl groups have an important role in the antiplasmodial activity of ellagic acid and structurally related compounds (Simões-Pires et al., 2009). Two compound, flavellagic acid and coruleoellagic acid obtained by adding more

hydroxyl groups into ellagic acid shown promising results *in vitro* and primary *in vivo* studies. They represent then interesting antimalarial lead compounds (Sturm et al., 2009).

VI.2. EFFECT OF *TERMINALIA MOLLIS* AND *ZANTHOXYLUM CHALYBEUM* ON OTHER PARASITES

T. mollis and *Z. chalybeum* exhibited an interesting activity against *Plasmodium falciparum*. We tried to evaluate the possible effect of the plants on other parasites such as trypanosoma, leishmania and amoeba.

Aqueous and methanolic root bark extracts of *T. mollis* exhibited a promising antitrypanosomal activity *in vitro* leading to further investigation. Ellagic acid antitrypanosomal activity was slightly high compared to the activity of crude extract, suggesting a very possible intervention of other compounds in this activity. The fact that aqueous crude extract was also active indicates that other polar compounds intervene in the activity of the plant. Punicalagin and gallic acid were reported to be active against trypanosome (Asres et al., 2001; Hoet et al., 2004; Koide et al., 1998) and they are more water soluble than ellagic acid. Considering the low solubility of ellagic acid in water (Bala et al., 2006) we may say that punicalagin and gallic acid play also an important role in the activity of the aqueous extract.

All *T. mollis* extracts tested present a negligible antileishmanial activity. Even the activity found with ellagic acid (IC_{50} = 18.43 µg/ml) as pure compound is very low. This clearly explains that the plant is inactive against leishmania.

T. mollis exhibited a moderate antiamoebic activity *in vitro* but the fact that metronidazole used as standard was less active indicates possible resistance of the *Entamoeba histolytica* strain used to metronidazole. Considering the known antiamoebic activity of gallic and ellagic acids (Alanís et al., 2003; Derda et al., 2009), those compounds may intervene in the activity of the plant. Further investigation using standardised *E. histolytica* strain will be of interest to determine the very possible interesting activity of the plant as well as its active ingredients. Moreover, we call special attention to possible metronidazole resistance in the study area for further appropriate management of this disease.

Methanol root bark extract of *Z. chalybeum* shown a remarkable antileishmanial activity *in vitro* (IC_{50} = 1.82 µg/ml) which may be attributed to its benzophenanthridine alkaloids mainly

chelerythrine, dihydrochelerythrine, nitidine and dihydronitidine (Fuchino et al., 2010). Chelerythrine and dihydrochelerythrine have a very interesting IC_{50} values (6 and 69 ng/ml respectively) and deserve to be further investigated. Nitidine and dihydronitidine may contribute to the activity of the plant by synergism. However, as nitidine and chelerythrine are quaternary alkaloids and then are poorly absorbed, dihydrochelerythrine and dihydronitidine (easily absorbed) may be more interesting for *in vivo* studies.

The activity of *Z. chalybeum* against trypanosome was moderate (IC_{50} = 19.38 µg/ml) but good enough to show the interest of the plant for further antitrypanosomal studies. The antitrypanosomal activity of this plant may be due lignans such cubebin already isolated in other *Zanthoxylum species* (Freiburghaus et al., 1996) but this plant may contain other antitrypanosomal substances.

Based on the classification of antiamoebic activity proposed by Tona et al., the amoebicidal activity of *Z. chalybeum* is low (MIC = 156.25 µg/ml) (Tona et al., 1998). As it is the case for *T. mollis*, the observed very low antiamoebic activity of the recommended amoebicidal drug, metronidazole leads to suspicion of possible local metronidazol resistance. Further investigation is of interest to clarify this case.

VI.3. ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory activity of pure compounds or plant extracts with interesting antiparasitic activity is an additional and important value; because those kinds of diseases are always accompanied with inflammation response. Inflammation is a normal protective response of our organism vis-à-vis to invading pathogens. However, it can sometimes cause tissue injury even death (Kumar et al., 2009; Serhan et al., 2010) hence the interest to control inflammation especially in case of infectious diseases (Clark et al., 2006). To evaluate the anti-inflammatory activity of our plants, three tests were carried out:

- ABTS assay which indicates the capacity of the plant sample to scavenger free radicals in general;
- Chemiluminescence (CL) assay which illustrates the effect of the sample on total ROS produced by stimulated neutrophils;
- SIEFED assay which demonstrates the ability of the sample to interact with myeloperoxidase activity, enzyme largely implicated in the inflammation process.

The ABTS assay of *T. mollis* and *Z. chalybeum* extracts revealed the significant antioxidant activity of the two plants comparable to the activity of green tea used as control. All plant samples inhibited ABTS radical activity in a dose dependent manner. A significant inhibition was observed for all plant samples from concentration of 2 µg/ml. Those encouraging results lead us to analyse the effect of the same plant samples on stimulated equine neutrophils.

The results from CL assay demonstrated the high capacity of our plant extract to inhibit total ROS produced by stimulated equine neutrophils. CL technique used in this study targets superoxide anions (produced by NADPH oxidase) from which other ROS are generated. Aqueous extract of *Z. chalybeum* and green tea showed quite similar activity whereas the activity of aqueous and hydroethanolic extracts from *T. mollis* were slightly high. Considering the important role of myeloperoxidase on inflammation response, we were interested in the evaluation of the capacity of our plant samples to interact with this enzyme. For this purpose, SIEFED technique was used.

Based on the results from SIEFED assay, it was clear that methanolic root bark extract of *Z. chalybeum* weakly interacts with myeloperoxidase (IC_{50} > µg/ml). It was previously reported that aqueous extract of the plant acts on 6 lipooxygenase (Muller-Jakic et al., 1993), enzyme implicated also in the inflammation process. Furthermore, the plant sample showed good capacity to scavenger ROS produced by stimulated neutrophils, suggesting that the plant may act on NADPH oxidase, superoxide anion or on other enzymes such as NO synthase implicated in ROS production. In contrast, all *T. mollis* extracts analysed exhibited a noticeable ability to interact with the activity of this enzyme. This technique is advantageous to specifically detect the inhibition of myeloperoxidase by the plant sample without any interference from unwanted compounds. This means that either the plant extract interacts directly with the enzyme, modifies its structure, or hides its active site and then limits or blocks the fixation of substrate (Boly et al., 2011; Franck et al., 2006; Tsumbu et al., 2012). The interesting inhibition of myeloperoxidase activity observed with *T. mollis* extracts could be correlated with its high total polyphenol content especially punicalagin, ellagic and gallic acids (analysed also in this study) as well as their derivatives present in the plant. Overall, the two plants have an interesting anti-inflammatory activity which will help in the control and management of the parasitosis for which those plants are active. Indeed, in case of chronic infection, PMNs continually stimulated produce pro-inflammatory cytokines largely implicated in systemic infectious diseases (Clark et al., 2006; Gimenez et al., 2003). The

observed positive effect of *T. mollis* and *Z. chalybeum* on stimulated PMNs could help in the control of inflammatory cytokines advantaging the management of concerned infections. Recently, a study tried to test the effect of ellagitannins from the fruit rind of pomegranate (including ellagic acid and punicalagins, present also in *T. mollis*) on the pathways involved in onset severe malaria. The study revealed that the antiplasmodial activity of these compounds may results not only in their direct anti-inflammatory activity but also in their capacity to limit the excess inflammatory response of the host, minimising then the risk of progression of more severe form of malaria (Dell'Agli et al., 2010). Moreover, ellagic acid, the antimalarial compound form *T. mollis*, was previously reported to possesses the capacity to reduce the levels of the inflammatory cytokines such as TNF-α (Beserra et al., 2011), the essential mechanism of systemic disease caused by infectious agents (Clark et al., 2006).

