School of Advanced Studies - University of Camerino

Curriculum in Life and Health Sciences – One Health

Malaria and Human Development

Ph.D. Thesis

Identification and pre-clinical characterization of medicinal plants used to prevent and cure malaria in pregnant women in Cameroon

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Abstract

In the midst of transient victories by way of insecticides against mosquitoes or drugs against the parasitic disease, the most serious form of malaria, caused by *Plasmodium falciparum*, continues to be a major public health problem. Malaria in pregnancy compromises the health of both the mother and child and can be fatal for both. Particularly, malaria during pregnancy constitutes the major cause of low birth-weight and stillbirth in endemics countries. In Cameroon malaria is one of the major public health problems. The absence of an effective vaccine makes the parasitic disease very difficult to be eliminated and impossible to be eradicated. Treatment and prevention of malaria during pregnancy is currently a challenge for maternal health responsibles, due to the emergence of multi-drug resistant malaria parasites (e.g. to sulfadoxine-pyrimethamine used for IPT). However, there is, yet, very little and conflicting evidence on the safety of artemisinin-based combination treatments (ACTs) in particular during first term pregnancy.

Traditional medicines have been employed to treat malaria for thousands of years. With the problems of increasing levels of drug resistance and difficulties in poor areas of effective antimalarial drugs being affordable and accessible, traditional medicines remains an important, sustainable source of treatment. Moreover, the development of evidence based safe and effective standardized herbal formulations for pregnant women with curative and transmission-blocking properties would be a major breakthrough to decrease malaria burden in women and their newborns.

This Ph.D thesis aimed to **a**) identify medicinal plants used to prevent and cure malaria during pregnancy in Menoua West Cameroon; **b**) assess the activity of extracts from identified plants *in-vitro* against *Plasmodium falciparum* asexual blood-stages and gametocytes and against sporogonic stages of *Plasmodium berghei in-vitro* and *in-vivo;* **c**) estimate their cytotoxicity *in vitro* and **d**) design an evidenced based (safe and effective) plant combination (herbal remedy) with multistage effects on *Plasmodium,* suitable for women in pregnancy.

Results show that 19 antimalarial plants are known to be employed in the study area to manage malaria during pregnancy and of these 11 were cited as

commonly used. Of the latter 10 plants were possible to be collected (11 plant part materials) and 22 extracts were prepared (water and methanol). Seven, namely methanol and water extracts of *D. edulis* stem bark, methanol and water extracts of *E. globulus* leaves, *P. americana* stem bark and *V. africana* leaves methanol extracts, and methanol extract of *C. citratus* leaves were found to reduce by 50% *in vitro* asexual blood stage development at concentrations < 20µg/ml, on both *P. falciparum* parasite strains used. These results are in line with the one found in the literature. The stem bark methanol extract of *P. americana* was able to reduce the viability of mature gametocytes by 94% at the primary screening concentration of 100µg/ml. Its IC_{50} value was estimated to be 34.7 µg/ml. Comparing the anti-plasmodial activity of methanol and aqueous extracts of the tested plants, consistently higher activity was found in the former. Similarly, the methanol plant extracts were more active *in vitro* on the development of the sexual stages (early sporogonic development) that evolve in the mosquito vector. Two extracts, namely the stem bark methanol extract of *P. americana* and *D. edulis*, exhibited promising inhibitory effects against the insect transmissible stages of the parasite showing IC_{50} values in the range of 6 to 13 µg/mL *in vitro* and *in vivo* a reduction of oocyst numbers by more than 70%, after feeding on gametocytemic mice treated with 150 mg/kg. The results on transmission-blocking are promising since they are similar to the one obtain with the standardized herbal NeemAzal. Moreover, none of the 22 plant extract was found to have cytotoxic effects on normal human cell lines (*NHF-A12*-human dermal fibroblast and *EA.hy926*-endothelial). Interestingly, almost all the 22 extracts displayed some anti-tumor effects, reducing cell viability of melanoma and/or human breast cancer cells. Furthermore, the methanol stem bark extract of *P. americana* and *D. edulis* were found to possess some mosquitocidal potential when tested on the *Sf9* insect cell line, but not as evident as NeemAzal® a azadirachtin A rich product used as reference.

The findings of this doctoral research put in evidence antiplasmodial activity in about one-half of the tested medicinal plants against one or different life cycle stages of the parasite. Thus, based on this parasitological evidence, herbal remedies can be suggested to be used as a tool for the management of malaria in pregnant women. Further ethnobotanical studies including more

villages and larger variety of respondents in the community will allow to identify additional medicinal plants uses, since our study was limited to a limited number of easy accessible villages. Moreover, our findings allow the design of rational combination formulations with antiplasmodial multi-stage activity. Preclinical studies will allow to assess efficacy and safety of such plant combinations, validating the concept of curative and preventive transmission-blocking herbal remedies.

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List of Abbreviations

ACT: Artemisinin based combination therapy

CI= Confidence interval

CQ: Chloroquine

CTRPp.GFP: Circumsporozoite and Thrombospondin-related adhesive protein [TRAP] related protein. Green Fluorescent Protein

CSA: Chondroitin sulfate A

DFA: Direct feeding assay

ESS: Early sporogonic stages

G6PD: Glucose-6-phosphate dehydrogenase

IC_{50}: Concentration required to inhibit by 50% the maximal response

INS: National Institute of Statistic, Cameroon

ip: Intra-peritoneal

IPTp: Intermittent preventive treatment in pregnancy

IRS: Indoor residual spraying

ISTp: Intermittent screening treatment in pregnancy

ITN: Insecticide treated bed nets

IUGR: Intra-uterine growth retardation

LBW: Low birth weight

MDA: Mass drug administration

MeOH: Methanol

MINSANTE: Ministry of Public Health, Cameroun

MIP: Malaria in pregnancy

MMV: Medicines for malaria venture

MPLC: Medium pressure liquid chromatography

NMR: Nuclear magnetic resonance

ODA: Ookinete development assay

PABA: Para-amino benzoic acid

PAM: Pregnancy associated malaria

PCR: Polymerase chain reaction

Pfdhps: Plasmodium falciparum dihydropteroate synthetase

pLDH : *Plasmodium falciparum* lactate dehydrogenase

PQ: Primaquine

RBC: Red blood cells

RDT: Rapid diagnostic test

RPMI: Roswell Park Memorial Institute medium

Sf9: Spodoptera frugiperda insect cell line

SMC: Seasonal chemoprevention

SP: Sulfadoxine-pyrimethamine

TM/CAM: Traditional medicine and Complementary Alternative Medicine

TCP: Target candidate profile

TPP: Target product profile

TPs: Traditional practitioners

WHO: World Health Organization

1. Introduction

1.1. Key notes on malaria disease

Malaria is a life-threatening vector-borne disease caused by protozoan parasites belonging to the genus *Plasmodium* and is spread to humans by the bite of infected female mosquitoes of the genus *Anopheles* (over 400 different *Anopheles* species occur). Malaria (from the Italian expression "*mal'aria"* which means *"bad air"*) is the most important eukaryotic parasitic disease, threatening the livelihoods of over 2.2 billion people (*World malaria report 2018*, 2018). There are five species of malaria parasites occurring in humans, namely *Plasmodium falciparum*, *P. malariae*, *P. ovale*, *P. vivax* and *P. knowlesi*. The latter, a zoonotic species, occurs in monkeys and infects occasionally people living at forest borders close to the habitats of monkeys (Cox, 2010; Kantele & Jokiranta, 2011). *P. vivax* which is widespread in Central and South America, Asia and Oceania, rarely leads to a lethal infection. It can exist latent inside hepatocytes, re-emerging after many months to several years. *P. vivax* is an important cause of low birth weight and in pregnancy is associated with severe complications. Though commonly transmitted, morbidity due to *P. ovale* is rare as immunity is established early after infection and parasitaemia remains low. It is found principally in Africa causing less than 0.5% of malaria cases (Desai et al., 2007). *P. malaria* is found worldwide but with a very patchy distribution. It causes renal

complications including chronic nephropathy and if left untreated the patients may remain carriers though asymptomatic for years (Barsoum, 2000).

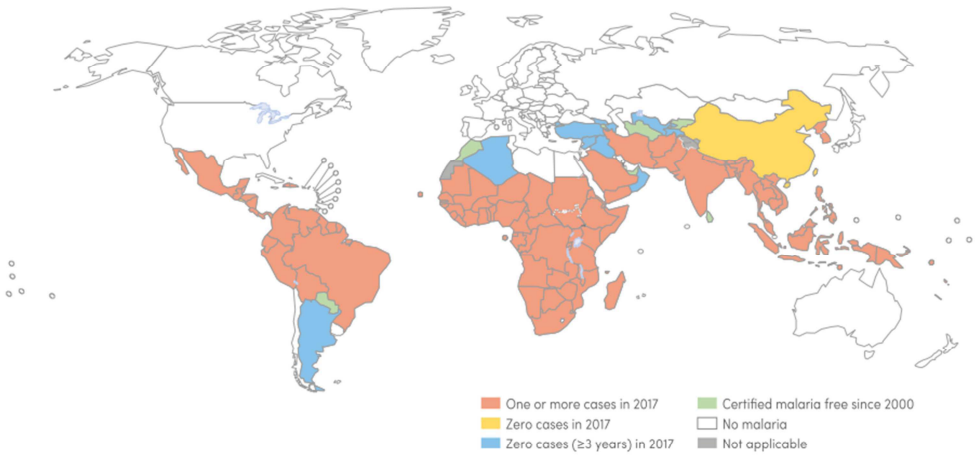
P. falciparum is the most virulent species and causes more than 90% of malaria deaths worldwide. Africa presents the highest endemicity of *P. falciparum*. The high-risk groups include children below the age of five years, pregnant women (especially in the first pregnancy) travelers, migrants from non-malarial regions moving into endemic regions, and individuals with immune response disorders (Menéndez et al., 2007).

About seven days after an infective mosquito bite, the clinical symptoms typical of malaria such as fever, headache, chills and vomiting appear. Malaria remains one of the most difficult diseases to control and to eliminate in endemic areas due to the wide diffusion of its vectors , highly anthropophylic mosquitoes belonging to the genus *Anopheles*, and due to the *Plasmodium* parasite which is extremely well adapted to its human and vector host.

If not recognized, diagnosed and properly treated, malaria is likely to develop severe clinical symptoms in high risk groups and most importantly, untreated individuals and asymptomatic gametocyte carriers contribute to maintain high levels of transmission of the disease.

1.2. Epidemiology and burden of malaria in pregnancy

The recent World Malaria Report 2018 estimates the number of malaria cases that occurred in 2017 to be 219 million (95% confidence interval [CI]: 203 - 262 million) that lead to 435.000 deaths. Moreover, the report data highlight that 92 % of global malaria cases still occur in Africa as shown in Figure 1. When malaria cases in 2017 are compared with the 239 million (95% CI: 219 - 285 million) cases that occurred in 2010 worldwide, it emerges a decrease of 20 million cases in 2017 compared to 2010. However, more recent data sets, i.e. data for the period 2015 - 2017 highlight that no significant progress in reducing global malaria cases was made in the more recent timeframe (*World malaria report 2018*, 2018). This illustrates the fragility of malaria control and the need to maintain control programs efficient even if numbers of cases have been reduced substantially.



WHO: World Health Organization.

Figure 1 Countries with indigenous cases in 2000 and their status in 2017. Countries with zero cases over at least the past 3 consecutive years are considered to be malaria free. Countries in the WHO European Region reported zero indigenous cases in 2016 and again in 2017. Both China and El Salvador reported zero indigenous cases (*World malaria report 2018*, 2018).

Globally, an estimated 125 million pregnant women reside in areas where they are at risk of contracting malaria in the course of pregnancy (MIP), and MIP or pregnancy associated malaria (PAM) remains an important cause of adverse birth outcomes (Dellicour et al., 2010). In sub-Saharan Africa, where the majority of adverse birth outcomes attributable to malaria occurs, *P. falciparum* is the dominant species. However, more than half of the 125 million pregnant women that are potentially exposed to malaria live in Southeast Asia and the Western Pacific where *P. falciparum* and *P. vivax* coexist. Co-existence and hence co-infections of *P. falciparum* and *P. vivax* also occur in South America, where 3% of the global total of women at risk for MIP reside (Dellicour et al., 2010).

Risk factors for malaria in pregnancy

In areas where malaria transmission is high, the burden of malaria concerns in particular primigravidae, whereas, in areas of low transmission, women are at risk during all gravidities. In areas of high transmission, primigravidae develop antibodies to *VAR2CSA* protein produced by malaria parasites, and are partially protected during subsequent pregnancies. This tends not to happen in areas of low transmission (Moore, Fowkes, et al., 2017). Generally,

pregnant women living in areas with low or unstable (episodic) transmission have little or no immunity to malaria and are at a two-to-three times higher risk of severe disease compared to non-pregnant adults (Marchesini et al.,2004). Severe MIP has specifically been associated with *P. falciparum* infections. *P. vivax* is less frequently causing severe disease but more likely to occur in mothers with little acquired immunity (Rijken et al., 2012).

Maternal age also plays an important role in whether uncomplicated malaria during pregnancy develops to severe disease. Young mothers are at greater risk for severe malaria disease compared to older mothers, who have acquired to some degree clinical immunity against the development of severe symptoms (Desai et al., 2007). Hence, in high transmission areas, such as sub-Saharan Africa, primigravidae and secundigravidae are at greater risk for severe malaria infection compared to multigravidae, but this is not true in areas of low transmission, where multigravidae are likely not to have experienced any prior malaria exposure and hence have not developed clinical immunity (Staalsoe et al., 2004).

Effects in pregnant women

The clinical effects of malaria in pregnant women vary from mild symptoms to severe anemia and death. Women living in low malaria transmission areas who have a low degree of acquired immunity are more likely to experience complications such as renal failure, pulmonary edema and cerebral malaria. Despite this, the overall maternal mortality rates appear to be in the same

order of magnitude in low-transmission areas (0.6 - 12.5%) compared to high transmission areas (0.5 - 23%) (Desai et al., 2007).

Anemia is one of the most common symptoms of MIP. *Plasmodium* causes anemia through hemolysis, increased splenic clearance of erythrocytes (infected and healthy cells), and reduced erythropoesis. Severe anemia during pregnancy (hemoglobin < 7 g/dL) is usually multifactorial with significant nutritional causative factors, but malaria plays an important role (Brabin et al., 2001).

Pregnant women are three times more likely to develop severe malaria and must be taken in charge promptly with general intensive care measures and parental antimalarial treatment to avoid deadly outcome (Kovacs et al., 2015). WHO defines severe malaria as parasitaemia with evidence of end organ dysfunction. The signs and symptoms of severe malaria can include severe anemia, hypoglycemia, acute respiratory distress syndrome, renal failure and cerebral malaria. The estimated mean mortality of pregnant women due to severe MIP is 39% (range 8 - 100%) (Kovacs et al., 2015).

Effects of MIP on foetal development and new borns

Malaria is a significant cause of stillbirth in endemic countries (Figure 2). MIP contributes to 12- 20% of stillbirths in endemic regions of sub-Saharan Africa, with lower rates if the mothers are given treatment. Stillbirth risk is higher in areas of high endemicity (Moore et al., 2017). The risk of stillbirth can be attenuated by the implementation of suitable malaria control

interventions. For example, the use of insecticide-treated bed nets (ITN) has been shown to lower rates of placental malaria and stillbirth (risk ratio 0.67 [95% CI, 0.45 - 1.00] for stillbirth) (Gamble & Ekwaru, 2006).

MIP increases also the risk of low birth weight (LBW) (defined as birth weight < 2500 g). Approximately 20% of LBW cases in malaria-endemic areas are attributed to placental infection with malaria (Figure 2) (Guyatt & Snow, 2004). A mother with a malaria-infected placenta is twice as likely to have a baby with LBW. LBW in turn is associated with a 3 to 20 fold increase in the likelihood of infant mortality (Umbers et al., 2011).

MIP causes LBW due to intrauterine growth restriction (IUGR). Up to 70% of IUGR in endemic areas is due to malaria, as a result of compromised oxygen and nutrient delivery to the fetus (Guyatt & Snow, 2004; Umbers et al., 2011). The contribution of malaria to preterm births is also sizeable, with up to 36% of prematurity in malaria-endemic areas attributable to *Plasmodium* infection. Prematurity may also result from the host's immune response to malaria parasites triggering early labor (Steketee et al., 2001).

Congenital malaria

Congenital malaria occurs when malaria parasites infect the fetus by crossing the placenta during pregnancy or infect the baby during delivery. Congenital malaria is confirmed by the identification of *P. falciparum* parasites in the cord blood or peripheral blood of an infant during the first 7 days of life (Moya-alvarez, Abellana, & Cot, 2014), or later if there is no possibility of

postpartum infection by a mosquito bite (as would be the case in a non-malaria endemic area)(Menendez & Mayor, 2007). Only few estimates on the prevalence of congenital malaria are available. While it was previously thought to range between <1% and 6% (Alvarez M. et al., 2014), more recent studies have demonstrated a prevalence rate of up to 33% in highly endemic areas (Menendez & Mayor, 2007). Also, it remains little known how many of these congenital infections persist and whether they cause clinical illness. Most descriptive reports of congenital malaria are from infants who are born in non-endemic areas (Menéndez et al., 2007). Given that much of the literature on congenital malaria is derived from single case reports, more research is needed to better understand the epidemiology and pathophysiology of congenital malaria.

Effects of MIP in early childhood

The development and health status of children from mothers with MIP is affected up to the age of 5 years and possibly beyond (Figure 2). Malaria during pregnancy can have adverse effects such as abortion (1st and 2nd trimester), intra-uterine growth retardation (IUGR) and LBW from the second to third trimester, and stillbirth and preterm delivery (leading to LBW) during the 3rd trimester. Prenatal malaria exposure is associated with an increased risk of getting malaria in children four to six months of age. MIP and in particular placental parasitaemia is suggested to increase the risk of malaria infections in infancy and childhood through several mechanisms: MIP might impede maternal antibody passage to offspring, compromising

the general immunity status of the fetus and newborn and thus making the offspring susceptible to any infections including malaria (Alvarez M. et al., 2014). In addition, *in-utero* exposure to malaria induces the development of T_{reg} cells provoking fetal immune tolerance to malaria antigens that persists into childhood (Menendez & Mayor, 2007). MIP is also associated with anemia during infancy and the infant's risk for anemia with maternal peripheral parasitaemia at delivery is 11.8% and 9.2% with placental malaria infection (Accrombessi et al., 2015).

The burden of malaria in pregnancy has been exacerbated by the advent of HIV which increases susceptibility to malaria in pregnancy, reduces the efficacy of antimalarial treatments, and complicates the use of antimalarials because of potential drug interactions (Menéndez et al., 2007).

Overall, successful control of malaria in pregnancy might save lives of mothers and babies and is a high public- health priority in all endemic countries, although the most appropriate methods for achieving this may vary according to local conditions.

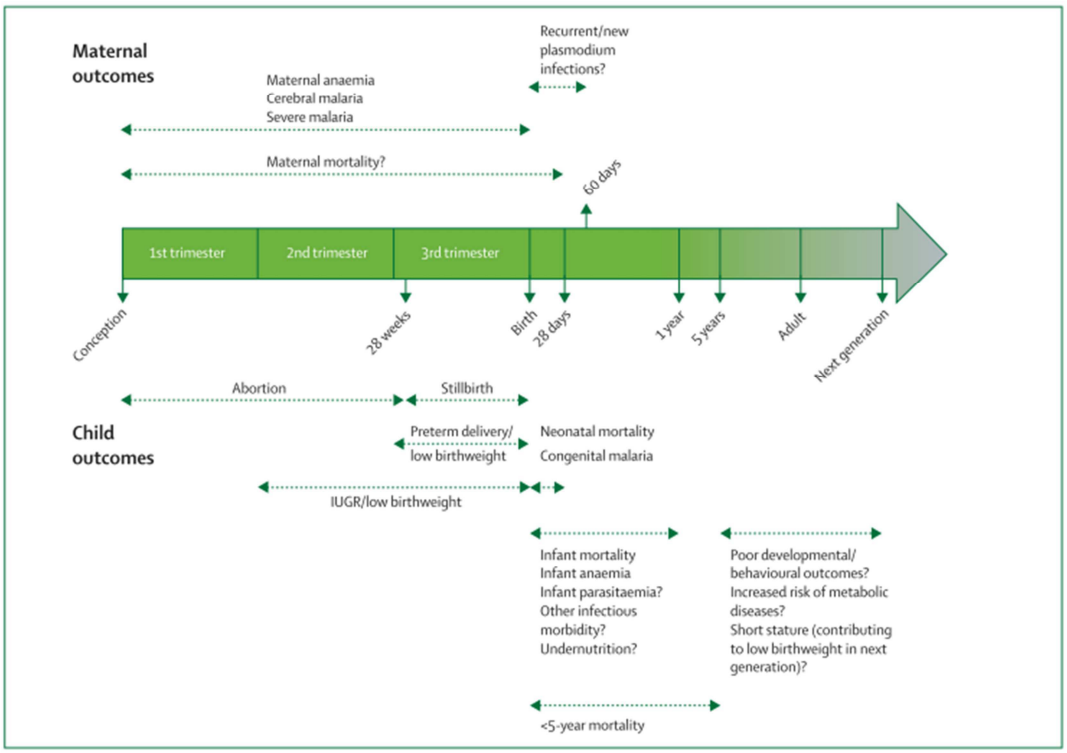


Figure 2 Effects of malaria in pregnancy through the life course. IUGR= Intrauterine growth retardation (Desai et al., 2007).

1.3. Pathophysiology of malaria in pregnancy

The clinical outcome of malaria infection involves many factors related to the parasite such as the species and strain of *Plasmodium*, parasite load, multiplication rate, cytoadherence or sequestration, but also to host factors such as state of immunity, age, genetic constitution, general health and nutritional status (Miller et al., 2002).

Malaria in pregnancy (MIP) leads to adverse effects on offspring, at least in part, through the preferential accumulation of parasites in the placental intervillous space. Placental sequestration is frequent in infections with *P.*

falciparum because asexual blood stages transfer a protein, *VAR2CSA*, to the red blood cell membrane that facilitates adherence to chondroitin-sulfate A (CSA) on syndecan-1, which is anchored in placental tissue (Pereira et al., 2016). This interaction is associated with the recruitment, retention and activation of mononuclear cells in the placenta and is thought to mediate malaria's effects on birth outcomes (Lucchi et al., 2008; Sharma & Shukla, 2017). Maternal antibodies against VAR2CSA have protective properties (Ataíde et al., 2014). Also *P vivax* can induce placental changes, but to date no studies have unambiguously documented the sequestration of *P. vivax* infected erythrocytes in the placenta (Rogerson et al., 2018).

A number of histological changes in *P. falciparum* infected placentae have been described, including the infiltration of mononuclear cells, deposition of malaria pigment, thickening of the trophoblast basement membrane, syncytial knotting and complement deposition (Rogerson et al., 2007). These histological and functional changes likely contribute to have a negative impact on fetal growth. Longitudinal Doppler data support the idea that malaria in the first half of pregnancy can lead to changes in umbilical artery blood flow and fetal growth during pregnancy, and this is influenced by both gravidity and nutritional status (Griffin et al., 2012).

1.4. Diagnosis

The diagnosis of MIP can be challenging due to placental sequestration of parasitized erythrocytes, leading to low levels of circulating parasites. Also

scarce resources (human and financial) limit accessibility to adequate diagnostic services and the adoption of advanced diagnostic techniques.

Microscopy: microscopic identification of *Plasmodium* parasites in the blood by an experienced technician remains the gold standard for malaria diagnosis (White NJ et al., 2014). Microscopy of stained blood smears is also broadly used to monitor the prevalence of malaria infections by national health services. For point-of-care testing, rapid diagnostic tests (RDTs) revealing circulating malaria antigens, like histidine-rich protein-1 (PfHRP2), are another frequently adopted tool for malaria diagnosis (Doctor M. et al., 2017; Kyabayinze et al., 2016; White NJ; et al 2014).

RDTs are easier than microscopy to carry out in low-resource settings, because they are not dependent on highly trained technicians and equipped laboratories. Therefore, RDTs might be the most suitable point of care testing method for symptomatic mothers in low-resource areas (D'Alessandro et al., 2018; Kyabayinze et al., 2016). However, the use of RDTs is not fully reliable for diagnosing MIP in women with asymptomatic infections, because RDTs need a higher circulating parasite density for detection than microscopy (Takem & Umberto, 2013). Thus, RDTs are insufficiently sensitive to detect MIP because of placental parasite sequestration. In research studies, the gold standard for MIP diagnosis is histopathology, but for evident reasons this is not applicable in mother and child health care services. (Liu et al., 2016a; Liu et al., 2016b). Using microscopy with placental blood samples, the sensitivity is estimated to be

94% (95% CI 86 - 99) (D'Alessandro et al., 2018). A new generation of ultrasensitive RDTs is under development and will be soon evaluated in pregnant women; this might mitigate the current diagnostic shortcomings (Das et al., 2017).

Polymerase chain reaction (PCR): molecular techniques, such as PCR are extremely sensitive, with quantitative PCR able to diagnose very low-density malaria infection (Britton et al., 2016). However, a specialized laboratory with trained staff is required, and assays are relatively time-consuming. Loop-mediated isothermal amplification (LAMP) has similar sensitivity to PCR but is more rapid and robust, and potentially applicable at the point of care (Boeuf et al., 2013; Umbers et al., 2011). However, both are presently reserved for research settings.

Placental histology: histological examination of placental tissue at delivery is a sensitive method for the detection of active or past malaria infection. Past malaria infection is characterized by the presence of malaria pigment, hemozoin, most commonly in fibrin deposits. Active infection is revealed by the occurrence of leucocyte infiltrates, principally monocytes, termed intervillositis, a histological alteration especially found in first-time mothers with little pregnancy-associated malaria immunity (Menendez et al., 2000).

1.5. Management of malaria in pregnancy

Current control strategies adopted in most African countries include universal access and use of insecticide-treated mosquito nets (ITNs), indoor residual spraying (IRS) and larval vector control where appropriate and, importantly, people's education to promote good practices regarding mosquito bite prevention, prophylactic malaria therapies, prompt diagnosis with the use of rapid diagnostic tests (RDT) and malaria treatment with artemisinin-combination therapies (ACTs).

1.5.1 Prevention strategies

In malaria-endemic African countries - including Cameroon - the combination of vector control (preventing exposure to mosquitoes) and chemoprevention (preventive medication-based treatment) is promoted to prevent MIP. WHO advocates an integrated approach using a three-pronged strategy for control of MIP in Africa comprising case management (prompt treatment using highly effective drugs), use of insecticide-treated nets (ITNs) and intermittent preventive treatment (IPTp), i.e. the administration of a full treatment course of an effective antimalarial at periodic antenatal visits, possibly one month apart (WHO/AFRO, 2004). Since 2004, this policy has been accepted in Cameroon and is implemented with the objective of giving at least 3 Sulfadoxine-pyrimethamine (SP) doses between the 16th and the 36th week of pregnancy (starting the treatment as early as possible). Also in Cameroon to reduce malaria during pregnancy, it is recommended to pregnant

women not only sleep under an insecticide-treated mosquito net but also to take antimalarial drugs as a preventive measure during pregnancy. Sulfadoxine-pyrimethamine (SP), commercialized as Fansidar®, is the recommended medicine for IPTp in Cameroon (MINSANTE; INS, Cameroon 2018). According to the national health survey conducted in the country, (MINSANTE ESDC-V Cameroon 2018), three quarters (75%) of women aged 15-49 who had a live birth in the 2 years preceding the survey, took at least one dose of Fansidar, 54% took at least two doses and 32% three or more doses . The percentage of pregnant women who took at least three doses of Fansidar® during their most recent pregnancy is higher in urban areas (40%) than in rural areas (25%) (MINSANTE; INS, Cameroon 2018). However, increasing levels of drug resistance across South-East Asia and Africa including Cameroon have been reported (Mbacham et al., 2010; WHO, 2013). A study in Cameroon has shown that an important proportion of pregnant women was infected and had sequestered parasites in their placenta. In this cross-sectional survey conducted in South-west region of Cameroon including 306 pregnant women, *P. falciparum* infection was detected in 5.6%, 25.5% and 60.5% of the cases in peripheral blood, placental blood and placental histological sections respectively. Placental histology was found to be more sensitive (97.4%) than placental blood film (41.5%) and peripheral blood (8.0%) microscopy (Anchang-Kimbi et al., 2009). This high infection rate was not expected, given the efforts of the health system in the country to

extensively provide SP free of charge. Therefore, there is a need for continuous up scaling and monitoring of IPTp.

Insecticide-treated nets (ITNs) and indoor residual spraying (IRS)

ITNs work by supplying a physical barrier from mosquitoes, repelling or killing mosquitoes which touch the insecticide treated netting. It provides a personal protection to the individuals under the net and at a vector population level reduces mosquito density. Insecticide treatment assures the nets' effectiveness even after the integrity of the barrier is compromised by holes (Janko et al., 2018). The use of ITNs in Africa has been shown to impact on MIP reducing LBW by 23%, miscarriages and stillbirths by 33%, and placental parasitaemia by 23% (Gamble et al., 2007). In spite of the proven efficacy of ITNs, its adoption was estimated at only 39% in Africa between 2009 and 2011 (Van Eijk et al., 2013). From that time, ITN use has steadily increased, and an estimated 61% of pregnant women at risk for malaria slept under an ITN in 2017 (*World malaria report 2018*, 2018)(Figure 3). In Cameroon overall 42% of pregnant women aged 15 - 49 year sleep under an ITN. This percentage is higher in urban areas (48%) than in rural areas (37%) (MINSANTE; INS, Cameroon 2018). Mosquito resistance to pyrethroids, the most commonly used insecticide on ITNs has been reported across sub-Saharan Africa and might impair efficacy in the near future (Janko et al., 2018).

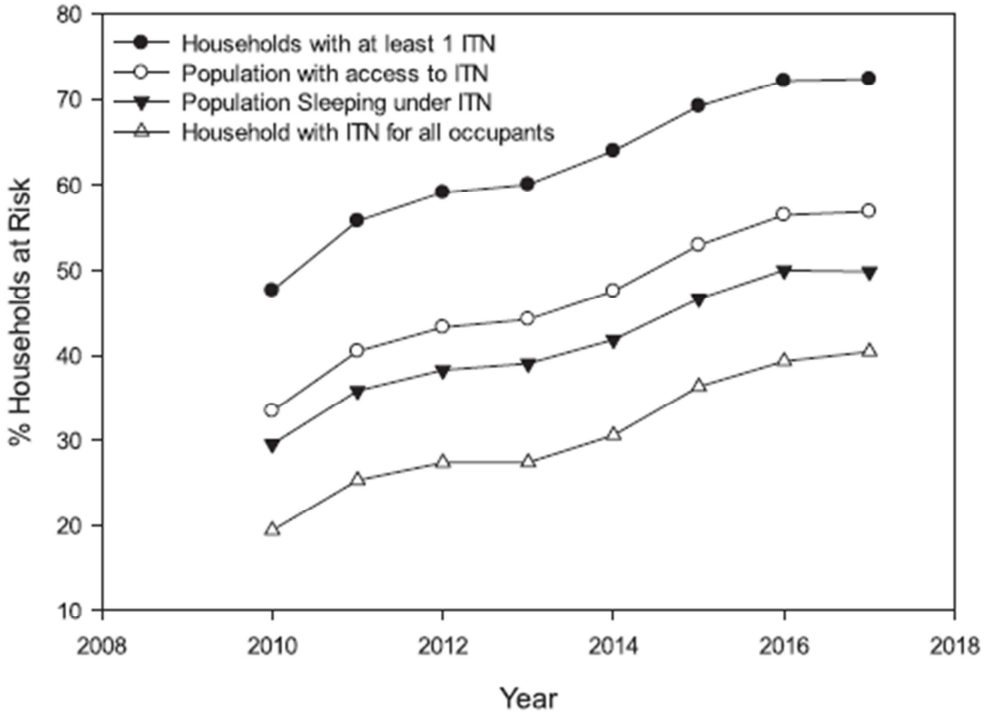


Figure 3 Uptake of ITN: percentage of people (households) in sub-Saharan Africa at risk for malaria with access to ITNs and using ITNs. ITNs: Insecticide Treat bed Nets (*World malaria report 2018*, 2018).