VI.4. REFERENCES

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CHAPTER VII. CONCLUSIONS AND PERSPECTIVES

The spread of drug resistance in falciparum malaria is becoming a serious health problem. Moreover, populations living in developing countries especially in Sub-Saharan Africa have the highest risk of acquiring malaria (WHO, 2011), and the implementation of policies for adequate prevention, treatment and management of this disease requires high costs. The use of artemisinin combination therapy was so far considered as the best than ever solution, since the emergence of chloroquine and sulfadoxine-pyrimethamine resistance. However, the appearance of the resistance to artemisinin in Western Cambodia and its possible extent in other malarious areas constitutes a worrying threat (Phyo et al., 2012; White, 2010). Inevitably, the development of new antimalarial drugs is one of the appropriate alternatives; because all antimalarial treatments under phase II and phase III clinical study are artemisinin based combination therapies (Burki, 2009) and an effective malaria vaccine still needs some years to be available (Zhang et al., 2012). In order to improve the chance to achieve this goal, we preferred to focus on traditional medicinal plants which offer an opportunity to develop standardised plant extracts, cheaper and readily accessible locally.

In this context, an ethnobotanical study was carried out in Rwanda on traditional medicinal plants locally used in the treatment of malaria. As results, 13 plants, mainly from Asteraceae family, were selected, from which 19 samples were collected and 46 extracts analysed *in vitro*. The selected plants were *Aristolochia elegans*, *Conyza aegyptiaca* (L.) Aiton (Asteraceae), *Fuerstia africana* T.C.E. Fr. (Lamiaceae), *Markhamia lutea* K. Schum (Bignoniaceae), *Microglossa pyrifolia* (LAM.) O. Kunte (Asteraceae), *Mitragyna rubrostipulata* (K. Schum.) (Rubiaceae), *Rumex abyssinicus* Jacq (Polygonaceae), *Rumex bequaertii* De Wild (Polygonaceae), *Solanecio mannii* (Hook.f.) Jeffrey **(**Asteraceae), *Terminalia mollis* (Combretaceae), *Tithonia diversifolia* (Hensl.) A Gray (Asteraceae), *Trimeria grandifolia* (Warb.) (Flacourtiaceae), *Zanthoxylum chalybeum* Engl. (Rutaceae).

The majority of plant samples analysed *in vitro* for antiplasmodial activity were active and 16 % exhibited a high activity (IC_{50} < 5 µg/ml). This allowed us to confirm the traditional use of the selected plants in the treatment of malaria. The best antiplasmodial activities *in vitro* (IC_{50} < 15 µg/ml) were obtained with methanol and dicholoromethane leaf and flower extract of *Tithonia diversifolia*, dicholoromethane, methanol and aqueous leaf extract of *Microglossa*

pyrifolia, methanol root bark extract of *Z. chalybeum*, dicholoromethane root extract of *Rumex abyssinicus*, methanol leaf and stem extract of *F. africana* and the methanolic root bark extract of *T. mollis*. Based on their selectivity index, four plants, *Z. chalybeum*, *T. mollis*, *F. africana*, and *R. abyssinicus*, were selected for *in vivo* assay.

The best results from *in vivo* antiplasmodial assay were obtained with the methanolic leaf and stem extract of *F. africana* whose active ingredient was already known and found to be cytotoxic. Two other plants, *Z. chalybeum* and *T. mollis*, highly active *in vitro* and moderately active *in vivo* after oral administration, were then submitted to a bio-guided fractionation to isolate active compounds.

Methanolic root bark extracts of the selected plants were bio-fractionated using different chromatographic techniques and identification of isolated pure compounds was done with NMR and mass spectrometry. Nitidine and ellagic acid were identified as the main active ingredients of *Z. chalybeum* and *T. mollis*, respectively. The presence of nitidine in *Z. chalybeum* was already known (Neuwinger, 1996), but to our knowledge, this study revealed for the first time the role of this compound as the main active antiplasmodial ingredient of the plant. Similarly, some ellagic acid derivatives were already reported in the stem bark of *T. mollis* (Liu et al., 2009) but, ellagic acid in pure form as the active ingredient responsible for the antiplasmodial activity of the plant is also reported for the first time. Since the two plants presented optimistic results to fight against malaria, we were attracted to search the effect of those plants on other parasites as well as their possible anti-inflammatory activity.

The root bark extract of *T. mollis* and *Z. chalybeum* were tested for antitrypanosomal, antileishmanial, antiamoebic and anti-inflammatory activity *in vitro T. mollis* is a good candidate for the development of new antitrypanosomal drugs. Polyphenolic compounds present in this plant such as ellagic acid, punicalagine and gallic acid may act by synergism in this activity. This plant was found to be inactive against leishmania even if ellagic acid in pure form exhibited a negligible antileishmanial activity. The root bark of *T. mollis* exhibited a moderate antiamoebic activity on an *Entamoeba histolytica* strain obtained from a patient, and this strain was suspected to be resistant to metronidazole used as reference. Moreover, this plant is rich in polyphenolic compounds among which we can cite ellagic and gallic acid and their derivatives. Consequently, it has an interesting antioxidant activity and a remarkable anti-inflammatory activity. The results from this study demonstrate that the anti-

inflammatory activity of this plant is due to its capacity to reduce ROS produced by stimulate neutrophils and its ability to interact with myeloperoxidase, enzyme implicated in the inflammation process. However, *T. mollis* may also exhibit its anti-inflammatory activity by targeting other sites of action.

Z. chalybeum has a very promising antileishmanial activity *in vitro* which may mainly result from the presence of benzophenanthridine alkaloids, especially chelerythrine, and dihydrochelerythrine. The plant showed a moderate antitrypanosomal activity but it can contain interesting antitrypanosomal compound such cubebin present in other *Zanthoxylum species* and already reported to be active against trypanosoma (Bastos et al., 1999). This plant did not present any interesting antiamoebic activity *in vitro.* Indeed, as explained above, the activity of the recommended drug, metronidazole was also low, suggesting a possible resistance to this drug. *Z. chalybeum* has an interesting antioxidant and anti-inflammatory activity and nitidine, the antiplasmodial compound of the plant, may play a role in this activity. Previous study reported that the plant acts by reducing the activity of 5-lipooxygenase (Muller-Jakic et al., 1993). Additionally, this study showed that the plant has a high capacity to reduce ROS produced by neutrophils but has negligible effect on myeloperoxidase; suggesting the possible capacity of this plant to target other substances implicated in the production of ROS such as NO synthase.