Globally, the implementation of IRS has declined and only 3% of the population was protected by IRS in 2017, which might be related to the need to shift from pyrethroids to more expensive chemicals (*World malaria report 2018*, 2018). IRS remains an important tool of integrated vector control and might contribute to improve birth outcomes in particular in urban settings of malaria-endemic regions.

Intermittent preventive treatment in pregnancy (IPTp)

Chemoprophylaxis historically has been successfully employed to prevent adverse health outcomes related with MIP. IPTp consists of giving

monthly doses of antimalarial medication to pregnant women initiating in the second trimester (at least 3 Sulfadoxine-pyrimethamine (SP) doses between the 16th and the 36th weeks of pregnancy). Women who take at least 2 courses of IPTp have a reduced risk for presenting the following MIP signs and symptoms of moderate to severe anemia (risk reduced by 40%), antenatal parasitaemia (by 61%), placental parasitaemia (by 55%) and for LBW (by 27%) (Radeva-Petrova et al., 2014).

Despite WHO recommendations, in 2017, only 22% of pregnant women received three or more doses of ITPp in sub-Saharan Africa (*World malaria report 2018*, 2018) (Figure 4).

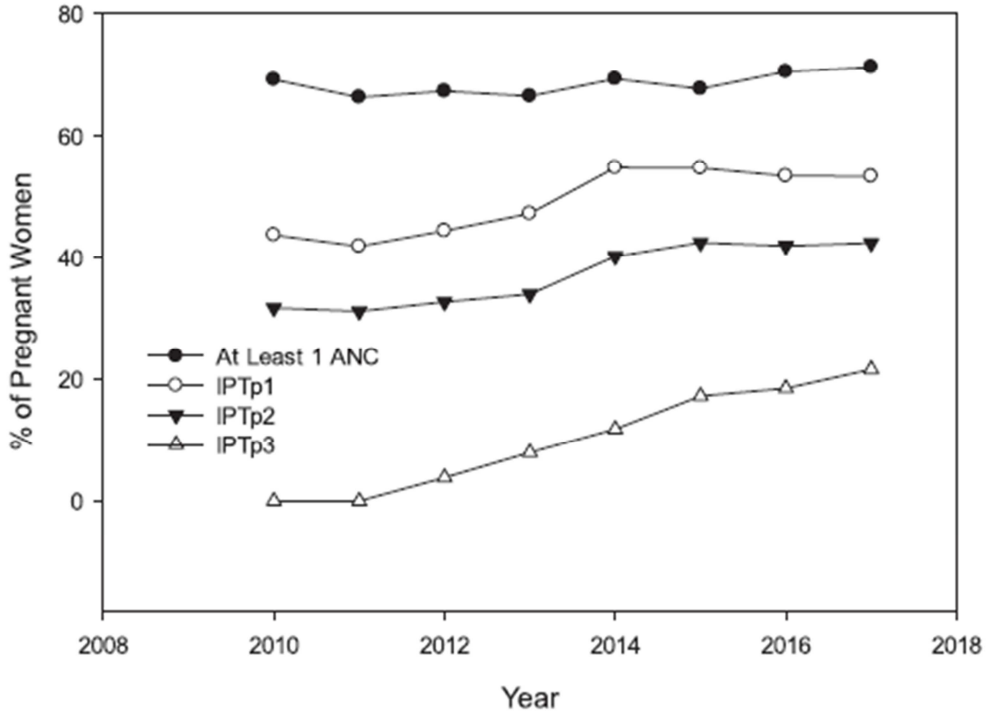


Figure 4 Uptake of IPT: percentage of pregnant women receiving IPTp, by dose in sub-Saharan Africa, 2010 - 2017. ANC: antenatal care, IPTp: intermittent preventive treatment in pregnancy.

Plasmodium resistance to SP has emerged through multiple mutations in the *P. falciparum* dihydrofolate reductase (*Pfdhr*) and dihydropteroate synthetase (*Pfdhps*) enzyme, with the diffusion of this haplotype in some areas of greater than 90%. Due to increasingly more resistant parasite populations, IPTp with SP is less effective at inhibiting parasite establishment and proliferation and preventing fetal growth restriction (Desai et al., 2018). In light of this emergency, alternative strategies for chemoprevention are being tested, including IPTp with alternative medications and strategies of intermittent screening and treatment in pregnancy (ISTp). ISTp strategies recommend RDTs to diagnosis MIP at multiple time points and treat only when RDTs are positive. However, this strategy relies on accurate diagnosis of MIP and has not been validated yet whether it is an appropriate strategy and alternative to IPTp-SP also in highly resistant areas. IPTp with dihydroartemisinin-piperaquine (DP) might be a promising alternative to IPTp-SP, but more research is needed in this area (Desai et al., 2018).

In South America and Asia, where malaria transmission is typically lower than in Africa, data on chemoprophylaxis are limited. Prophylaxis with mefloquine or chloroquine has been efficacious for preventing MIP in pregnant women in Thailand (Villegas et al., 2007). Other chemoprophylactic strategies employed outside of Africa have included monthly IPTp-SP with azithromycin and ISTp-SP and artesunate. The

strategies show promise in the reduction of low birth weight or maternal parasitaemia (Desai et al., 2018).

1.5.2 Treatment of uncomplicated malaria

All MIP infections should be treated without delay to avoid complications for the mother and fetus. To establish safety of treatment in pregnancy, the WHO recommends trimester-specific treatment strategies for uncomplicated malaria.

For first trimester treatment against *P. falciparum*, currently WHO recommends a 7 day treatment course with quinine and clindamycin. However, in many places in particular in Africa, clindamycin is unavailable, and quinine monotherapy therefore is given (WHO, April 2019). Side effects of the seven day quinine course, such as tinnitus or fullness in the ears, result in poor compliance, interruption of treatment and the risk of recrudescence.

Second line treatment includes artemisinin-based combination therapy (ACT) or oral artesunate with clindamycin (WHO, April 2019). ACTs are considered as valid alternative to quinine, but establishing their safety in the first trimester has been challenging, especially because in animal studies, the drugs showed embryo-toxicity (Steketee et al., 2001). Pharmacovigilance is particularly important in pregnancy where both the mother and the developing fetus are at risk. A meta-analysis of prospective observational studies comparing the risk of miscarriage, stillbirth, and

major congenital anomaly (primary outcomes) among first-trimester pregnancies treated with artemisinin derivatives versus quinine or no antimalarial treatment, showed interesting results. Totally 1,664 well-documented pregnancies followed prospectively after artemisinin (717) or quinine (947) treatment in the first trimester, were included. Compared to quinine treatment, ACTs were not associated with increased risk of miscarriage, stillbirth or embryo-toxicity. While the data are limited, they indicate no difference in the prevalence of major congenital anomalies between treatment groups. The benefits of 3-day artemisinin combination therapy regimens to treat malaria in early pregnancy are likely to outweigh the adverse outcomes of not fully treated malaria, which can occur with oral quinine because of the known poor adherence to 7-day regimen. ACTs were not associated with increased risk of miscarriage, stillbirth or embryo-toxicity (Dellicour et al., 2017).

First trimester treatment of uncomplicated non-falciparum malaria consists of chloroquine or quinine for chloroquine-resistant infections.

Second and third trimester treatment of uncomplicated malaria follows the same guidelines as treatment for malaria in non-pregnant adults (D'Alessandro et al., 2018). Hence, first line treatments with ACTs can be used in MIP. ACTs include a short-acting artemisinin component and a longer acting partner drug, such as SP. The potent artemisinin reduces the number of parasites rapidly and SP (or other longer acting partner drug)

acts on the remaining parasites and provides a post-treatment prophylactic effect, preventing new infections (Tarning, 2016).

1.5.3 Treatment of severe malaria

Severe malaria has historically been attributed to infections with *P. falciparum*, but more recent evidence has included also *P. vivax* as a significant contributor to severe malaria (Kovacs et al., 2015). When a pregnant woman presents with severe malaria, the priority is to save her life. For pregnant patients with severe malaria, WHO recommends the use of parental artesunate at any time in pregnancy for both *P. falciparum* and *P. vivax* infections (WHO, April 2019). WHO also recommends treatment with primaquine after delivery to achieve cure and prevent relapse by eradicating *P. vivax* sequestered in the liver (Kovacs et al., 2015).

Artemisinins are founded to be the most effective drugs for severe MIP after the first trimester. Since there is still limited evidence on their safety in women (embryotoxic and teratogenic evidence in animal studies), WHO does not recommend their use in the first trimester for uncomplicated malaria. However, their use is recommended in the first trimester in cases of severe malaria given the high risk of maternal mortality. As illustrated above, data on the use of artemisinins in the first trimester in humans reveal no associated increased risk of adverse pregnancy outcomes (Kovacs et al., 2015). However, it would be essential to have data on eventual long-term neurodevelopmental damages studying the effects of different drug

combinations in order to express a judgement on the safety of ACTs administration in the first trimester.

Quinine is an alternative to artemisinin that has been used for centuries for the treatment of malaria. It is not considered embryotoxic or teratogenic in animal studies but it is not well-tolerated in humans (Kovacs et al., 2015). Quinine can extend the cardiac QT interval and is related with tinnitus, headache, blurred vision, altered acuity, nausea, diarrhea, and rarely, massive hemolysis (WHO, 2015). These side effects reduce compliance with treatment regimens and lead to higher levels of treatment failure (Kovacs et al., 2015).

1.5.4 Vaccines

Malaria, along with tuberculosis and HIV infection, is a disease in which all components of the immune response (both cellular and humoral) are involved, yet provide only partial clinical protection, which indicates that developing an effective vaccine is a most challenging task. The fact that adults living in high transmission areas acquire partial protective immunity indicates that vaccination is feasible (Phillips et al., 2017). An efficacious vaccine against malaria would be of particular benefit for pregnant women. One vaccine, RTS,S/AS01 (RTS,S) has recently been approved by the European Medicines Agency, and randomized controlled clinical trials are ongoing in four countries including Kenya and Ghana. RTS,S is the world's first malaria vaccine that has been shown to reduce

malaria cases in young children. The vaccine acts against *P. falciparum*, the most prevalent parasite species in Africa. Rigorous clinical testing in 7 African countries has shown its potential for malaria case prevention and to save lives. Among children who received 4 doses in large-scale clinical trials, the vaccine prevented 4 out of 10 malaria cases over a 4-year period and 3 out of 10 cases of life-threatening severe disease.

Efforts are also underway to develop a vaccine targeting the VAR2CSA antigen to protect women against pregnancy-associated malaria (PAM) (Pehrson, Salanti, Theander, & Nielsen, 2017).

1.5.5 New developments in malaria pharmacological control strategies: transmission blocking drugs

The intrinsic parasite's and vector's potential of transmitting malaria at very high level and the emergence and diffusion of vectors and parasites resistant to insecticides and drugs respectively, is contributing to the difficulty of eliminating malaria and keeping up the status malaria free in areas from which the parasite appears to be eliminated. WHO's Global Technical Strategy for Malaria (GTS), endorsed by the World Health Assembly in 2015, and the Roll Back Malaria (RBM) Partnership's Action and Investment to defeat Malaria (AIM) have embraced the goal of a "world free of malaria" and have put forward ambitious targets of reducing malaria case incidence and mortality rates globally by at least 90% by 2030 (Hemingway et al., 2016). One of the main reasons for not reaching

global elimination of this disease is the complex life cycle of the parasite involving two different hosts and numerous proteomically distinct parasite stages. Moreover, new medicines are urgently needed against emerging resistant strains of the parasite and these compounds must be active against all existing resistant strains. There is also a need for cost-effective malaria chemoprophylaxis for pregnant women that can be taken without risk during the entire pregnancy. The ideal antimalarial for the treatment of malaria cases should have activity against the asexual and sexual blood stages of the parasite (and against all species) as well as against the hypnozoite where present (*P. vivax* and *P. ovale*) and should have a half live of several days (Fairhurst & Dondorp, 2016; Hemingway et al., 2016).

Blocking the transmission of the parasite with compounds active against the transmissible stages of the parasite is one of the main effective intervention strategies for malaria control and elimination (Alonso et al., 2011). The currently established malaria control strategies – treatment with an ACT, vector control with ITNs and/or IRS and IPTp with SP - are not enough to interrupt transmission. An additional tool is required to achieve a higher level of malaria control leading to zero transmission. This can be a drug/vaccine with transmission-blocking properties. Antimalarial drugs with transmission-blocking property, i.e. which affect the sexual stages of the parasite in the human host (gametocytocidal activity) or development of the parasite in the mosquito (sporontocidal activity), are valid tools to reduce transmission (Ponsa et al., 2003).

Asexual erythrocytic stages are the major focus of drug discovery projects because these parasite stages are causing the clinical symptoms of malaria in infected individuals. Such drugs allow to eliminate the parasites and provide relieve to the clinical presentation of malaria. Due to artemisinins' partial efficacy against gametocytes, subjects treated with an ACT can still transmit malaria (Sowunmi, Balogun, Gbotosho, & Happi, 2009). Gametocytes are crucial for the transmission of malaria infection from humans to mosquitoes. Preventing or reducing the development, function or survival of gametocytes in human hosts can interrupt transmission. Thus, drugs able to block transmission could be complementary when combined with other malaria transmission blocking interventions (Eziefula et al., 2012). So an additional, or more effective, gametocytocidal drug is needed if transmission is to be prevented. Currently, the approved drug that can prevent transmission of *P. falciparum* is primaquine (PQ). In 2010 WHO recommended a single dose of PQ of 0.75 mg/kg as a gametocytocidal agent (not sufficient for hypnozoite clearance in *P. vivax* infections), in combination with ACT making it the first transmission-blocking strategy to be endorsed for field application. However, this dose level has restrictions as it should not be given to pregnant women or small children and because there is the risk of hemolysis in individuals affected with glucose-6-phosphate dehydrogenase (G6PD) deficiency. However it must be said that the deficiency is characterized by different genetic variants that translate into different phenotypes (from mild to severe) that

must be taken into account when determining PQ safety in such patients. Finally, in 2012, WHO revised its recommendation to 0.25 mg/kg in a single dose, but this is still not advised for pregnant women and infants (Goncąlves & Hunziker, 2016). Therefore, identification of novel, safe and effective transmission blocking drugs is urgently needed.

Thus, drugs not only are important to cure patients, save lives and prevent malaria in individuals, but are essential public health tools to impact on the intensity of malaria transmission and thus reduce the overall burden of malaria. In line with global frameworks from WHO and the United Nations, Medicine for Malaria Venture (MMV) - a product development partnership in the field of antimalarial drug research and development – puts one of its strategic focus on bringing forward new tools to counteract resistance, reduce transmission and ultimately to achieve malaria elimination (Medicines for Malaria venture, 2017). Based on an integrated drug development model, MMV recently proposed to orient R&D efforts on two Target Product Profiles (TPP) and six different Target Candidate Profiles (TCP) (Burrows et al., 2017). Among them, TCP 5 and TCP 6, focus on transmission blocking drugs and TPP 1 defines drugs (combinations) that are effective against resistant strains, can cure clinical malaria, stop transmission and prevent relapse in a single encounter (Medicines for Malaria venture, 2017).

2. *Plasmodium life cycle: antiplasmodial targets for pharmacological control and transmission blocking strategies*

The responsible for malaria disease is the apicomplexan parasite *Plasmodium* of which *P. falciparum* represents the species causing most severe morbidity and mortality.