Definitely, the results from the first part of this study allowed us to confirm the traditional use of selected plants in the treatment of malaria. In the second part, ellagic acid and nitidine were found to be the main active compounds responsible for the antiplasmodial activity of *T. mollis* and *Z. chalybeum,* respectively, and those compounds may play a major role not only in the activity of the plants on other parasites tested but also in their interesting anti-inflammatory activity. However, both ellagic acid and nitidine have poor bioavailability and their structure modification could help to benefit from the remarkable therapeutic properties of those compounds. Nevertheless, the fact that those molecules have poor absorption is an advantage for their use in the treatment of intestinal parasites against which ellagic acid and nitidine are active. Although, the results obtained helped to achieve our objectives, further studies will be of interest to exploit the beneficial properties of the plants investigated and need then to be recommended.

Based on the results obtained from *in vitro* antiplasmodial activity assay, some plants such as *Conyza aegyptiaca* (the most cited by traditional healers) and *Solanecio mannii*, exhibited

moderate activity (IC_{50} between 15 and 25 µg/ml) but were not selected for *in vivo* test. Further *in vivo* analysis and the determination of their active ingredients are recommended.

Fuerstia africana gave promising antiplasmodial activity *in vivo.* Although ferroginol the previously isolated antiplasmodial compound (IC_{50}= 1.95 mg/ml) (Koch et al., 2006) was also cytotoxic, this plant may have other safer active ingredients which need to be isolated.

In this study, HPLC methods developed to investigate the active ingredients of *T. mollis* and *Z. chalybeum* need to be validated.

The present work tried to determine the activity of the selected plants on some neglected diseases. Unfortunately, except for malaria, we were not able to do any *in vivo* assay or to isolate active ingredients responsible for the observed antiparasitic activity. Moreover, those plants could be also active against other parasites, especially intestinal pathogens for which the poor oral absorption of ellagic acid and nitidine is not a handicap. If those plants show promising activity, the development of standardised extracts will be of interest for local use in developing countries such as Rwanda.

According to the results obtained from the *in vitro* antiamoebic activity of *T. mollis* and *Z. chalybeum*, the resistance to metronidazole is possible in Eastern Province of Rwanda. Further studies using a standardised *Entamoeba histolytica* strain is highly recommended not only to certify the activity of the plants on this parasite, but also to uncertain the suspected resistance to metronidazole.

This study illustrated the very interesting anti-inflammatory activity of *T. mollis* and *Z. chalybeum* and attempted to give some ideas about the possible targets of the plants extracts in the inflammation process. However, the determination of the anti-inflammatory activity of the plant samples on other aspects of the inflammation will be of interest. Previous studies reported the use of plant extracts in the treatment of cancer (Zacharia, 2007). Considering the relation between cancer and inflammation and the promising anti-inflammation activity of our plants, further investigations on their possible anticancer properties is recommended. The anticancer property is already known in other *Terminalia species* (Kandil et al., 1998; Yang et al., 2010) and it was already reported that ellagic acid and ellagitannins such as punicalagins present in this plants possess anticancer activity (Seeram et al., 2005). Similarly, the anticancer activity of nitidine found in *Z. chalybeum*, is very known (Kinnamon et al., 1998). Some plant extracts were already used in the treatment of cancer; in case those plants

have an interesting anticancer activity (Zacharia, 2007); the use of standardised extracts from those plants may be considered.

Rwandan traditional healers believed in their medicines and claim to cure several diseases among which we can cite malaria for which a recommended modern therapy should be used. However, a public awareness campaign is needed so as to avoid the threatening *Plasmodium falciparum* resistance. We are planning to give a feed back to those traditional practitioners in the beginning of July 2012, and this will be an occasion to talk about the use of the medicinal plant in the treatment of parasitosis especially malaria, as well as the advantages of close collaboration between us.

We hope that the results from this work will help for further investigations on Rwandese medicinal therapy in general and particularly on plants used in the treatment of various parasitosis and inflammation. As those medicinal plants have been used for a long time, they are often safe and cheap clinical studies are possible (Graz et al., 2007); we should consider doing them in order to benefit from their interesting potentialities especially in the treatment of infectious diseases.

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ANNEXES

ANNEXE 1 HPLC ANALYSIS OF *T. MOLLIS*

1. HPLC METHOD

Stationary phase **:** ODS C18 column HYPERSIL 250/4.6 mm, (5 µm) (Alltech);

Mobile phase **:** Acetonitrile (ACN) and trifluoroacetic acid (TFA) 0.05 % in gradient mode (see below)

Flow rate **:** 1 ml/min;