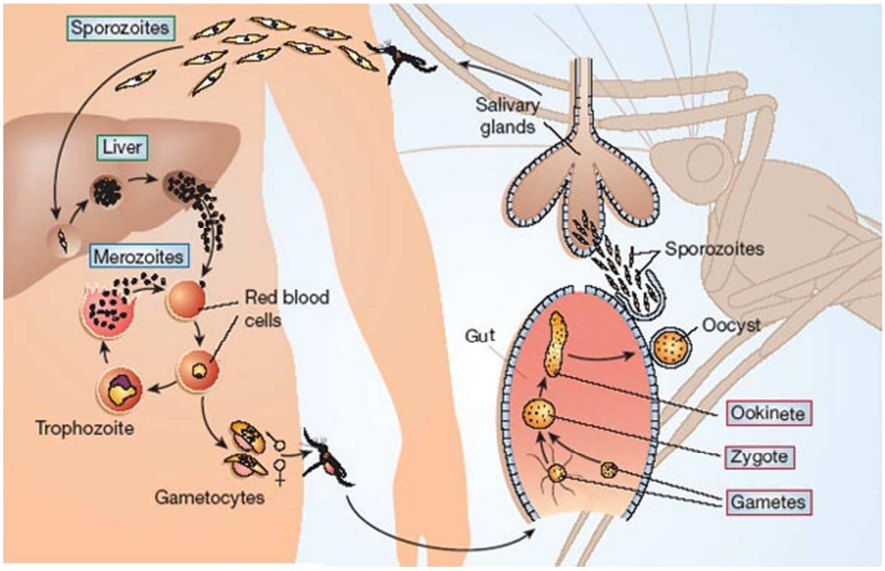


Figure 5 The Plasmodium life cycle. In the human host, *Plasmodium* reproduction occurs first in the liver (exoerythocytic schizogony) and in a second phase in red blood cells (erythrocytic schizogongy). Gametocytes are the infective parasite stages for the invertebrate vector. In the mosquito the parasite replicates through sporogony which initiates in the midgut (gamete to zygote to ookinete development), leads to the formation of oocysts that produce sporozoites, the stages infective to the human host. (Picture from: "Medicine: Knockout malaria vaccine?", (Ménard, 2005).

Plasmodium is transmitted to humans by the bite of an infected *Anopheles* mosquito that releases sporozoites into the human bloodstream (Derbyshire et al., 2011). The sporozoites (haploid parasitic forms competent of invading liver cells) then migrate to the liver, invade hepatocytes and develop to

mature schizonts giving rise to thousands of merozoites. After completion of the asexual pre-erythrocytic cycle in the liver, merozoites are released into the blood circulation and undergo schizogonic reproduction in red blood cells. The asexual erythrocytic cycle includes the following stages: invading merozoites, rings, trophozoites, schizonts and egressing merozoites (Garcia et al., 2006). In the course of ongoing asexual replication in erythrocytes, a small proportion of merozoites commits to sexual differentiation developing into dimorphic haploid forms known as gametocytes, namely micro- and macrogametocytes. (Sinden RE. et al., 2012). Gametocytes mediate transmission from the vertebrate to the invertebrate host once ingested by a blood feeding mosquito. Inside the mosquito midgut an increase in pH (7.4), decrease of temperature and the presence of xanthurenic acid, trigger the development of male and female gametes. During this process, each male gametocyte, after three rounds of mitosis, undergoes exflagellation releasing eight motile microgametes whereas each female gametocyte forms a single macrogamete. Gametogenesis and fertilization of female gametes by flagellated males to form a diploid zygote occurs within 2 -4 h after the blood meal and ookinete maturation is completed after 22-24 h. Banana-shaped, elongated and motile ookinetes pass the midgut epithelium and develop on the outer gut wall into oocysts. Mature oocysts rupture and deliver thousands of sporozoites into the hemocoel that migrate to the salivary glands from where they will be injected into human hosts during mosquito

feeding (Sinden et al., 2012). Currently, the majority of antiplasmodial drugs target asexual blood stages, responsible for the clinical symptoms of malaria.

2.1 Mechanism of merozoite invasion and intra-erythrocytic development of the parasite.

In 48 hours *Plasmodium* blood stages replicate by schizogony giving rise to at least 16 new merozoites at each round, which egress from the erythrocyte and invade other red blood cells (Gilson & Crabb, 2009). At the moment at which merozoites are freely exposed to the blood, ionic compounds present in the plasma are able to activate merozoite membrane surface proteins (MMPs) which have been shown to be essential for red blood cell invasion (Singh et al., 2010). After MMPs activation, the merozoite coming in contact with the erythrocyte surface attaches to it and turns to situate its apical pole towards the host cell forming a tight junction (Aikawa et al., 1978; Dvorak et al., 1975). The parasite enters the host cell by endocytosis involving an active process of erythrocyte-surface depression followed by the formation of a parasitophorous vacuole membrane (PVM). During the process of invasion, several proteins are involved in the complex interaction of the parasite with the erythrocyte cell surface, considered trigger factors for downstream cascades. Among the many proteins, apical membrane antigen 1 (AMA1), a protein which is conserved across apicomplexans and plays an essential role in the invasion process (Leykauf et al., 2010; Treeck et al., 2009), is considered a possible target for new antimalarial drugs or vaccines. Another

target for antimalarial drugs was highlighted in recent studies suggesting that *PfRh*/EBL proteins mediate merozoite attachment to the erythrocyte surface, committing the parasite to host cell invasion (Riglar et al., 2011). After the parasite has entered the erythrocyte by forming the parasitophorous vacuole, it develops into a trophozoite, undergoes schizogonic replication to become a mature schizont that releases multiple, invasive daughter merozoites. These asexual forms are responsible for the clinical symptoms of malaria and are currently the main targets of antimalarial drugs.

2.2 Gametocytogenesis: targeting the transmissible stage of the parasite in the vertebrate host

A small proportion (0.1%–5%) of asexual parasites develop into male and female sexual stages called gametocytes, which are the only stages transmissable to the mosquito vector and which do not directly contribute to disease pathology (Meibalan & Marti, 2017; Ngotho et al., 2019). Due to the complexity of the biological and molecular mechanism underlying gametocytogenesis, targeting gametocytes in humans is still one of the major issues of the malaria eradication agenda (The malERA Consultative Group on Drugs, 2011). The knowledge of how the sexual commitment is trigged in the human host is fundamental for the specific design of transmission blocking strategies. Reviewed studies (Day et al., 2003; Ngotho et al., 2019; Williams, 1999) reveal that *P. falciparum* gametocytogenesis can be induced in response to stress and environmental factors, such as depletion of nutrients in *P. falciparum in vitro* culture media, which is currently the

method used for *in vitro* production of gametocytes, or by cell to cell communication with the active role of extracellular vehicles (EVs) secreted by infected RBCs (Pierre-Yves Mantel, 2013). Transcriptomic studies suggest that sexual differentiation in *Plasmodium* is regulated by an apicomplexan conserved transcriptional factor named *P. falciparum* ApiAP2-G (Kafsack et al., 2014; Ngotho et al., 2019)(Figure 6). This factor is responsible (under the epigenetic regulatory activity of histone deacetylase 2 (PfHda2) and heterochromatin protein 1 (PfHP1)) for gametocyte formation or repression depending on environmental conditions (Brancucci et al., 2014). Other two members of the AP2 family, namely AP2-O and AP2-Sp, are fundamental for oocyst and sporozoite formation in the mosquito vector while AP2-L, is required for *Plasmodium* liver stage development in the mammalian host, emphasizing the important role of this family of DNA-binding proteins, that might be considered as a valid targets for drug development against *Plasmodium* and more generally, against *Apicomplexan*.

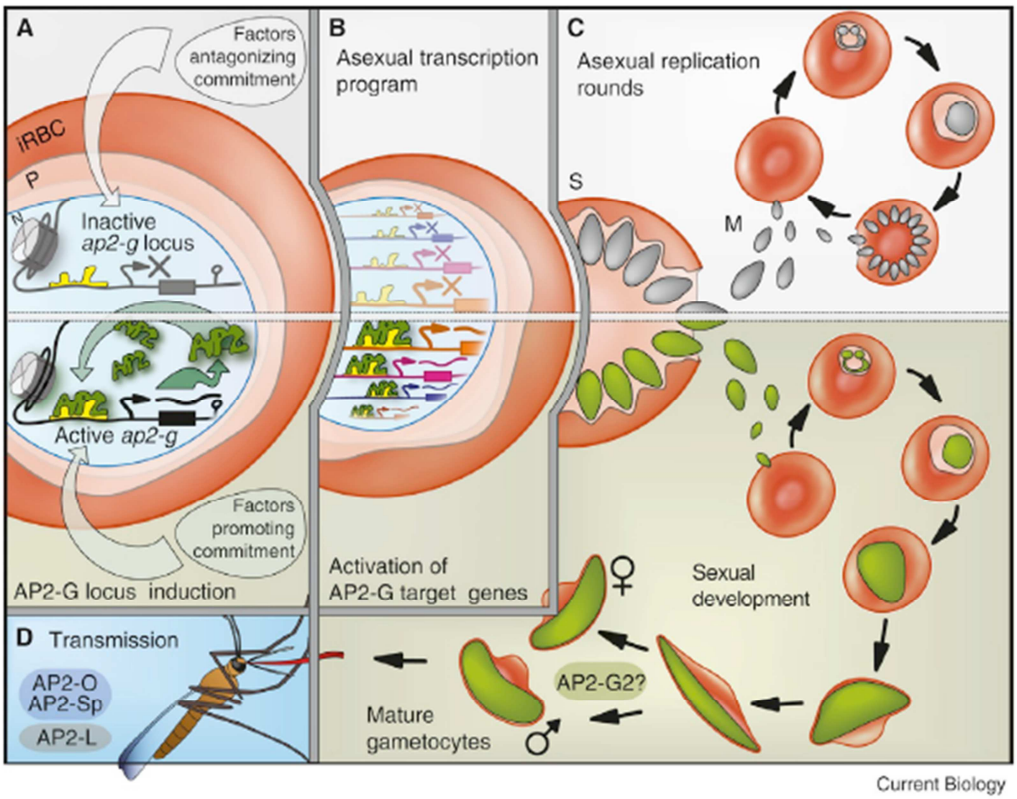


Figure 6 Schematic illustration of AP2-G-induced sexual differentiation in *P. falciparum*. The ap2-g locus is shown in the transcriptionally inactive (upper part of the panels) or in the active state (lower panels). AP2-G protein (in green) binds to upstream DNA motifs (in yellow). AP2-G controls the activity of numerous target genes that, in turn, are required for the early sexual pathway. When the protein is absent, parasites fail to induce a gametocyte-specific gene cascade. (C) Non-committed merozoites (in gray) continue asexual reproduction after schizogony and erythrocyte re-invasion. In contrast, committed progeny (in green) enter sexual development. AP2-G2 may at some stage contribute to determining the sex ratio between transmissible gametocytes. (D) Following the mosquito blood meal, zygotes are formed and continue parasite development within the insect vector. Two members of the AP2 family, AP2-O and AP2-Sp, were previously shown to be essential for oocyst and sporozoites formation, respectively. After transmission to the mammalian host, another AP2 factor, AP2-L, is required for liver stage development, emphasizing the important role of this family of DNA-binding proteins in the developmental cycle of *Plasmodium* spp., and potentially of other Apicomplexan. (iRBC, infected red blood cell; P, parasite; N, parasite nucleus, S, schizont; M, merozoites (Kafsack et al., 2014).

2.3 Early sporogonic stage development in the mosquito mid gut: targeting Plasmodium in the Anopheles vector

Among all *Plasmodium* development stages, one of the most promising targets for malaria transmission control might be the early sporogonic stages of the parasite in the mosquito midgut (Sinden, 2004)(Figure 7). The current

"gold standard" test of the ability of a compound to prevent transmission of *P. falciparum* to mosquitoes is the standard membrane feeding assay (SMFA) (Burrows et al., 2014), which provides a laboratory model for getting a better understanding of the molecular mechanisms of early sporogonic stage development (from ingested gametocytes to the formation of ookinetes), oocyst and sporozoite formation. In particular, early sporogonic development spanning from gametogenesis, fecundation, zygote formation to ookinete maturation, provides a large spectrum of targets for the development of new transmission blocking tools (Angrisano et al., 2012). Several factors influence the initial sexual processes of *Plasmodium* in the *Anopheles* mosquito mid gut after the infective blood meal: the drop in temperature to 5 °C below the vertebrate temperature, the rise in pH from 7.4 to approximately 7.5–7.6 (under *in vitro* conditions as much as pH 7.8–8.0 can be tolerated) (Billker et al., 2004) and most importantly the presence of secondary metabolites in the mosquitoes gut among which xanthurenic acid is known to trigger the molecular mechanisms by which the male gametes egress from the infected cells and fertilize the female gametes. Several enzymes have been identified and shown to be involved in the regulation of microgamete formation namely Serine/Arginine-rich (SR) protein kinase (SRPK) (Tewari et al., 2010), calcium-dependent protein kinase, CDPK4 and mitogen-activated kinase, MAP2 (Tewari et al., 2005). Once the male gametes egress from the cell, within 6 to 8 h the zygote is formed and in 24 h ookinetes are fully developed and recognizable due to the peculiar banana-shaped form.

Relevant transmission blocking targets identified among the molecular mechanisms regulating zygote to ookinete development are enzymes of the Serine/Threonine NIMA-related (never in mitosis/Aspergillus) protein kinase family, such as Nek-2 and Nek-4, which are both essential in the *Plasmodium* genome replication before meiosis (Reininger et al., 2005, 2009). Further possible drug targets are represented by proteins regulating the development of the cytoskeleton and constituting the mature ookinete pellicle formed by adhesin-substrate complexes in the apical part (containing micronemes with high protein) and the plasma membrane situated on the inner membrane complex (IMC)-anchored myosin, comprising the outer alveolar membrane and the inner alveolar membrane (Baum et al., 2008). In this process calcium/calmodulin dependent protein kinases play an essential role (Silva-Neto et al., 2002) by forming actin and myosin fibers (Siden et al., 2000) which are also responsible of its peculiar motile characteristic.

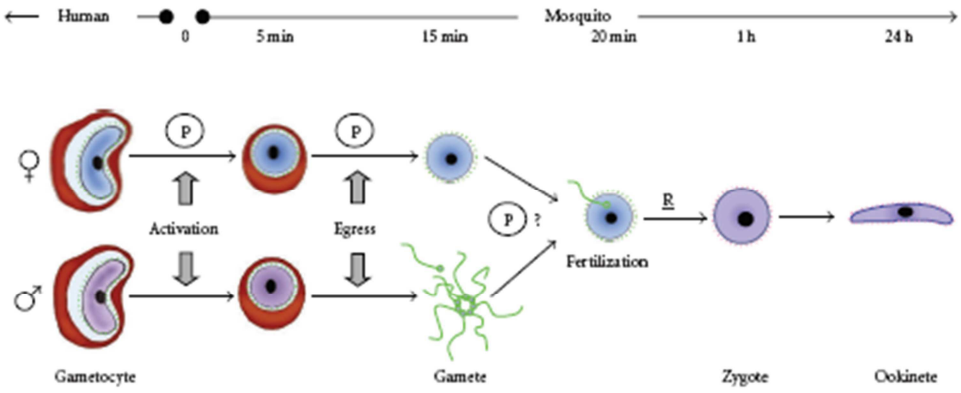


Figure 7 Schematic *Plasmodium* early sporogonic stages development (Kuehn & Pradel, 2010)

3. African and Cameroonian Traditional Medicine.

Traditional medicine (TM) is "the sum total of knowledge, skills and practices on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose or treat physical and mental illnesses" (WHO, 2002). Herbal medicine involves the use of herbal remedies. "Herbal remedies include herbs, herbal materials, herbal preparations and finished herbal products that contain as active ingredients parts of plants or other plant materials or combination thereof"(WHO, 2002). Today, about 80% of the world population relies on traditional medicine as a source of medication for primary health care. For many individuals in Africa, particularly those living in rural areas, this is the only available, accessible and affordable source of health care (CIFOR, 2005).