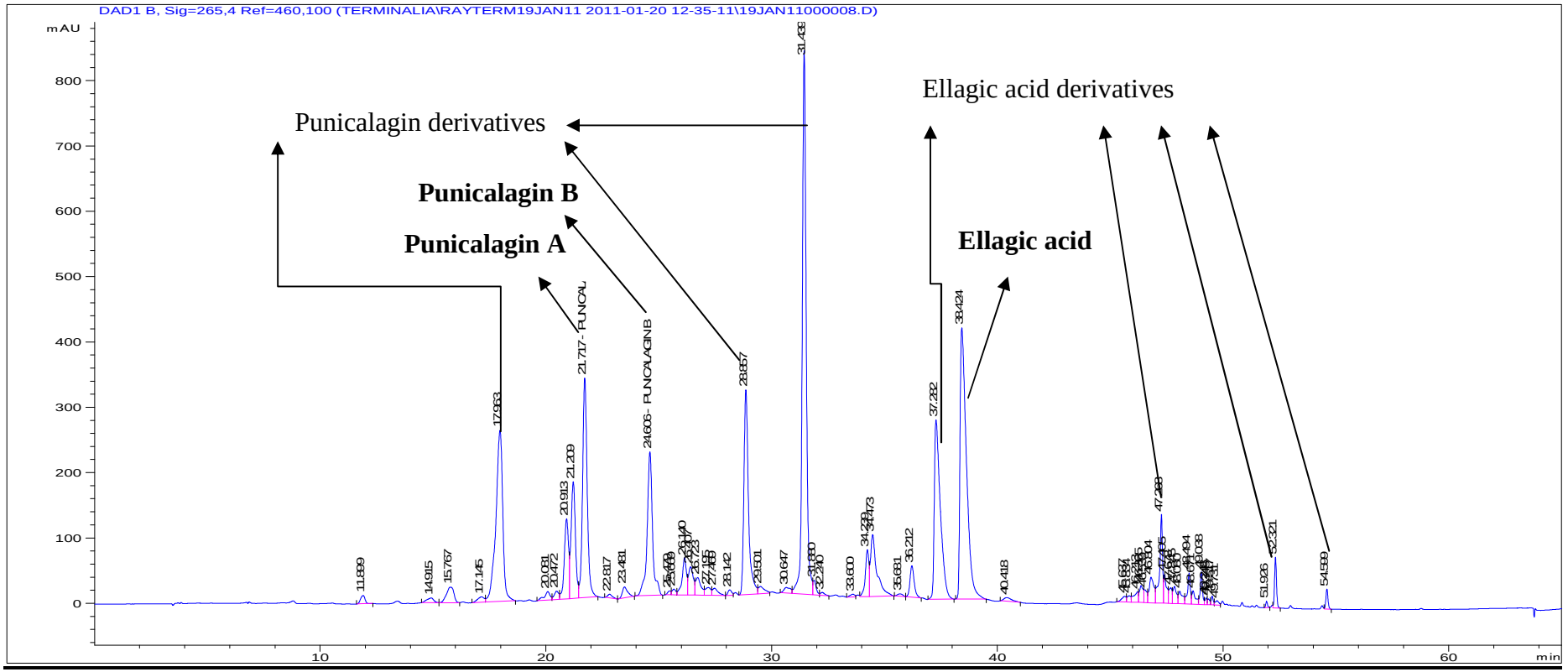
Injection volume **:** 10 µl

Detector **:** UV diode array

Gradient time **:** 65 min

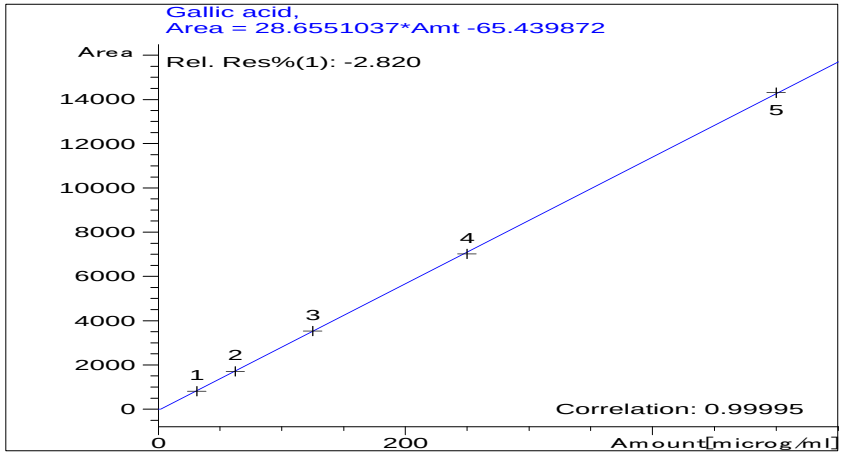
Time (min)	ACN (%)	TFA 0.05 %
0.00	0.0	100.0
30.0	20.0	80.0
40.0	20.0	80.0
50	60	40
60	90	10
61	0	100

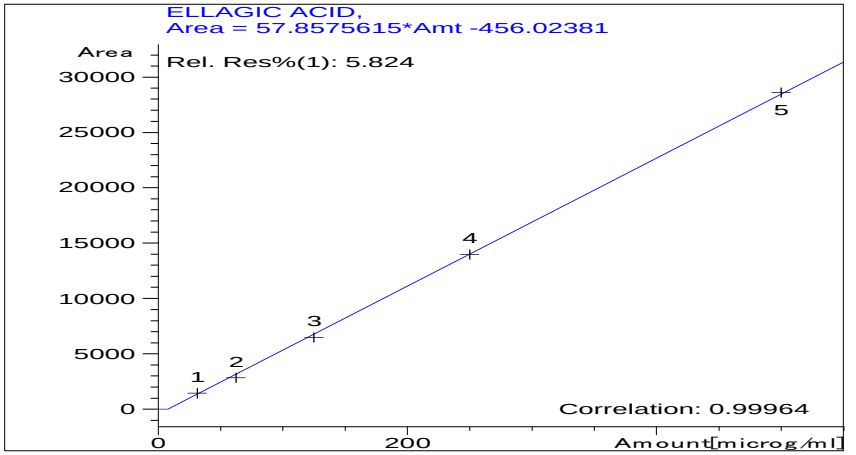
2. HPLC CHROMATOGRAM

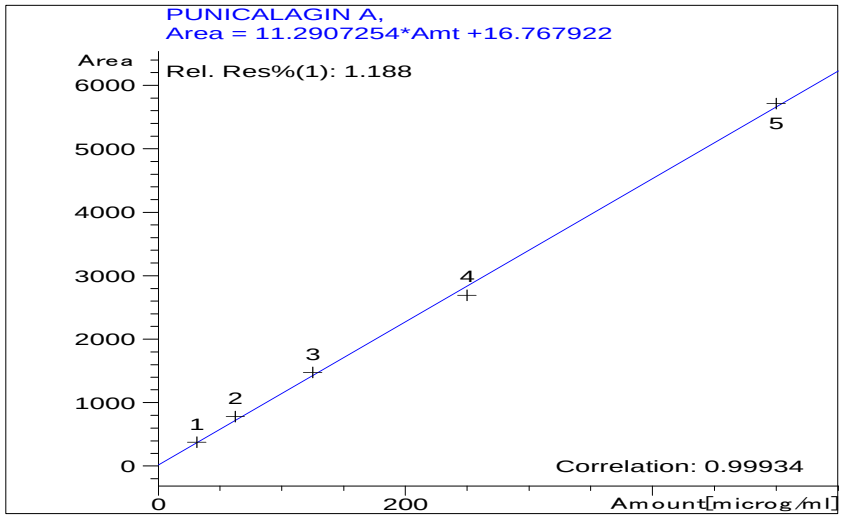


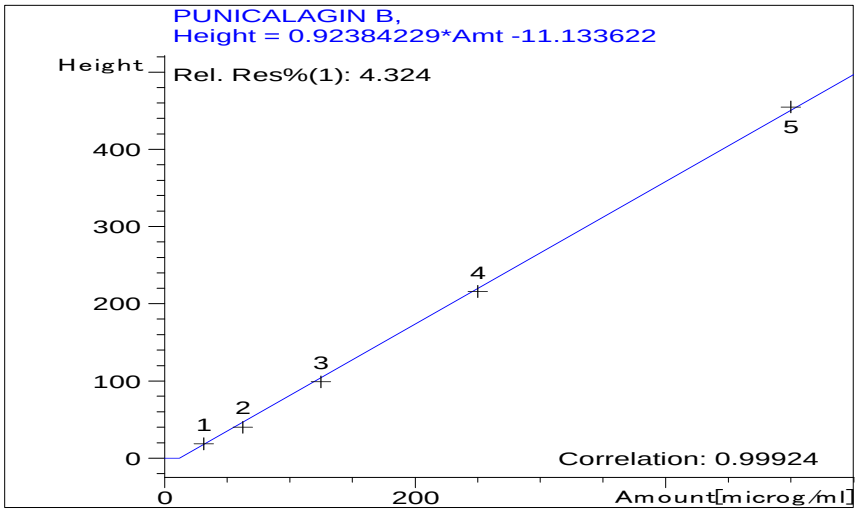
HPLC chromatogram of the methanolic root bark extract of *T. mollis*

3. CALIBRATION CURVES (quantification of pure compounds from *T. mollis*)

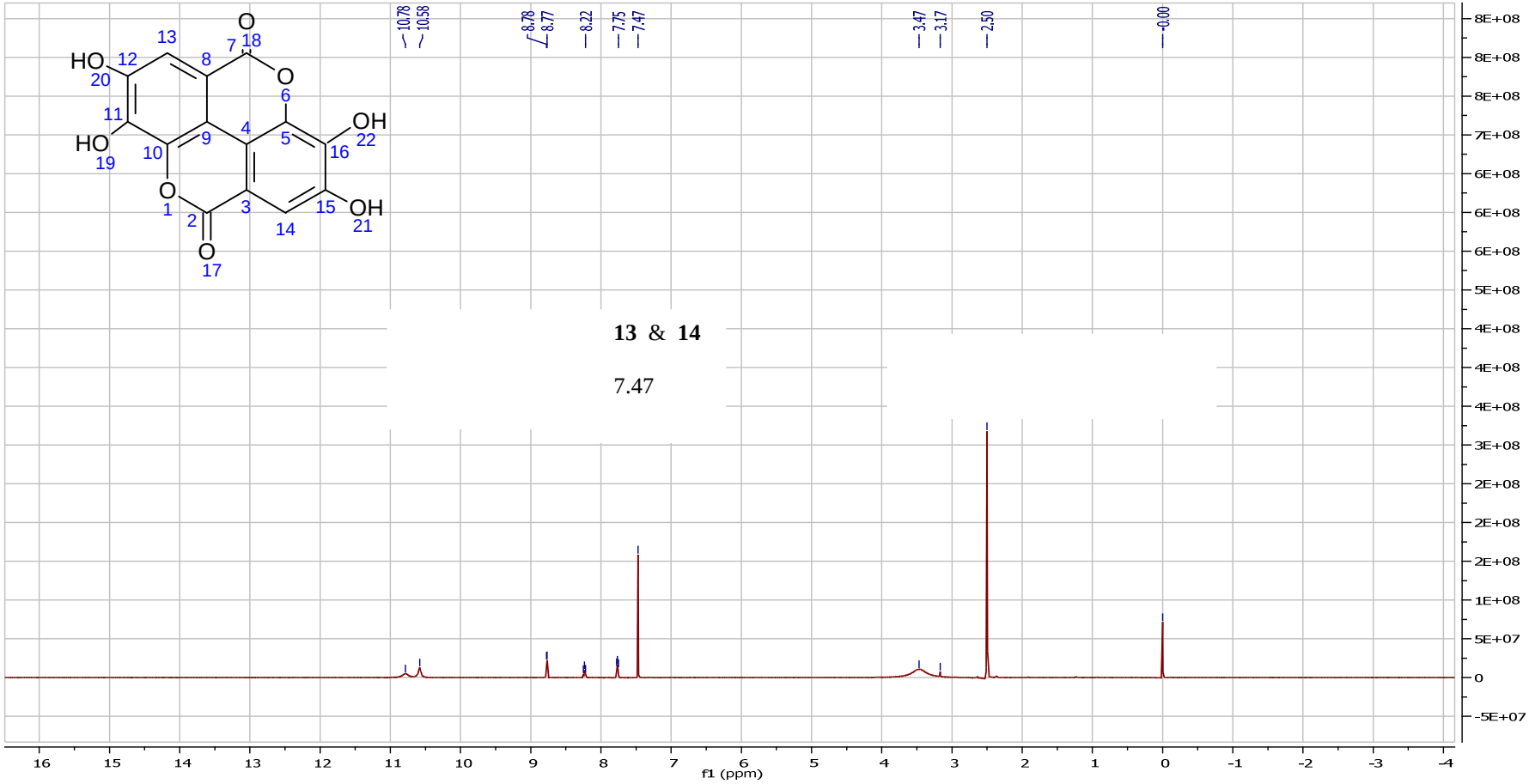




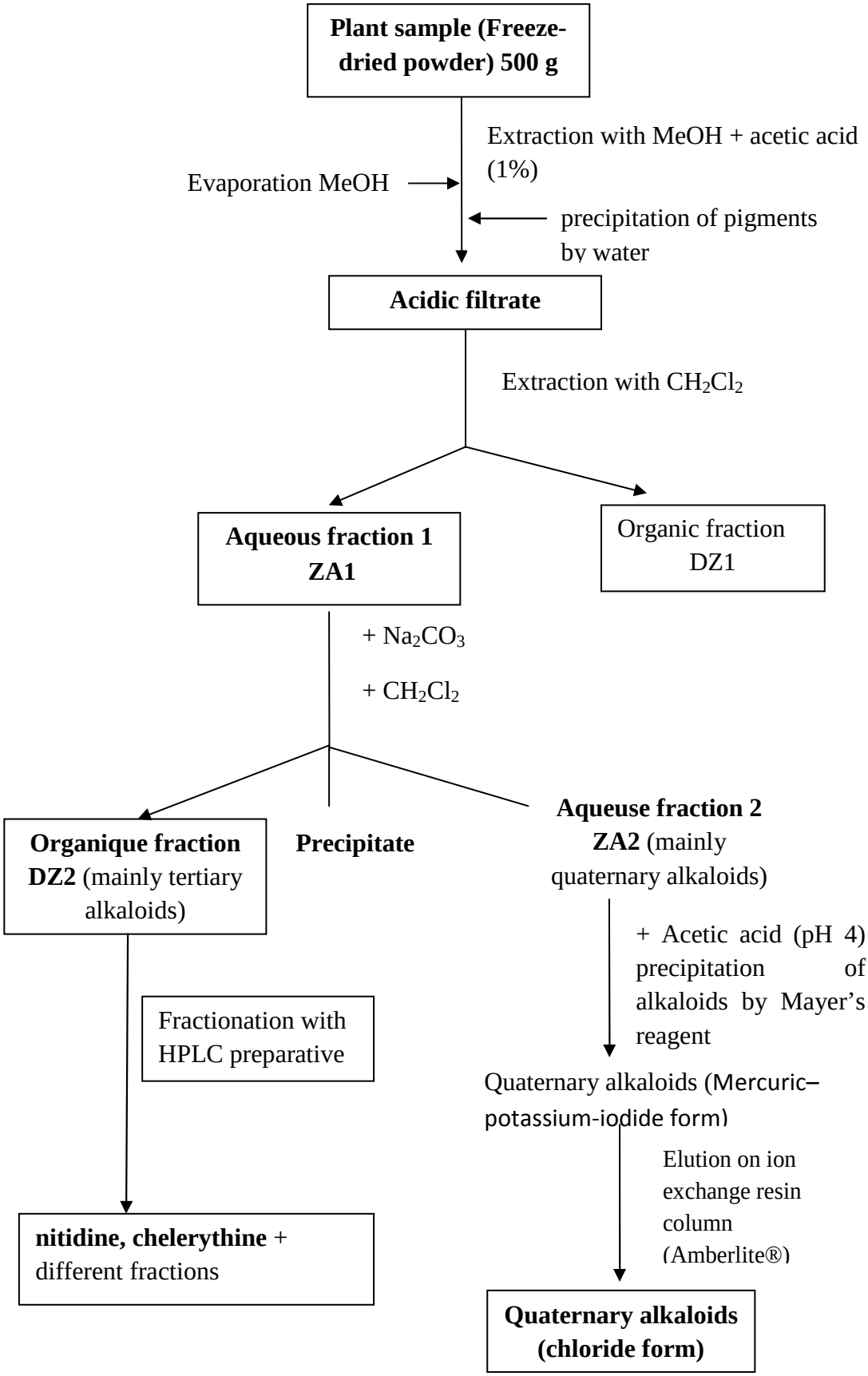




^{1}H NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTRUM OF ELLAGIC ACID IN DMSO