In Africa south of the Sahara including Cameroon and probably in many parts of the tropical world, populations use and rely on traditional medicines more than on modern medicine. This is probably because traditional medicine and traditional health care are easily accessible to the majority of the populations whether urban or rural. In addition, because traditional healers live within and are part of the community, they have a higher distribution and a lower patient-healer ratio in rural areas than modern medical practitioners. They spend more time with their patients and knowing the background information of the persons puts them in a better position to deal with the patient in a holistic approach. Modern medicine is a

comparatively foreign, “technical” culture and is only well known to the scholars and doctors who practice it (Fokunang et al., 2011).

3.1 Policy of traditional medicine.

In Cameroon, a national policy, laws and regulations, and a national program on traditional medicine, complementary and alternative medicine (TM/CAM) have been developed. The health system in Cameroon is organized on three levels, representing a form of pyramid. At the summit is the central level, followed by the intermediate level and the operational level that constitutes the base. Currently, Cameroon recognizes at all level of its health care system, a non-structured or poorly organized organogram of traditional medicine (Fokunang et al., 2011). Nevertheless the will of the government to integrate and implement traditional medicine has been clearly expressed and remains a priority.

The conference of Regional Governors organized in 1976, by the Ministry of Public Health for creating an organizational structure to valorize traditional medicine permitted the establishment of 3 organs in operation such as the national commission for TM, in charge of supporting the government in the definition and exploitation of TM, the permanent committee of TM in charge coordinating the research activities and practices of TM and the Medical Institute for the study of Medicinal Plants (IMPM) in charge of operational research.

The political engagement was validated by the ministerial decision No. 031/D/MSP/DS/BT of 31 july 1979 creating and organizing the TM sector

within the jurisdiction of the Ministry of Public Health (Fokunang et al., 2011). This decision originated from the Declaration of Alma-Ata in 1978 on primary health care, which recognizes traditional medicine and traditional practitioners as essential partners to reach the health for all goals.

The decree of 4th December 1979 constituting the General Delegation for technical and scientific research (DGRST) formerly known as National Office of Technical and Scientific Research (ONASREST)-IMPM was created in 1974. IMPM was charged with drawing up a realizable research program on TM in order to improve the health conditions of Cameroonians. The IMPM is composed by four centers among which the center for the study of medicinal plants (CEPM) elaborates and promotes research programs geared towards the development and production of drugs and adequate therapeutics using local natural substances.

The creation of an association of TM enacted by the freedom of association law *No. 90/053* of 19 December 1990 was followed by a circular note *No.: D26/NC/MSP/SG/DMPR/DAMPR/SDMR/SSCMT* of 16th September 1991 that authorizes and encourages conventional medicine practitioners to collaborate closely where possible with traditional practitioners. The decree No.93/405 of 04 August 1993 defined the reorganization of IMPM, changing the name from CEPM to CRPMT (Centre de Recherche en Plantes Médicinales et en Médecines Traditionnelles). In the late nineties arrived the prime ministerial decree *No. 98/405 PM*, instructing the homologation strategy to put in the market pharmaceutical products. This led to the

creation of the National Drug Commission, which harbors the Commission specialized in pharmacovigilance and traditional pharmacopoeia and the Commission specialized in phototherapy and alternative therapeutic techniques. The presidential decree No. 2002/209 of August 2002 organizing the Ministry of Health, put in place a sub-directory in charge of primary health care, the service of traditional social-health care (based on of the office Ethics and Social Health Care Deontology) and the Bureau of Welfare and Legislation Control. This decree also created a division of operational research made up of Scientific Network Cells in charge to support medicinal plant research (Fokunang et al., 2011). Local initiatives are in progress by different non-governmental organization (NGO's) and public institutions to train traditional practitioners in the basic conversation techniques and preparation of their herbs within the frame work of quality assurance.

3.2 Safety, efficacy and quality control

The safety and efficacy of plant based traditional medicine as well as quality control of the preparation processes, have become important issues for both health authorities and the public. Scientific evidence from studies performed to evaluate the safety and effectiveness of medicinal plants, herbal products and traditional practices is limited. While evidence is available that acupuncture, some herbal medicines and some natural therapies (e.g. massage) are effective for specific ill-health conditions, validation studies on the majority of commonly used products and practices still need to be

affected. Requirements and methods for research are complex, given the multiple factors that determine the quality of a herbal product.

The main advantage of making "improved phytomedicines" is that they can be developed much faster and more inexpensively than new modern drugs. The "improvement" lies in the pharmacologic evidence of safety and efficacy, the standardized dose and quality control. Improved traditional medicine has been categorized by M. Willcox et al., 2012 (Merlin Willcox et al., 2012) as follows:

Category 1: Traditional medicines that are prepared by a TP for an individual patient with fresh or dried raw materials, with a short shelf life.

Category 2: Traditional medicines currently used in the community that are prepared in advance and composed of crude raw plant materials.

Category 3: Standardized plant extracts prepared in advance and supported by scientific research.

Category 4: Isolated pure compound molecules from traditional medicines following scientific research.

In addition, the safety, effectiveness and quality of standardized products (phytomedicines, herbal remedies) depend on the quality of their primary materials (which can include hundreds of potentially bioactive constituents), and how the materials are handled throughout the production processes (World Health Organisation, 2008).

3.3 Role of medicinal plants in the development of antimalarial drugs

There is a strong association between medicinal plants and the development of antimalarial drugs since 1820, when quinine was isolated from *Cinchona* bark by Pelletier and Caventou (Honigsbaum & Willcox, 2004). One and half century later, Chinese scientists isolated qunghaosu now known as artemisinin from qing haosu (*Artemisia annua*) (Klayman, 1985). *Cinchona* bark and *Artemisia annua* plants were used traditionally to manage malarial manifestations. Since then, much research has been done in exploring plant-based (herbal) antimalarials, insect repellents and other vector control agents (insecticides, larvicides), used in malaria control (Willcox & Bodeker, 2000) (Mueller et al., 2000).

When dealing with traditional medicine, it is important to bear in mind that healers basically treat the symptoms of the disease, especially those that are apparent to them. As malaria can elicit a wide variety of symptoms, over 1200 plant species are involved in the treatment of this disease. The history of antimalarial drugs and currently used drugs demonstrate that plants are an important source for the development of new drugs against malaria.

Medicinal plants considered effective in the treatment of malaria are therefore those observed by healers to alleviate or prevent one or more recognized symptoms of malaria. Since malaria can occur concurrently with

other infectious diseases, accurate diagnosis is difficult be achieved and patients present with complex symptomatic manifestations. Ethno-medical believes of populations also plays a role in the choice of plants for the treatment of malaria.

Willcox and Bodeker reviewed the potential advantages of traditional plant-based medicines: "(1) they are inexpensive and easily available, particularly if people grow them themselves; (2) there is no record of resistance to whole-plant extracts, possibly due to the synergistic action of many constituents (isolation and administration of a single active agent greatly facilitates the evolution of resistance in parasites and mosquitoes); (3) it is plausible that phytotherapy produces fewer adverse effects than chemotherapy, because there are many active agents, each at a smaller dose than that required when a single agent is administered" (Willcox & Bodeker, 2000).

3.4 Standardized preventive and therapeutic herbal remedies

A review published in 2010 by Merlin Willcox mentioned that antimalarial herbal preparations are widely used by African populations, and finally a few of them have actually been developed to standardized improved remedies (M Willcox et al., 2011).

For example in Burkina Faso the standardized product SAYE, can be found in pharmacies all over the country. It is a combination remedy prepared from *C. planchonii* roots, *Cassia alata* leaves and *Phyllanthus amarus* whole plant.

The remedy was tested in *P. berghei* infected mice, and was found to suppress parasitaemia by 74.15% when given at a daily dose of 250 mg/kg for 4 days (O. Da et al., 2014). Similarly, a parasitaemia suppression of 52% was observed in a chemoprophylaxis experiment in which mice under SAYE treatment were challenged with infective mosquito bites by (Yerbanga et al., 2012). *In vitro* assessments on *P. falciparum* blood stage cultures (*3D7* chloroquine sensitive strain), however, showed week activity of the extract (IC50 = 80.11 µg/ml) (Traore et al., 2008).

In Mali, the standardized phytomedicine "Soumafoura Tiemoko Bengaly" (SBF) can be found, a product based on *Argemone mexicana L.* (*Papaveraceae*) and developed by the Department of Traditional Medicine in Mali. It has been named in honor of the traditional healer Tiemoko Bengaly who participated in the development of this product (Graz B. et al., 2010). A clinical trial of STB infusion in children showed that 35% and 72.5% of patients treated with a low (13ml/kg one dose daily for three days) and high (152 ml/kg twice daily for seven days) doses (Willcox et al., 2007), respectively, had adequate clinical response, an outcome measure defined in the study as the absence of parasitemia on day 14 irrespective of axillary temperature or axillary temperature <37.5 °C irrespective of the presence of parasitemia, without having previously met the criteria for treatment failure.

Graz and colleagues (2010) conducted a randomized controlled trial of *A. mexicana* and artesunate-amodiaquine- measuring the rate of second-line treatment need and deterioration to severe malaria. Second-line treatment

was not required for 89% of patients on *A. mexicana* versus 95% on ACT. Deterioration to severe malaria was 1.9% in both groups in children aged less than 5 years. The investigators recommended that *A. mexicana* can be used as first-aid treatment when there is no access to other antimalarials (B Graz et al., 2010).

N'Dribala, is a further standardized Burkinabe preparation based on *Cochlospermum planchonii* roots. This remedy showed therapeutic efficacy in a clinical study conducted in adults. In this study it was evidenced that 52% of the N'Dribala treated patients had no microscopic detectable parasitaemia after the end of treatment course (Traore M. et al., 2008).

Artavol® is a standardized phytomedicine approved by the government of Uganda for the prevention of malaria. It consists of *Artemisia annua*, lemongrass extract and ground kernel of avocado (Van Der Kooy & Sullivan, 2013). Artavol® was born from a research evaluating medicinal plants in form of tea to prevent malaria revealing *Artemisia annua* tea taken once a week to be safe, to be able to reduce fever cases by 80% and laboratory diagnosis confirmed malaria by 16.7%. In addition it was found to boost malaria specific immunity (Ogwang et al., 2011). Phytochemical investigation revealed that the preventive effects of *A. annua* tea are possibly due to flavonoids rather than artemisinin (Ogwang et al., 2011).

Totaquina is based on a powder of *Cinchona ledgeriana* bark and has been developed by the "Institut Malgache de Recherches Appliquees", Madagascar.

The ethanol extract of this product was active *in-vitro* against *P. falciparum* with an IC50 less than 10ug/ml. Moreover, Totaquina was found to suppress parasitaemia in *P. berghei* infected mice by 100% (Totaquina given orally at a dosage of 500mg/kg daily for 4 days). In addition, a clinical trial involving 586 patients was carried with Totaquina and quinine as control in Madagascar. Giving Totaquina to patients at a dosage of 1.2 g daily for 5 days cleared parasitemia in > 90% of patients after the 5 days treatment course, similarly to quinine controls (M Willcox, 2011). In the thirties, the period of development of this product, the Malaria commission of League of Nations, forerunner of the United Nations, recognized Totaquina to be as effective as quinine and bear the advantage of being cheaper (League of Nations: Malaria Commission of Health Organization, 1938).

In view of the above mentioned, it is important to notice that although there are already these improved traditional medicine (ITM) in different African countries, and that their development and marketing are authorized, there is no indication or contraindication on their use for women during pregnancy.

3.5 Medicinal plants used in Cameroon to manage malaria.

In Cameroon, there is a rich tradition in the use of herbal medicines for the treatment of several ailments and a large proportion of population (data not available) depends on traditional medicines for their primary health care needs (Kuete & Efferth, 2010). Traditional medicine includes the use of

medicinal preparations from plants, material of animal origin and minerals, as well as spiritual healing, traditional midwifery, hydrotherapy, massage, cupping, counter-irritation, surgery and bone-setting (WHO, 2001). Cameroon presents about 90% of the African ecosystems which includes; the Sahelian areas, humid tropical forests, coastal and mountainous eco-regions. The country is characterized by a huge diversity of flora and fauna ranking 5th of African countries following the Democratic Republic of Congo, South Africa, Madagascar and Tanzania (Jiofack et al., 2009). This rich biological biodiversity is associated with a wide diversity of ethnic groups; each contributing a unique ethno-pharmacopoeia to the national therapeutic patrimony of Cameroon which is recognized as one of the richest countries in the continent (Fokunang et al., 2011).

Ethno-botanical and ethnopharmacological studies conducted in different areas of Cameroon document a wide variety of plants used for malaria treatment. Scientific reviews on the antimalarial potential of medicinal plants used to treat malaria in Cameroon reported 217 species (Table 1) (Kuete & Efferth, 2010; VIncent P.K. Titanji et al., 2008). Some of these plants (about 1/3) have been investigated to assess their *in vitro* activity against *P. falciparum* and more than 100 bioactive compounds have been isolated (Kuete & Efferth, 2010; Titanji et al., 2008).

As it is the case for other ailments, single or combinations of plants are utilized to make traditional antimalarial preparations. With the aim to develop improved traditional medicines from herbs, many studies have been

done to demonstrate the efficacy of such preparations using *in-vitro* and *in-vivo* approaches for validating antimalarial properties of single plants and combinations. In Cameroon, the polyherbal product "Nefang" composed of *Mangifera indica* (bark and leaf), *Psidium guajava*, *Carica papaya*, *Cymbopongon citratus*, *Citrus sinensis*, and *Ocimum gratissimum* (leaves) has been deeply investigated. The *in-vitro* EC_{50} of Nefang determined against the *P. falciparum* CQ-sensitive *3D7* strain and the multidrug resistant *Dd2* strain was found to be 96.96 and 55.08 µg/ml, respectively (Arrey Tarkang et al., 2014). No significant cytotoxicity of Nefang and each of its constituent plants was recorded in tests conducted with two human cell lines, Hep G2 and U2OS (Tarkang et al., 2014). At the highest tested (*in-vivo*) dose of 600 mg/kg against *P. berghei* and *P. chabaudi* parasite strains, Nefang exhibited a parasitaemia suppression of > 80% employing the standard Peter's 4-day test. The prophylactic activity was found to be 79.5%, slightly lower than that exhibited by chloroquine (86.9%) and similar to pyrimethamine (78.4%). Furthermore, using the Rane's test, the schizontocidal activity of Nefang on established infection was evaluated. There was a dose-dependent chemo-suppression exhibited by Nefang at 600 mg/kg body weight compared to that of chloroquine (10 mg/kg) and artesunate (5 mg/kg), both used as positive controls. Nefang aqueous extract at 600 mg/kg dosage also demonstrated a protective effect when considering as measure of outcome animal survival. Given the above illustrated evidences regarding efficacy and safety of the Nefang, the polyherbal aqueous extract

represents a promising remedy for further standardization and eventual country based commercializing (Tarkang et al., 2014).