ANNEXE 2 FRACTIONATION SCHEME OF THE ROOT BARK OF *Z. CHALYBEUM*



ANNEXE 3 HPLC ANALYSIS OF *Z. CHALYBEUM*

1. HPLC METHOD

Stationary phase **:** Pursuit 5 Diphenyl SS 250 x 4.6 mm, (5 µm) (Varian);

Mobile phase **:** Acetonitrile (ACN) and trifluoroacetic acid (TFA) 0.05 % in gradient mode (see below)

Flow rate **:** 1 ml/min;

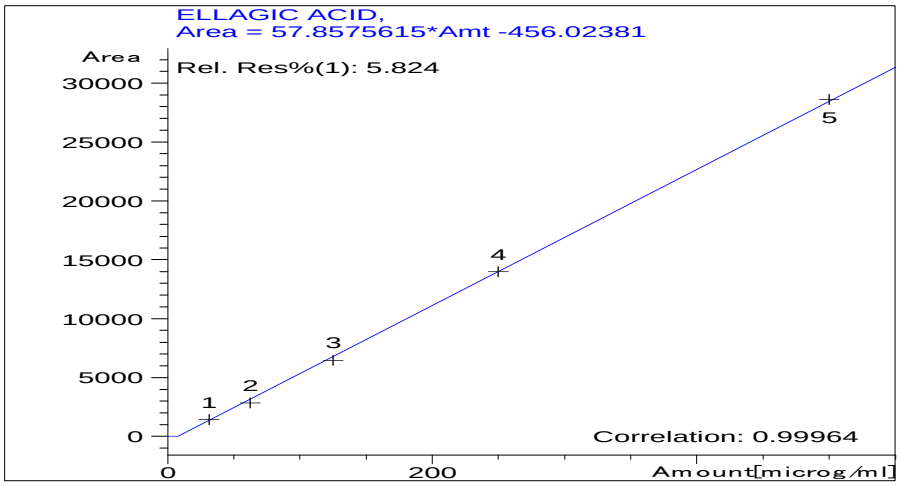
Injection volume **:** 10 µl

Detector **:** UV diode array

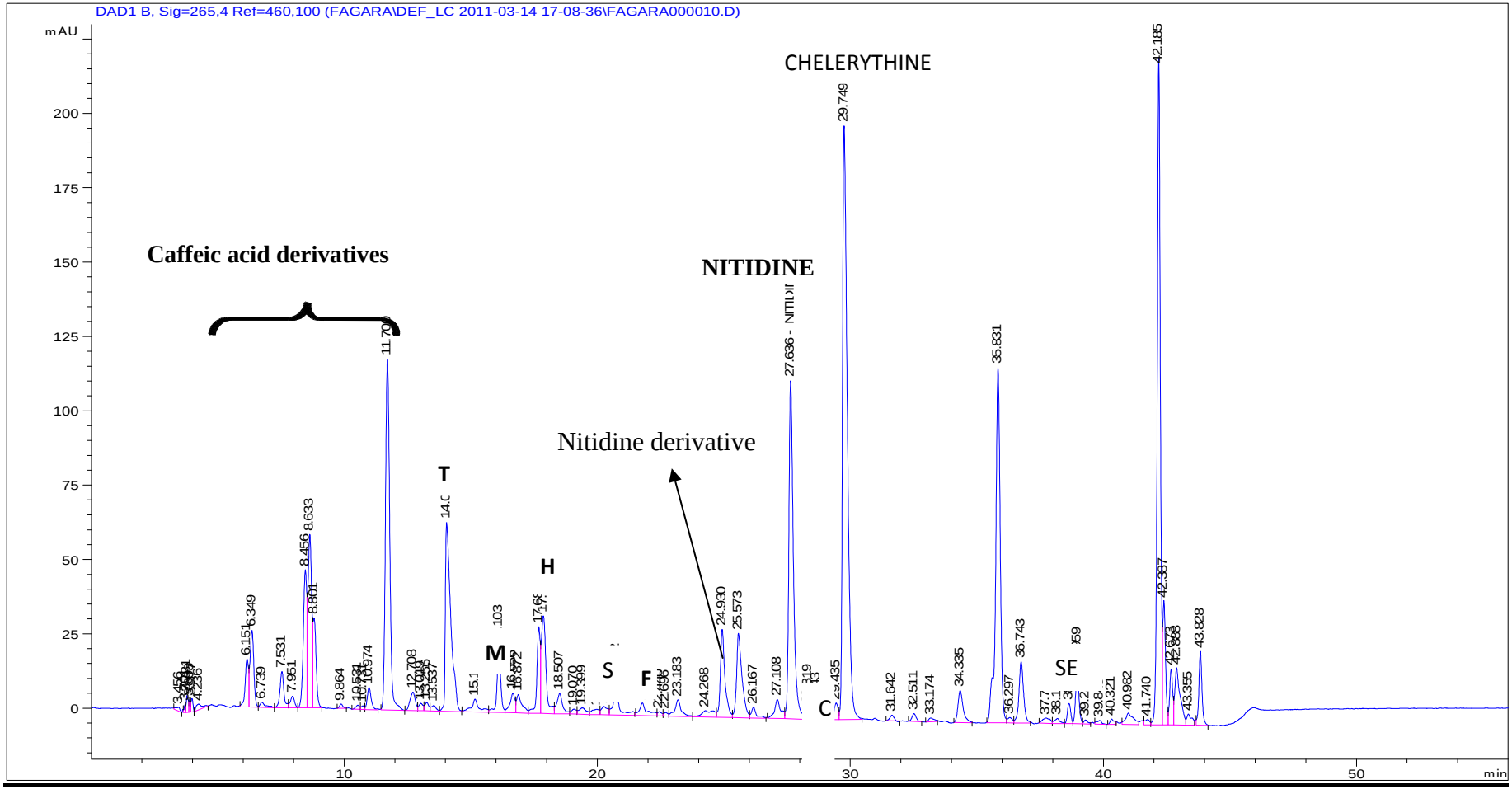
Gradient time **:** 56 min

Time (min)	ACN (%)	TFA 0.05 %
0.0	10.0	90.0
30.0	40.0	60.0
40.0	60.0	40.0
41	10.0	90.0