Table 1 List of Cameroonian medicinal plants reported to be used in traditional medicine to treat malaria (Vincent P.K. Titanji et al., 2008)**.**

No	Family	Scientific name	Local/common name	Parts used
1	Acanthaceae	*Eremomastax speciosa* *Justicia insularis* *Justicia flava* *Thomandersia hensii**	Pèkidjum (Bandjoun) Oyem ze (Ewondo) Ngoka (Baka)	Leaves Leaves Leaves Leaves, stem bark
2	Annonaceae	*Annona murica** *Cleitopholis patens* *Enantia clorantha** *Hexalobus crispiflorus** *Monodora myristica* *Pachypodanthium confine** *Uvaria chamae* *Uvariodendron spp.* *Xylopia parviflora** *Xylopia phloidora** *Xymolox monosperma**	Feb (bulu) Poivre (french)	Leaves Stem bark Stem bark Leaf, seed Seed Leaf Leaf Leaf Seed Seed Leaf, stem bark
3	Anarcardiaceae	*Amaranthus viridus* *Mangifera caesia* *Mangifera indica**	Mango	Leaf, stem bark Leaf, stem bark Leaf, stem bark

		Spondias monbin slutea *Tricoscypha ferruginea*	Amvut (Beti)	Leaf Leaf, steam bark
4	Apocynaceae	*Alstonia boonei** *Alstonia congensis* *Catharathus roseus* *Holarrhea floribunda** *Picralima nitida** *Rauvolfia macrophylla* *Rauvolfia obscura* *Rauvolfia vomitoria** *Tabernaemontanan crassa* *Tabernaemontana penduliflora* *Voacanga africana*	Ekuk (Ewondo) Bokuka (Douala) Ba'ab (Bakweri) Etoe (Ewondo) Obenton (bulu)	Stem bark Leaf, stem bark Roots Stem bark Root, stem bark, fruit rind, seed and leaf Stem bark Stem bark Steam bark Stem bark Roots Leaf, stem bark
5	Asteraceae	*Acanthospermum hispidum* *Ageratum conyzoides** *Aspilia africana* *Bidens bipinata* *Bidens pilosa** *Conyza sumatrensis* *Emilia coccinea*	King grass (English) Black jack (English) Alo mvu (ewondo)	Leaf Whole plant Leaf, tops and stem bark Stem bark Leaf Soft aerial part Leaf

		Lagera alata *Microglossa angolensis* *Microglossa pyrifolia* *Spilanthes acmella/oleracea* *Tithonia diversifolia* *Triplotaxis stellulifera* *Vernonia amygdalina* *Vernonia conferta* *Vernonia guineensis*	Ondondon si (ewondo) Fleur jalousie (French) Ndole (yemba, batcham) Abayak (ewondo) Ginseng	Leaf Whole plant Aerial part Leaf, flowers Leaf , flowers Aerial part Leaf Fruit Rhizome
6	Arecaceae	*Cocos nucifera*	Coconut	Leaf
7	Basellaceae	*Basella alba*		Leaf
8	Bignoniaceae	*Markhamia gellatiana* *Markhamia sessilisSpathodea campanulata**		Leaf Leaf Leaf, stem bark
9	Bixaceae	*Bixa arellana*		Leaf
10	Bombacaceae	*Bombax flammeum*		Stem
11	Boraginaceae	*Chretia cymosa*		Leaf
12	Cannaceae	*Canna indica*		Stem, leaf
13	Capparidaceae	*Buchholzia coriacea* *Cadaba farinose* *Cataeva adansonii* *Cleone rutidosperma**		Leaf, seeds Leaf Stem Leaf
14	Caricaceae	*Carica papaya** *Polycarpa glabrifolia*	Paw paw or papaya	Leaf, pulp, rind, seed Leaf

15	Cleomaceae	*Cleome ciliate*		Stem bark
16	Clusiaceae	*Allanblackia monticola**		Stem bark
		Mammea africana	Abodzok (ewondo)	Stem bark
17	Combretaceae	*Anogeius leicarpus*		Leaf
		Combretum glutinosum		Leaf
		Combretum latialatum		Leaf
		Combretum micranthum		Leaf
		Combretum platysterum		Leaf
		Combretum spinesis	Banga school (English)	Leaf
		Guiera senegalensis		Leaf
		Terminalia ivorensis		Leaf
		Terminalia macroptera		Leaf
		*Terminalia superba**		Leaf
18	Commeliaceae	*Commelina benghalensis*		Leaf
		Palisota hirsutePollia condensata		Leaf Leaf
19	Curcubitaceae	*Momordica charantia**		Whole plant
		Momordica condensata		Leaf
20	Euphorbiaceae	*Alchornea cordifolia**	Aboué (Ewondo)	Young shoots, leaf
		*Alchormaanes difformis**		Leaf
		*Antidesma lacinitum**		Leaf
		Bridelia micrantha		Leaf
		*Euphorbia hirta**		Whole plant

		*Euphorbia poinsonni**		Leaf, stem bark
		*Mallotus oppositofolius**	Ricin (French)	Leaf
		Manihot esculenta		Leaf
		Manniophyton fulvum		Leaf
		*Neoboutonia velutina**		Leaf stem bark
		*Phyllantus muellerianus**		Leaf, stem bark
		Ricinus cumunis		Leaf, seeds
21	Fabaceae	*Cajanus cajan**		Roots, leaf
22	Hypericaceae	*Harungana madagascariensis**		Stem bark
		Psorosperuns febrifugum		Leaf
23	Labieae	*Lantana camara*		Leaf
		Menthe sylvestris		Leaf
24	Loganiaceae (?)	*Strychnos icaja**		Root
25	Lamiaceae (Labiatae)	*Hoslundia opposite*	Masepu or cotimanjo	Leaf, root bark
		*Ocimum gratissimum**		Leaf
26	Lecythidaceae	*Napoleona vagelli*		Leaf
27	Leguminosae-Casesalppinoideae (Caesalpinaceae)	*Albisia zigia**	Simgang (Bassa)	Leaf
		Cassia alata		Stem bark, leaf and root
		Cassia hirsute	Ngom (Ewondo)	Leaf
		Cassia occidentalis	Lem (Bafang)	Root
		Disthmonanthus benthamianus	Bubinga (Ewondo)	Leaf
		Guibourtia tessmannii		Stem bark, leaf
		Senna hirsute		Leaf

		Senna occidentalis		Leaf
28	Leguminoceae-papilionoideae	*Millettia griffoniana* *Milletia laurentii* *Melletia sanagana* *Pennisetum purpureum* *Pterocarpus soyauxii*		Leaf, stem bark Roots, leaf Roots Leaf Stem bark
29	Liliaceae	*Allium sativum**		Whole plant
30	Loganiaceae	*Anthocleista schweinfurthii* *Anthocleista vogellii*		Leaf Stem bark
31	Malvaceae	*Gossipium spp.** *Hibiscus asper* *Hibiscus tiliaceus* *Sida acusa* *Sida rhombifolia* *Sida urens*	Cotton	Cottonseed Leaf Leaf Leaf Leaf Leaf
32	Meliaceae	*Azadirachta indica** *Cedrela odorata* *Enthadrophragma angolense** *Hydrangea sp.* *Khaya gradifoliola** *Khaya senegalensis** *Trichilia emetia*	Nemier (French) Mokwe (Baweri)	Leaves Leaves Stem bark, leaf Leaves Leaves Seed Leaves

		Trichilia gilleti *Turreanthus africanus**		Leaves Seed
33	Meliantaceae	*Bersama engleriana**		Leaf
34	Menispermaceae	*Peniantus longifolius** *Trichilisia gilletii*		Stem bark Leaf
35	Mimosaceae	*Cylicodiscus gabunensis** *Terapleura tetraptera*		Leaf, stem bark Leaf
36	Monimiaceae	*Glossocalys brevipes**		Leaf
37	Moraceae	*Ficus exasperate** *Ficus thonningii** *Milicia excels* *Musa paradisiaca** *Musa sapientum*	Keghawous (Oku) Abang (Ewondo) Plantain Odzoe (Ewondo)	Leaf Leaf Leaf Leaf Leaf
38	Myrtaceae	*Eucalyptus globulus* *Eucalyptus grandis* *Eucalyptus robusta** *Psidium guajava** *Pycnanthus angolensis**	Ntsedock (Bafang) Eucalyptus (French) Guava tree; Afele	Leaf Leaf Leaf, stem bark and fruit Leaf, stem bark Leaf, stem bark
39	Nympheaceae	*Numphea lotus*		Leaf
40	Ochnaceae	*Lophira alata*		Leaf
41	Passifloraceae	*Passiflora foetida*		Leaf
42	Pentadiplandraceae	*Pentadiplandra brazzeana**	Liane blanche (French)	Leaf, stem bark
43	Piperaceae	*Piper nigrum** *Piper unbellatum** *Peperomia pellucida*		Seed Leaf Leaf

		*Peperomia vulcanica**		Leaf
44	Poaceae (Gramineae	*Cymbopongon citratus**	Fever grass or fibergrass	Leaf
45	Polygonaceae	*Rumex Abyssinia* *Rumex abyssinicus*		Leaf Leaf
46	Rubiaceae	*Cinchona calisaya* *Cinchona ledgeriana* *Cinchona succirubra* *Coffea arabica* *Crossopteryx febrifuga* *Metacarpus scaber* *Morinda confuse* *Morinda lucida** *Mytragina ciliate* *Mytragina stipulosa* *Schumanniophyton magnificum**		Leaf Leaf Leaf Leaf Leaf Leaf Leaf, stem bark and root bark Leaf, stem bark and root bark Leaf Leaf Stem bark
47	Rutaceae	*Araliopsis tabuensis** *Citrus limon* *Citrus sinensis* *Fagara macrophylla* *Zanthoxylum leprieurii* *Zanthoxsylum lemarei*	Citronier (French)	Stem bark Roots, leaves Roots, stem bark Fruit Leaf, stem bark -

48	Sapindaceae	*Dedonaea viscosa* *Lecaniodiscus cupanoides*		Leaf Root, leaf
49	Scrophulariaceae	*Scoparia dulcis**		Whole plant
50	Simaroubaceae	*Brucea antidysanterica* *Harrrisonia abyssinica* *Odyendyea gabonensis** *Quassia africana*	 Ozhéng (Ebolowa)	Roots, leaf Roots, leaf Leaf, stem bark Roots, leaf
51	Ulmaceae	*Celtis cf. tessmannii* *Trama guineensis*		Leaf Leaf
52	Verbenaceae	*Clerodendron scandens* *Stachytaphera cayenensis**		Leaf Leaf
53	Vitaceae	*Cissus quadrangularis*		Leaf
54	Zingiberaceae	*Aframomum citratum** *Aframomum melegueta** *Aframomum latifolium** *Aframomum sceptrum** *Aframomum zambesiacum** *Costus dubius* *Reneimia cincinnata** *Zingiber officinale**	Alligator pepper Ndong (Ewondo)	Fruit Fruit Fruit Fruit Fruit Fruit Fruit Leaf

Note: * medicinal plants already screened for their antimalarial activity.

4. Rationale

Malaria remains the leading cause of morbidity in Cameroon, and is among the top five causes of mortality. In Cameroon, which is among the most affected countries, 71% of the population lives in high-transmission areas, with pregnant women and children under 5 years being the most affected groups (The Global Fund, 2016). MIP is a major cause of stillbirths and LBW. The use of SP for IPT in Cameroon was adopted in 2004, and at least 3 SP doses are recommended to pregnant women to be taken between the 16th and the 36th week of pregnancy (MINSANTE; INS, Cameroon, 2018). However, it is important to emphasize that progress in reducing malaria case incidence has historically been volatile in most African countries. Effective elimination/eradication strategies have been elusive, primarily owing to the complex life cycle of *Plasmodium* and the emergence of drug-resistant *P. falciparum* strains (E.A. Ashley et al., 2015; Lu Feng et al., 2017). Against this background and in the absence of an effective vaccine, there is an urgent need to discover new, potent, safe, and affordable drugs or to develop standardized herbal remedies or phytomedicines capable to reliably prevent and cure malaria in all patients including the high risk groups of children and pregnant women and that are also capable to reduce transmission intensity.

Traditional medicine (complementary and alternative medicine) is commonly used by various nations globally (Tilburt & Kaptchuk, 2008). The rich ethnopharmacological history of traditional knowledge and usage associated with medicinal plants represents a huge collection of bioactive

substances as gifts of nature to mankind. The approach of retrieval of information from folk use of plants has often shown to yield interesting knowledge, e.g. on new antimalarial compounds structure, on chemical classes more frequently harboring active compounds and on possible mode and mechanisms of actions. Such information is essential for guiding medicinal chemistry studies aimed at the development of new drugs but also for the design of improved antimalarial phytomedicines (Boyom et al., 2011; Cragg et al., 1997; Tsabang et al., 2012).

As illustrated above, in the current national health policy of Cameroon the implementation and organization of traditional medicine practice is considered a priority of the government, with the objective to encourage the development of standardized herbal medicines (with affordable prices) as a means of guaranteeing access to treatment to all. As recommended by Fokunang et al., among the strategies to translate the policy into action, attention should be focused on: making an inventory of the various medicinal plants and herbs which are used to treat common diseases (including malaria); coordinate, encourage and support scientific research into traditional medicine therapies (Fokunang et al., 2011).

Nowadays, there are many research institutes in the tropics - in Cameroon there is LABOTHERA, KAMSU-KOM and AFRICAPHARM - which are dedicated to the development of improved and standardized herbal remedies and their local commercialization after government approval, in a vision of

using them in an integrated approach together with conventional, modern drugs (Fokunang et al., 2011).

Indeed, some well-known examples of the seminal contribution of ethno-medicine to the treatment of malaria by modern medicine methods are quinine and artemisinin, isolated from *Cinchona* tree and *Artemisia annua*, respectively (Musoke & Ghee, 2016). This testifies the enormous potential of medicinal plants as a source for the development of new drugs or standardized herbal remedies that target multiple stages of *Plasmodium*.

As mentioned above, traditional knowledge is source for both, standardized remedies and antimalarial drugs to be used for the management of malaria and targeting primarily the asexual blood stages of the parasites. However, in order to achieve the set malaria control goals comprising malaria elimination, identification of novel compounds capable of interrupting parasite transmission from the human to the mosquito host, needs to be recognized as a target of high priority (Delves et al., 2012).

The use of medicinal plants by women during pregnancy to treat themselves is a common and well established practice in African countries including Cameroon. However, ethnobotanical studies have always marginalized women especially pregnant women, although their particular condition during pregnancy should be given special attention. In Cameroon, only two studies reported have being focused on women health. The first one involving 24 TPs and 179 women identifies medicinal plants used for the management of pregnant women's health conditions in general. Eighty-eight medicinal

plant species and 24 health conditions (malaria not included) were recorded. Among the 88 medicinal plants, ten, namely *Aloe buttneri*, *Cymbopogon citratus*, *Crassocephalum bauchieuse*, *Sida veronicifolia*, *Nelsonia canescens*, *Hibiscus noldea*, *Aframomum letestuanum*, *Crassocephalum bauchieuse*, *Ipomoea tenuirostris*, *Commelina benghalensis* and *Ageratum conyzoïdes* presented high frequencies of citation (Yemele et al., 2015). The other study is focused on herbal remedies used for treating reproductive health care problems. This study involved 74 respondents including 40 TPs. The results showed that a total of 70 plant species are used in the treatment of 27 reproductive ailments, with the highest number of species (37) being used against venereal diseases, followed by female (29) and male infertility (21), respectively. *Acanthus montanus*, *Dyschoriste perrottettii*, *Eremomastax speciosa*, *Crinum jagus*, *Ageratum conyzoides*, *Laggera alata*, *Vernonia ambigua*, *Spathodea campanulata*, *Senna alata*, and *Cissus quadrangularis* were among the most frequently utilized species (Tsobou et al., 2016). In Mali a study conducted by Nordeng et al. identified 48 medicinal plants used to treat fever of malaria in pregnant women (Nordeng et al., 2013) and this information was subsequently confirmed by Nergard Sogn et al (Nergard et al., 2015). In the Nordeng study 9 medicinal plants, namely *Combretum micranthum*, *Trichilia emetica*, *Lippia chevalieri*, *Vepris heterophylla*, *Parkia biglobosa*, *Combretum glutinosum*, *Opilia amentacea*, *Sarcocephalus latifolius*, and *Mitragyna inermis*) have emerged as the most commonly used ones.

To the best of our knowledge not any study has addressed up to present the plants used in Cameroon to manage malaria in women during pregnancy. We here aim to finally close this gap.

In the current Ph.D. thesis research, we conducted an ethnobotanical study in the Menoua division West Cameroon, in order to identify medicinal plants commonly used by TPs to prevent and treat malaria in pregnancy; then they were evaluated for their *in vitro* activity against asexual blood-stages, gametocytes and sporogonic stages of *Plasmodium* parasites to validate their antimalarial activity and in an attempt to design and develop an improved and standardized herbal remedy also for women in pregnancy, targeting multiple stages of the *Plasmodium* life cycle.

5. Objective of the thesis

The overall objective of the present Ph.D. thesis is to identify medicinal plant species mostly used by TPs in West Region of Cameroon for the prevention and treatment of malaria in women during pregnancy, and characterize the antiplasmodial properties and safety of the identified plants in a vision to contribute to the standardization of preventive, curative and transmission blocking herbal remedies for pregnant women.

5.1. *Specific objectives*

- To identify plants used by women during pregnancy for prevention and treatment of malaria.

- To assess the way of preparation and use of the identified plants and explore their perceived beneficial and adverse effects.

- To characterize *in vitro* the inhibitory effects of water and methanol plant extracts on asexual blood stages and stage V gametocytes of *P. falciparum* parasite strains.

- To characterize the inhibitory effects of water and methanol plant extracts on early sporogonic development (gamete and zygote formation and ookinete maturation) of the parasite using the *P.*

berghei CTRPp.GFP strain in the microgamete exflagellation and Ookinete Development Assay (ODA).

- To evaluate the *in vivo* transmission-blocking properties of selected (most active *in vitro*) plant extracts, by measuring oocyst numbers in *Anopheles stephensi* after having fed on *P. berghei GFPcon* infected gametocytemic *BALB/c* mice treated with the plant extracts.

- To assess the effects of the most active plant extracts on *P. falciparum* field isolates, performing the Direct Membrane Feeding Assay (DMFA) with gametocytemic blood from children living in an endemic area.

- To assess the cytotoxic activity of plant extracts on normal cell lines *NHF-A12*-human dermal fibroblast and *EA.hy926*-endothelial cells), an insect cell line (*Sf*9 cells) and tumor (*MDA-MB231*, *A375*) cells lines.

- To design syrup-based standardized herbal remedy formulations with the most active plants.

6. *Materials and methods*

6.1. Ethnobotanical study

The study is a descriptive interview-study and has been performed in the Menoua division, West Cameroon. Interviews have been conducted with key informants, namely traditional practitioners (TPs) and groups of women from local women associations. The study was performed in the period lasting from October 2017 to January 2018.

Study area:

Cameroon is a central African country comprising 10 regions which are subdivided into 58 divisions, among which there is Menoua division. Menoua is one of the 8 divisions of the West Region in Cameroon and its main city is Dschang. It covers an area of 1380 km^2. The division spreads from Santchou (altitude 600 m), Dschang (altitude 1400 m) to the Nkong-Ni (Djuttitsa) plateau at an altitude of 1965 m. The climate is tropical with an average annual temperature of 20.4°C (14.8 to 27.1) and average rainfall of 1936 mm each year. The area is characterized by a dry season lasting from mid-November to mid-March and a rainy season which extends from mid-March to mid-November. Menoua division is administratively divided into 22 villages (Figure 8). The health care delivery system in Cameroon has the objective to render Primary Health Care (PHC) accessible to the entire population through decentralization of the health management services to the health district level. Health policies and strategies are elaborated at the

central level and implemented at the district level by the District Health Service (DHS).

The health system comprises three organizational levels, representing the form of a pyramid: At the summit there is the central level represented by the Minister of Public Health and the three general and reference hospitals, a university teaching hospital and four assimilated central hospitals. At the intermediate level figure Regional Health Delegations and Regional Hospitals one for each of the 10 regions of the country. These serve as technical support to the health districts.

The operational level at the base consists of 173 Health District Services. They are responsible for the implementation of the national programs. This level includes care structures represented by district hospitals, district medical centers and integrated health centers. Moreover, currently Cameroon has incorporated at all levels, a non-structured or poorly organized organogram of traditional medicine.

The Health District is a geographic area that covers a population between 30 000 to 400 000 inhabitants. It is divided into health areas covering 5000 to 30000 inhabitants. In each health area, the Integrated Health Centre (IHC) is in charge of providing the Minimum Package of Activities (MPA) (package of health care activities provided by health centers at the lowest level of the health system). The prevention of diseases such as malaria in pregnant women and the availability of essential drugs for the prevention and cure of malaria in pregnant women are part of this package.

The Dschang Health District is one of the largest districts in the west region of Cameroon, covering a surface of about 1060 km2. It comprises 22 health areas, and 54 health facilities. It counts 182 persons in his staff including 124 health personnel (medical doctors, nurses and assistant nurses) and others with different disciplinary background, including sociologists, psychologists, anthropologists, statisticians and economists.

The landscape of the district is characterized by hills and slopes, with a poor road infrastructure that renders geographical accessibility difficult. This situation is worse during rainy season characterized by heavy down-pours that make the roads muddy, slippery and sometimes unusable. The poor conditions of health facilities and proximity to the forest are favoring the use of herbal medicines by people in these communities.

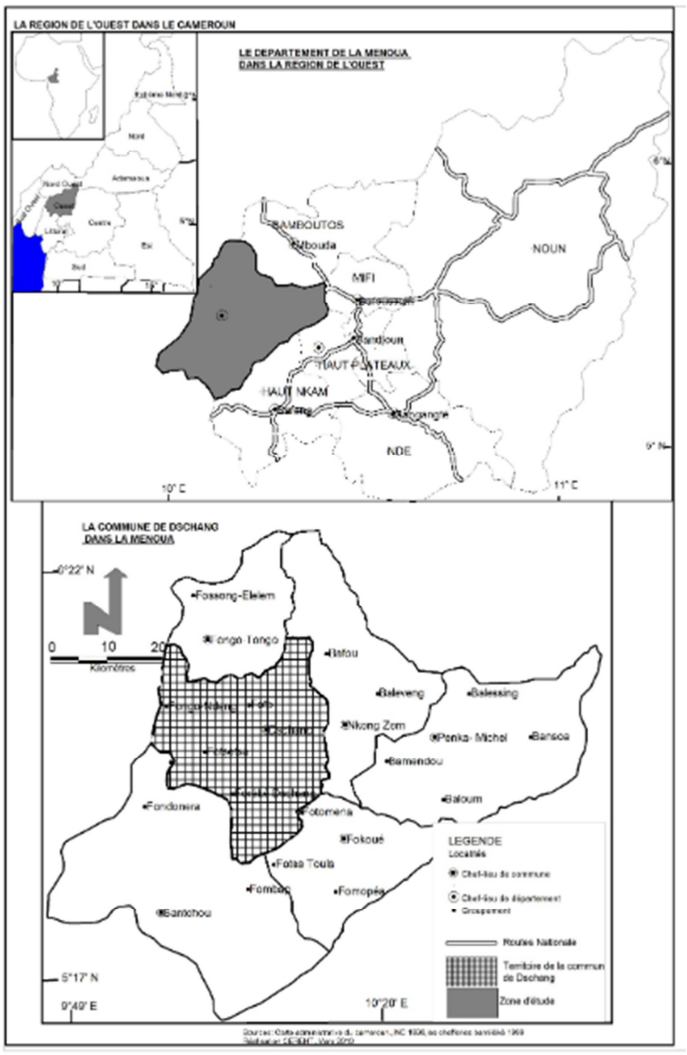


Figure 8 *Map of Cameroon showing the study division Menoua, situated in West Cameroon*.

The total population of the Dschang health district was estimated at 218 006 habitants in 2012. This population belongs to the Bamileké ethnic group and their main activity is agriculture, small trade and animal husbandry. The study area includes 6 communities (villages) namely Bafou, Baleveng, Fongo-tongo, Santchou, Foto and Foreke. The latter two constitute Dschang town and surroundings. These 6 villages were selected due to the geographic proximity to Dschang, and a well-established collaboration of traditional

practitioners with the Dschang Health District (DHD). In addition, at the level of Dschang, there is an association of traditional healer named REPLAMET, pursuing the objective to valorize traditional medicine and promote research collaborations of the study of medicinal plants.

Ethical clearance of the ethnobotanical investigations

This study was approved by the National Ethics Committee of Cameroon in Yaoundé (No. 0152/CRERSHC/2018) (Annex 1). Subsequently, the study was authorized by the chief in charge of the Health District of Dschang and by the director of the Dschang Health Hospital. All participants in this study were provided with information on the nature of the study, benefits and risks involved. Those who agreed to participate gave oral or signed a written consent at the beginning of the interview.

Data collection of ethnobotanical information

Interviewers

Interviews were conducted by Alain Tenoh (principal investigator, pharmacist and Ph.D. candidate), Armand Tsiotsa Tsapi (Master in Public health and Ph.D. candidate at the University of Dschang/Rome la Sapienza), and Kevin Edingue, Franck Nguetsa , Prudence Babia and Mispa Kwasenwi (all are Master student in Public health and epidemiology at University of Dschang). Especially the interviews with TPs were conducted by A.T and A.T.T. The questionnaire was read, discussed and approved by the

interviewers. Interviews were performed in French or local language, since all interviewers were able to speak and understand both languages. Among interviewers, there were 3 men and 2 women. The discussion groups with women were conducted by both male and female interviewers.

Interviews with TPs:

All traditional healers in the 6 communities of the study area were potentially eligible for the study. The first contact with TPs was taken by the president of the traditional practitioner association (REPLAMET), who informed them on the study. Subsequently, the responsible of the study (Alain Tenoh, Principal investigator) was given the opportunity to explain in detail the study objectives to TPs during a monthly upgrading course organized for traditional practitioners in the Dschang Health District. Out of the 18 course participants, 11 accepted to get involved in the study. With each TP having accepted, an appointment was taken at his home to administer the questionnaire. Before initiating the interview consensus was obtained that the data collected will be used for a doctoral thesis and published in scientific journals (Annex 2).

The interview addressed questions about the plants used for the prevention and cure of malaria in pregnant women, name of the plant in local langue and French, parts of the plant employed, mode of preparation and administration (dosage), duration of treatment and administration in which period of pregnancy. The questionnaire was based on open questions, with

the exception of the questions aimed at sociologically framing the interviewees. (Annex 2). The traditional healers received a small bottle of red wine as compensation for lost work time and cola nuts as it is tradition in the Bamiléké culture. A total of 12 traditional healers (one traditional healer participated in the study with his wife who practices the same profession) were included in the study and interviews conducted with them covering all 6 study communities.

Group interviews with members of local women associations

Thirty-eight women from 4 different local women associations were involved in the study. 1. "Women 4 better Health" whose main objective is to promote the integration and insertion of women into the community; 2. "Femmes en Politique de la ville de Dschang" - Women in politics, trains women on how to take part in politics in society and especially in the city of Dschang; 3. Women from *PIPAD* - "Programme Intégré pour la Promotion de l'Auto développement" Integrated Program for the Promotion of Self Development, whose one of the objective is to promote women self-employment and helping vulnerable people (disabled, HIV-infected mothers and children); 4. women from the "Association des Jeunes du quartier Nzenmeh-Foreke"- Nzenmeh-Foreke Youth Association, who promotes community development and self-employment. The group discussions took place in groups of 4 to 6 women organized after ordinary meetings of the local associations. The group

discussions were performed by two interviewers (at least one female), following a list of predefined questions (Annex 3).

Oral consent was obtained from each group of women before initiating the discussion. All were informed about the use of the collected data for a doctoral research, the objectives of the study and our concern to preserve the health of mothers and infants while valuing at the same time traditional medicine in the community.

Plant collection and plant species identification

Among the 11 frequently cited plants by TPs, 10 were harvested during the dry season in December 2017 and January 2018. The harvest took place by Alain Tenoh for the easily available plant and the remaining ones namely *Picralima nitida*, *Voacanga africana*, *Solanocia mannii* and *Mangifera indica* were provided by the president of TPs association. Collected plant material was air-dried in the shade and then ground to a fine powder in the laboratory.

A voucher specimen of each of the 11 plants selected was deposited at the "Herbarium National du Cameroun", Yaoundé.

6.2. Description *of the ten harvested plants*

Persea americana Mill (Lauraceae)*:* It is a tropical evergreen tree or shrub known as 'avocado', 'avocado pear' or 'alligator pear' that can grow 20-

30 m tall. Its common name is Avocado. *Persea americana* (*P. americana*) is cultivated in most tropical countries, particularly for its edible fruits. But all parts of the plant, including leaves, bark, root, seed and fruit pulp are used for medicinal purposes. Its oil has applications in cosmetics, in the form of topical creams, to manage various skin problems. In Cameroon, the leaves decoction is taken to treat toothache, high blood pressure, diabetes, malaria and to relief painful menstruations. The leaves and stem bark are also boiled together in water and the resulting liquid is taken to cure toothache, malaria and typhoid fever (Tene et al., 2016). The fruit pulp is eaten to lower bad blood cholesterol, and to prevent mental strain and cardiovascular diseases; while the aqueous extract of its seed is drunk against intestinal worms. This tree planted around the house serves as air purifier. Biological tests carried out on this plant revealed the following activities: antioxidant, anti-*helicobacter pylori*, anti-ulcer and gastro-protective, antibacterial and antimicrobial, anti-platelet and anti-thrombic, wound healing, hepatoprotective, vasorelaxant, analgesic and anti-inflammatory, hypoglycemic and antidiabetic, antihypercholesterolemic, anti-diarrheal, anti-arthritic, antimalarial, haemopoietic and cardiovascular (Gomez-Flores et al., 2008; Yukes & Balick, 2009). Falodun et al., found that seed (from unripe fruit) extracts contain fatty alcohol metabolites, which possess potent activity against chloroquine-sensitive (*D6*) and chloroquine resistant (*W2*) strains of *Plasmodium falciparum* (Falodun A. et al., 2014). More

interesting yet, it has been reported that its extracts can be safely taken to cure diseases till doses greater than 500 mg/kg (Tene et al., 2016).

Bioactive constituents such as alkaloids, flavonoids, phenolic compounds, saponins, steroids and tannins, significantly present in the *Persea americana* extracts, account for its various activities and uses in medicine, while the great amount of nutrients like high-monounsaturated fatty acid, sugar, phytosterols, dietary fiber, minerals and vitamins, present in the plant extracts explain its high nutritional value. Although some of the individual phytochemicals found in avocado have been well characterized (Akinpelu et al., 2015), many others are still to be discovered.

***Dacryodes edulis (G. Don.) H. J. Lam (Burseraceae)*:** *D. edulis* is one of those plants currently used by indigenous persons for its nutritional value and to manage various health problems. The plant belongs to the *Burseraceae* family. The common names of *Dacryodes edulis* in English are: african pear, african pear tree, bush butter, bush butter tree, bush fruit tree, eben tree, native pear; and in French: “safoutier” or “prunier”. *D. edulis* is a medium-sized, evergreen tree attaining a height of 18-40 m in the forest but not exceeding 12 m in plantations. It is generally branched from low down, with a deep, dense crown. The plant is cultivated widely, since it adapts well to differences in the duration of day light, temperature, rainfall, soils and altitudes. It is cultivated in most rural communities by the peasant farmers, mostly for its edible fruits (Olivier et al., 2016; Orwa et al., 2009a). All parts

of this tree, including leaves, bark, roots, resin, seeds and fruit pulp are used for medicinal purposes (Zofou et al., 2011). In Congo Brazzaville, the leaves are boiled with those of *Lanata camara*, *Cymbopogon* citratus and *Persea americana* in water to form a decoction for treating malaria. A steam bath can also be taken from the decoction to treat the same ailment. In western Cameroon, leaves and bark of *D. edulis* are associated to those of *Citrus limonum* and *Cymbopogon citratus*, and then boiled. The resulting liquid is drunk to cure malaria. Its leaves are also known in this part of the country to be effective against digestive disorders, toothache and earache. Its bark extract is equally used to cure dysentery and anemia (Olivier et al., 2016). Many studies have been carried out to characterize the chemical composition of *D. edulis*. Almost all parts of the plant are concerned by these researches, but leaves, stem bark and fruit seeds are the most studied. The results show the presence of lipids, volatile components and bioactive components (alkaloids, phenolic compounds, flavonoids, tannins, anthraquinones, cardiac glycosides and steroids. The *D. edulis* leaves showed the highest *in vitro* activity on asexual blood stages of *Plasmodium* (IC50 6.45 g/mL on 3D7 and 8.2 g/mL on DD2) (Zofou et al., 2011b). Another study conducted to identify the compounds responsible for the antimalarial activity of *D. edulis*, allowed to isolated 5 compounds from ethyl acetate and hexane extracts of the plant's stem bark. The most active compound identified was methyl 3,4,5-trihydroxybenzoate ($C_8H_8O_5$), with IC50 values of 0.37 and 0.55 µg/mL, against 3D7 (chloroquine-susceptible) and DD2 (multidrug-resistant) strains

of *Plasmodium falciparum* respectively. None of the tested compounds was cytotoxic against LLC-MK2 cells, suggesting their selective activity on the malaria parasite (Zofou et al., 2013).