2. CALIBRATION CURVE (quantification of nitidine)

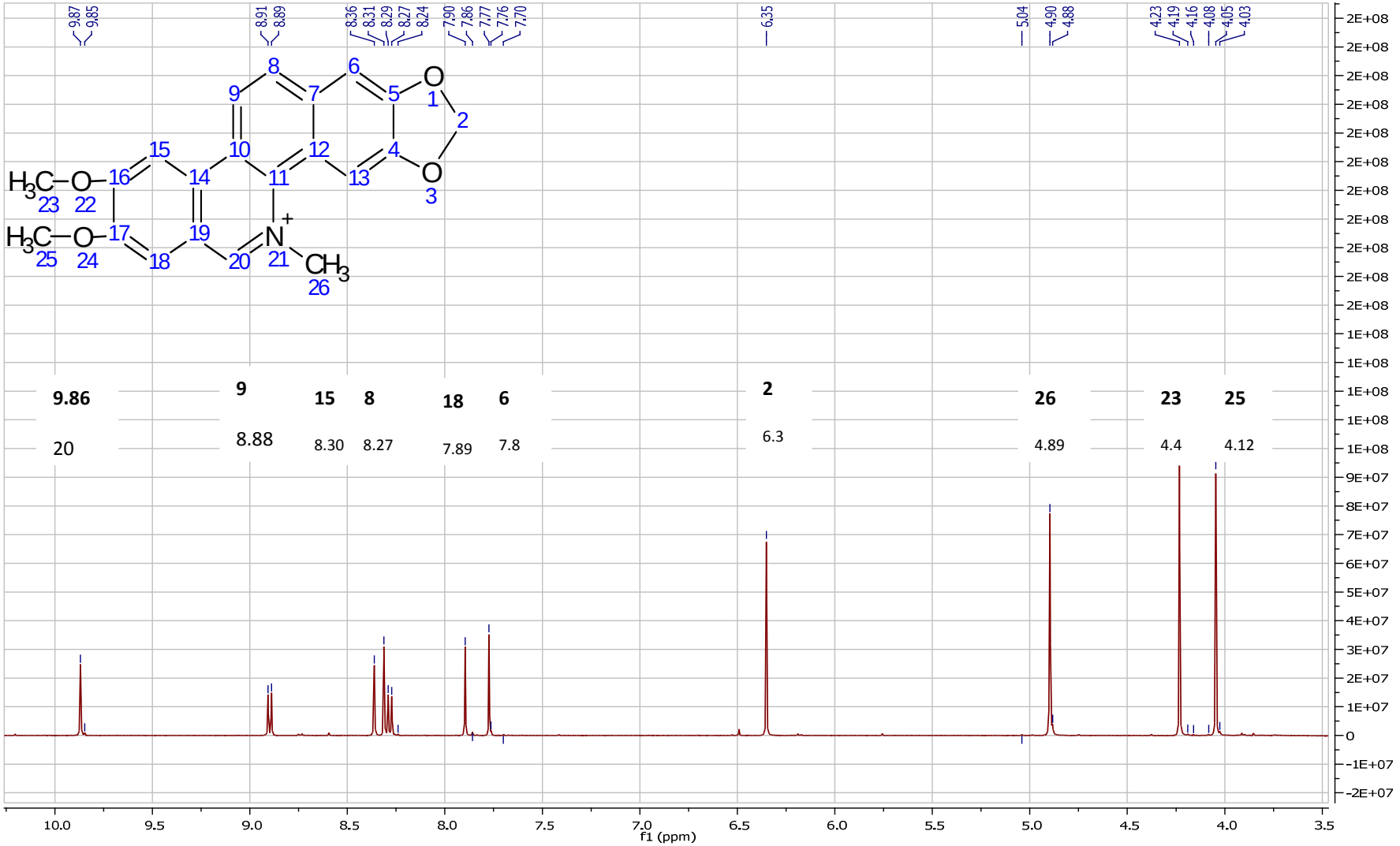


3. HPLC CHROMATOGRAM



HPLC chromatogram of the methanolic root bark extract of *Z. chalybeum*. Sample was dissolved in methanolic and then analysed using acetonitrile and 0.05% trifluoroacetic acid as mobile phase and Pursuit 5 diphenyl as stationary phase. T: tembetarine, M: magnoflorine, H: hesperidine, S: skimmianine, F: ɣ fagarine, C: methyl candicine, SE: sesamine

4. ^{1}H NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTRUM OF NITIDINE IN DMSO



ANNEXE 4 ADDITIONAL PUBLICATIONS

Toxicity of some Rwandan Medicinal Plants used in the treatment of Malaria

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PSE, YSM, CRETE June 2011

Abstract N°

INTRODUCTION

Malaria remains a major public health problem especially in sub tropical regions. New antimalarial drugs are highly needed because of a real increase of parasites' resistance to current drugs[1]. Medicinal plants constitute an interesting source of new drugs but sometimes, their toxicity may compromise their medicinal use. Previous studies reported a very promising antiplasmodial activity for some Rwandan medicinal plants[2] but few is known about their toxicity.

The aim of our study was to evaluate the antiplasmodial activity and the toxicity of some Rwandan medicinal plants used in the treatment of malaria. Samples from selected plants were dried and extracted with methanol, dichloromethane and water. Crude extracts obtained were tested *in vitro* for their antiplasmodial activity. The most active extracts were evaluated for cytotoxicity assay and for in vivo antiplasmodial activity. The eventual toxicity of those plants was also determined.

METHODOLOGY

• Plant extracts: 5 g of dry plant powder was macerated 3 times in 50 ml of methanol or dichlorometahne for 30 min with constant shaking and evaporated to dryness. The aqueous extracts were obtained using 100 ml of distilled water for 2 g powdered plant material and let to boil for 1 hour. After filitration of the solution, filtrate was freeze-dried.

• *In vitro* antiplasmodial assays: continuous cultures of Plasmodium falciparum, chloroquine sensitive (3D7) and resistant (W2) strains, were assessed following the procedure already described by Frédérich et al.[3]. Each extract sample was applied in a series of eight 3-fold dilutions (0.09 -200 µg/ml) on 2 rows of a 96-well microplate and was incubated with a parasitic suspension (hematocrit= 1 %, parasitaemia = 2 %) for 48 h at 37°C, under an atmosphere of 5% CO_2. Parasite growth was estimated by determination of lactate dehydrogenase activity as described by Kenmogne et al.4.

• *In vitro* cytotoxic assays: human normal fetal lung fibroblasts (**WI-38**) were maintained in continuous culture in a humid atmosphere at 37°C and 5% CO_2 in DMEM medium. After 24 h incubation of a 96-well microplate seeded with 200 µl medium containing 8000 cells/ well, the medium was replaced by six 3-fold dilutions of **plant extract (0.82- 200 µg/ml)**. After 48 h incubation, cell viability was determined by **colorimetric method** using WST-1 cleaved by cellular enzymes (absorbance at 450 nm).

• *In vivo* antiplasmodial assays, based on the classical **4-day suppressive test**[5] : female Swiss mice (5 mice/group) were infested by ***Plasmodium berghei*** *NK173* 4h before treatment. Treatment doses (**200 and 300 mg/kg of plant extract**) were given intraperitoneally and orally once daily from Day 0 to Day 3. On Day 4 and 7, thin mouse tail blood smears were prepared and stained with Giemsa. **Parasitaemia** was determined **counting at least 500 erythrocytes** under microscope. Mortality was controlled during 20 days. Chloroquine at 4 mg/kg doses was used as a positive control and physiological serum (**T0**) was used as a negative control.

• *Toxicity of the extracts*: to determine the toxicity of the extract the survival-time was calculated and animal deaths was monitored.

• *Identification and quantification of aristolochic acid*: HPLC method was used to identify and to quantify aristolochic acid in *Aristolochia elegans* seed. Aristolochic acid was used as reference.

Selected species, their scientific names, parts used, voucher number and collection place (altitude)

Scientific name of the plant (family), date	Plant part[a]	Voucher number	Collection place (altitude)
Aristolochia elegans MAST (Aristolochiaceae), 2007	Seed	2007V39	Huye (1 684 m)
Conyza aegyptiaca (L.) AIT (Asteraceae), 2007	L	2007V38	Huye (1 684 m)
Markhamia lutea K.SCHUM (Bignoniaceae), 2008	L	2008R17	Nyaruguru (1 753 m)
Microglossa pyrifolia (LAM.) O.KUNTE (Asteraceae), 2007	L	2007R1	Bugesera (1 413m)
Mitragyna rubrostipulata (K.SCHUM.) (Rubiaceae), 2008	L&SB	2008R19	Nyaruguru (1 648 m)
Fuerstia africana T.C.E. Fr. (Lamiaceae), 2008	L&S	BR-S.P.502885	Huye (1 719 m)
Rumex abyssinicus JACQ (Polygonaceae) , 2008	R	2008R15	Huye (1 670 m)
Rumex bequaertii DE WILD (Polygonaceae), 2008	R	2007R3	Huye (1 600 m)
Solanecio mannii (Hook.f.) JEFFREY (Asteraceae) , 2008	L	2007R2	Musanze (2 033 m)
Terminalia mollis LAWSON (Combretaceae), 2007 and 2008	L, SB &RB	BR-S.P.508716	Ndego (1 592 m)
Tithonia diversifolia (HENSL.) A GRAY(Asteraceae), 2008	F&L	2007R12	Huye (1 680 m)
Trimeria grandifolia (HOCHST.) (Flacourtiaceae), 2008	L	2008R16	Nyaruguru (1 722 m)
Zanthoxylum chalybeum ENGL. (Rutaceae), 2007 and 2008	SB&RB	BR-S.P.508726	Ndego (1 377 m)

[a] F = flower; L = leaves; R=root; RB= root bark; S = stem bark; SB = stem bark

Areas of plant's collection: regions where malaria is most endemic: East, South and South-West of Rwanda

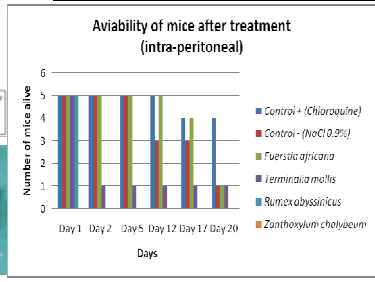
RESULTS

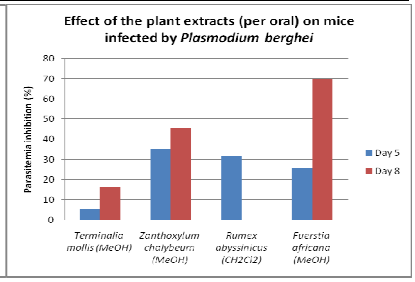
➢ Aristolochic acid was present in *Aristolochic elegans* seed at a rate of 0.04%. Even if this concentration is not so high, chronic use of this plant in prevention of malaria should be avoided. Indeed, aristolochic acid is already known to induce intestinal fibrosis, tubular atrophy and renal failure. [5]

➢ For *in vivo* antiplasmodial assays using intraperitoneal route, three of four plants tested namely *Zanthoxylum chalybeum, Terminalia mollis* and *Rumex abyssinicus,* presented some toxicity in mice. Most of the time, the viability of mice treated with the extracts from those plants did not exceed 4 days.

IC_{50} values expressing promising therapeutic activity toward *P. falciparum* (3D7 and W2 strains) and Selectivity Index (SI) corresponding to the ratio between cytotoxic (WI-38) and antiparasitic (3D7) activity.

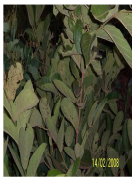
Species	Plant part	Extract	Yield (%)	3D7, IC_{50} (µg/ml)	W2, IC_{50} (µg/ml)	WI-38, IC_{50} (µg/ml)	SI
Zanthoxylum chalybeum	Root bark	MeOH	16.8	**4.2 ± 2.7**	**1.9 ± 0.5**	40.0 ± 8.5	9.5
Rumex abyssinicus	Root	CH_2Cl_2	7.4	**4.3 ± 2.0**	**3.1 ± 1.2**	13.3 ± 0.9	3.1
Fuerstia africana	Leaf & seed	MeOH	13.3	**6.9 ± 2.3**	**4.1 ± 1.6**	13.0 ± 2.3	1.9
Terminalia mollis	Root bark	MeOH	26.3	**11.7 ± 0.9**	**18.9 ± 1.6**	77.2 ± 8.9	6.6





Zanthoxylum chalybeum

Stem et root bark of the plant are used to treat malaria and intestinal parasites in Rwanda



Terminalia mollis

Root bark is used to treat intestinal parasites in Rwanda and to treat malaria in Burundi.

Fruestia africana

The whole plant without root is used to treat malaria and the leaf is used to treat gonorrhoea

Rumex abyssinicus

The root of the plant is used to treat malaria, gonorrhoea, constipation, poison, hepatitis and the leaf is used to treat cough, gastric ulcer, etc.

CONCLUSION

The use of *Aristolochia elegans* seed to prevent malaria should be avoided. Although most of plants tested showed an antiplasmodial activity some of them may be toxic. On one hand, the use of those plants at low dose could be safe but a chronic toxicity is still possible. On another hand, compounds responsible for the beneficial activity of those plants may not be the toxic ones. Therefore, further investigations are needed to clarify which compound is responsible for each activity.

References

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[2] Muganga et al., *Journal of Ethynopharmarcology*, **2010**, 128, 52-57
[3] Frederich et al. *Journal of Natural Products* ,) **2002,** 65, 1381-1386.
[4] Kenmogne et al., *Phytochemistry,* **2006,** 67, 433-438.
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[6]Pozdkiz et al., *Kidney international*, **2008**, 73, 595-607

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