***Ocimum gratissimum L.* (Lamiaceae):** *O. gratissimum* belongs to the family of *Lamiaceae* and is commonly known as clove basil or lemon basil. It's an aromatic shrub, perennial herb, 0.5 - 3 m tall. *O. gratissimum* is grown for the essential oil in its leaves and stems. Eugenol and to a lesser extent thymol extracted from the oil are substitutes for clove oil and thyme oil. The essential oil is also an important insect repellent (Orwa et al., 2009d). The whole plant and the essential oil have many applications in traditional medicine, especially in Africa and India. Preparations from the whole plant are used as stomachic and in treating sunstroke, headache and influenza. The essential oil is applied against fever, inflammations of the throat, ears or eyes, stomach pain, diarrhea and skin diseases. It is being evaluated as an antibiotic. The chemical composition of the oil is variable and at least 6 chemotypes have been reported, characterized by the main components of the essential oil: eugenol (1-hydroxy-2-methoxy-4-allylbenzene), thymol, citral, ethyl cinnamate, geraniol and linalool. The methanol leaf extract shows the presence of flavonoids, alkaloids, tannins, terpenoids, phlobatannins and cardiac glycosides with steroidal ring (Pandey, 2017; Tchoumbougnang et al., 2005). Essential oils from the leaves of *O. gratissimum* were tested against local isolates of *P. falciparum* in

Cameroon and found to be highly active, with an IC_{50} value ranging from 6.9 to 14.9 µg/mL. Ngemenya et al. (2004), recorded an IC_{50} of 29.5 µg/mL of *O. gratissimum* leave essential oil on *P. falciparum F32* strain (Titanji et al., 2008).

***Solanocia mannii* Hook. F. (Asteraceae):** *S. mannii* is a plant (shrub or tree of 12 m) not very well known and scarcely documented. The plant is common in East Africa and also in South Africa. In Cameroon, the plant is traditionally used by TPs for the treatment of diarrhea, fever and vomiting of bile (Hubert et al., 2013). The dichloromethane/methanol (1:1) leave extract of the plant revealed activity on the promastigote of *Leishmania donovani* parasite *in vitro* (Hubert et al., 2013). No study reported either on the phytochemical composition of plant extracts or the antimalarial properties of this plant.

***Eucalyptus globulus* Labill (*Myrtaceae*):** *E. globulus* (also known as blue gum) from the family *Myrtaceae*, is a large to very large evergreen tree, 40-55 (max. 60) m tall, with a straight, massive trunk 0.6-2 m in diameter; narrow, irregular crown of large branches and drooping aromatic foliage (Orwa et al., 2009c). In West Cameroon, *E. globulus* is very widely cultivated and used for firewood and construction poles. *Moreover,* it's widely employed by traditional healers of Western Cameroon for the management of malaria. The leaves are valuable for the extraction of eucalyptol, a commercially important *Eucalyptus* component (oil contains cineole). The

oils are used as an inhalant with steam and other preparations for relief of colds and influenza symptoms. Because of the refreshing odor of the oil and its efficiency in killing bacteria, it is also used as an antiseptic (Orwa et al., 2009c). It helps to treat lung infections, gastrointestinal ulcers and angina. The antiplasmodial activity of *Eucalyptus globulus* was previously reported. The IC_{50} from the crude extract of the leaves using methylene chloride/methanol (1:1) solvent mixture of *E. globulus* was found to be 16.80 µg/ml and 26.45 µg/ml against the *P. falciparum 3D7* and *Dd2* parasite strain respectively, supporting the wide use of *E. globulus* as antimalarial by endogenous traditional healers of Western Cameroon (Zofou et al., 2011b).

***Cymbopongon citratus* Stapf. (*Poaceae*):** also known as Lemon grass or fibergrass, *C. citratus* is a herb widely found and used in tropical countries, especially in Southeast Asia; it's an evergreen, perennial grass that is aromatic and grows up to 1.5 m tall. The plant belonging to the *Poaceae* family is used as a fragrance and flavoring agent and in folk medicine as an antispasmodic, hypotensive, anticonvulsant, analgesic, antiemetic, antitussive, anti-rheumatic, antiseptic agent and for the treatment of nervous, gastrointestinal disorders and fevers (Shah et al., 2011). The plant is also used as an antibacterial, antidiarrheal and antioxidant, but the mode of action for the different bioactivities has not been studied in detail. *C. citratus* contains various phytoconstituents such as flavonoids and phenolic compounds, terpenoids and essential oils, which may be responsible for the different biological activities (Shah et al., 2011; Tchoumbougnang et al.,

2005). In Western Cameroon, the leaves of *C. citratus* are boiled with those of *D. edulis* and *M. indica* in water to form a concoction against malaria (Titanji et al., 2008). A study conducted by Tchoumbougnang et al., 2005 reported that the essential oils extracted from fresh leaves of this plant were active in the four-day suppressive *in-vivo* test on *P. berghei* in mice. The oil demonstrated significant suppression of parasitaemia (62.1 - 86.6%) at the oral dosage range of 200 to 500mg/kg of mice body weight (Tchoumbougnang et al., 2005). Bidla et al. (2004) also investigated *C. citratus* leaves and they recorded an inhibition of 57.9% of *P. falciparum*, *in vitro* by a 20 μg/mL chloroform/ethanol (1:1) extract (Titanji et al., 2008).

***Voacanga africana* Stapf. (*Apocynaceae*):** *V. africana*, is a small tree in the dogbane family (*Apocynaceae)* that grows to 6 m in height. It is native to tropical Africa. Global interest in the plant as a source of useful chemicals has been growing. Also, today the plant is a major non-timber wood product exported from Ghana, Cameroon, Nigeria, and Cote d'Ivoire (Vilgiate, 2009). The root bark and seeds of this tree contain a number of alkaloids, including ibogaine (a hallucinogenic/aphrodisiac compound in bark), tabersonine (a major constituent of seeds), voacangine and other *voacanga* alkaloids, traditionally used in Africa for spiritual purposes. Recently, various products containing this plant (root bark and seeds) have been distributed on the drug market, exploiting its hallucinogenic/aphrodisiac effects. Until now, there has been no report that has provided quantitative analyses of these alkaloids

in the commercialized, hallucinogenic products and elucidated their botanical origins (Kikura-Hanajiri R. et al., 2009).

***Mangifera indica L.* (*Anarcardiaceae*):** commonly called "mango", *M. indica* belongs to the order *Sapindales* in the family *Anacardiaceae* which is a family of mainly tropical species. Native from Asia, it is a large evergreen tree, 10-40 m in height, with a dark green and umbrella-shaped crown (Mukherjee, 1971; Orwa et al., 2009d). Mango is cultivated for the fruit, which can be eaten in 3 distinct ways, depending largely on the cultivar: unripe (mature green, very popular in Thailand and the Philippines), ripe (the common way to enjoy mango throughout the world), and processed (at various stages of maturity, in the form of pickles or chutneys, dried slices, canned slices in syrup, juice and puree or paste) (Yadav & Singh, 2017). The fruit is surrounded by golden, juicy flesh, rich in vitamin A and C. Various parts of the *M. indica* tree have been used in traditional medicine for the treatment of different ailments, and a number of bioactive phytochemical constituents of *M. indica* have been reported, namely, polyphenols, terpenes, sterols, carotenoids, vitamins, amino acids, and so forth. Several studies have provided evidence of the pharmacological potential of different parts of mango trees such as leaves, bark, fruit peel and flesh, roots, and flowers. The various plant parts contain components with various biological properties: anticancer, anti-inflammatory, antidiabetic, antioxidant, antibacterial, antifungal, anthelmintic, gastroprotective, hepatoprotective, immunomodulatory, antiplasmodial, and antihyperlipemic (Ediriweera &

Samarakoon, 2017). Bidla et al (2004) evaluated the *in-vitro* antimalarial activity of *M. indica*, which is grown widely also in Cameroon for its fruits as food. The chloroform:methanol (1:1) extract showed *in-vitro* activity on *P. falciparum* with a growth inhibition of asexual blood stages of 50.4% at 20 µg/mL (Titanji et al., 2008).

***Senna alata L.* (*Fabaceae*):** *S. alata* also known as *Cassia alata* is a shrub belonging to the *Fabaceae* family. It's an important medicinal tree, as well as an ornamental flowering plant. It is native to Central America and is mainly encountered in the Caribbean area but has also been introduced into many tropical countries and islands whatever the continent. It is commonly known as candle bush, with reference to the shape of its inflorescences, or ringworm tree (Hennebelle et al., 2009). *S. alata* is often called the ringworm bush because of its very effective fungicidal properties, for treating ringworm and other fungal infections of the skin. In Africa, like in Cameroon *S. alata* is well renowned for its dermatological value to a minor extends for its purgative property. In Cameroon, the leaves are used both topically (as a powder) and orally (in decoction) against skin diseases. Other traditional uses include the management of gastro-intestinal disorders, infectious diseases (including malaria), diabetes and miscellaneous. The major constituents in leave extracts are linalool (23.0%), borneol (8.6%), pentadecanal (9.3%) and α-terpineol (5.9%). Also, flavonoids, anthraquinones, 10-hydroxyanthraquinone and alarone have been reported (Hennebelle et al., 2009). Screening of S. alata extracts on *P. falciparum*

evidenced antimalarial activity of the plant with IC_{50} higher than 5 µg/mL for root extract. The leaf extract was less active with IC_{50} higher than 50 µg/mL (Atindehou et al., 2004) (Mambu et al., 2005).

***Picralima nitida* (Stapf.) T.A Durand & Hook (*Apocynaceae*):** *P. nitida* is the only species of the genus *Picralima* and it is related to *Hunteria* and *Pleiocarpa*. *P. nitida* is commonly called picralima, akuamma, quinqueliba or pile plant; it belongs to the hunterieae tribe of the *Apocynaceae* family. The plant is widely distributed in high deciduous forest of West-Central Africa from Ivory Coast to West Cameroon and extending across the Congo basin and Uganda (Erharuyi et al., 2014). *P. nitida* is an understory tree which reaches up to 4-35 m in height. *P. nitida* bears white flowers (about 3 cm long) with ovoid fruits which at maturity are yellowish in color. *P. nitida* has widely varied applications in West Africa folk medicine. Various parts of the plant namely leaves, seeds, stem bark and roots are used by herbalists for the treatment of fever, hypertension, jaundice, gastro-intestinal disorders and for malaria (Erharuyi et al., 2014). The extracts from different parts of the plant have been found to exhibit a broad range of pharmacological activities which lends credence to its ethnomedicinal uses. A study conducted in Cameroon by Bickii et al., 2007 reported an IC50 < 30 µg/ml for the methanol and dichloromethane–methanol 1:1 extracts from the seeds and bark on *P. falciparum* W2 (Indochina I/CDC) chloroquine resistant strain (Kuete & Efferth, 2010;Titanji et al., 2008). Moreover, the review published by Erharuyi et al. 2014 illustrates the *in-vitro* antimalarial

activity of various *P. nitida* extracts. The seed, fruit, rind and stem bark methanol extracts showed inhibitory activity against drug resistant clones of *P. falciparum* at doses in the range of 1.23-32 µg/mL (Erharuyi et al., 2014). The methanol seed extract of *P. nitida* demonstrated significant activity against the chloroquine-resistant *P. falciparum W2* strain with an IC_{50} value of 10.9 µg/mL (Erharuyi et al., 2014). The *in-vivo* antiplasmodial activity of the ethanol seed extract of *P. nitida* was evaluated in chloroquine-sensitive *P. berghei* infected mice. The result showed that the ethanol seed extract of *P. nitida* exhibited *in-vivo* antiplasmodial activity in both early (4-Day chemosuppressive test) and established infections (Curative test): the ethanol seed extract produced a dose dependent chemosuppressive effect of 65.5%, 70.4% and 73.0% respectively treating mice at doses of 35, 70 and 115 mg/kg/day (Okokon et al., 2007). Indole alkaloids isolated from the seeds of *P. nitida* such as akuammine, akuammidine, akuammicine, akuammigine and pseudoakuammigine are interesting compounds with opioid analgesic activity (Erharuyi et al., 2014). The pharmacological potential of these alkaloids have only partially been investigated.

6.3. Preparation of plant extracts (methanol and water).

Plant materials were air-dried at room temperature under shade and ground using a blender. Plant powders (50 g) were macerated in absolute methanol (MeOH) for 24 h under stirring and filtered with filter paper Whatman® no.1. The methanol was removed using a rotary evaporator at 40 °C under

reduced pressure. The extracts were further concentrated by freeze drying and stored at -20° C till use.

For the preparation of aqueous extract (using distilled H2O), 50 g powder of each plant part were boiled in distilled water for about 30-45 minutes, allowed to cool and filtered. The filtrate was then freeze dried and stored at -20° C until use.

The yield (%w/w) of the aqueous and methanol extraction was determined, respectively.

6.4. Murine malaria model

Rodent host: Three to four weeks old, female BALB/c mice weighing (19 ±3 g) were used for the experiments and were reared, and maintained under standard conditions at 24 °C of temperature, 14 h light/10 h dark cycle and 70% humidity in the animal facility of the University of Camerino (Italy). Animal rearing and handling were in compliance with the Italian Legislative Decree on the "Use and protection of laboratory animals" (D. Lgs. 116 of 10/27/92) and in full adherence with the European Directive 2010/63/UE adopted on 22nd September, 2010.

Parasites strains: Two different strains of *P. falciparum*, *W2* (chloroquine resistant) and *3D7* (chloroquine sensitive) were used for testing the extracts' acitivity against asexual blood stages. The transgenic *P. falciparum 3D7* strain *3D7elo1-pfs16-CBG99* expressing the *Pyrophorus plagiophthalamus* CBG99 luciferase under a gametocyte specific promoter was used for the

assay on gametocytes. *P. falciparum* strains are maintained at the Department of Biomolecular and Pharmacological Science of the University of Milan, according to standard protocols (D'Alessandro et al., 2016, 2013).

The rodent parasite *P. berghei* is a well-established and widely used model parasite for both *in-vivo* and *in-vitro* investigation of parasite–host interactions and the parasite can be genetically modified with relative ease (de Koning & Waters, 2000).

Genetically modified *Plasmodium berghei ANKA* strains (chloroquine sensitive), which express a constitutive green fluorescent protein throughout the parasite life cycle (*PbGFPcon*) or specifically during zygote to ookinete development (*PbCTRPp.GFP*) (Vlachou et al., 2004), were used. Parasite strains have been kindly provided by R.E. Sinden (Imperial College, London) and were maintained following standard procedures (Ramakrishnan et al., 2012). Briefly, *PbGFPcon* and *PbCTRPp.GFP* infected blood was stored in liquid nitrogen (-70°C) with glycerol as a cryo-preservative. At occurrence, capillary blood was unfrozen, diluted in PBS and i.p. inoculated to mice. Parasite propagation was effected through acyclic passage from infected to healthy mice by i.p. administration of parasitized red blood cells. Cyclic passages, from mice to mosquitoes and from mosquitoes to mice were routinely performed every three to four months to preserve parasite infectivity to mosquitoes. Mosquitoes infected with *P. berghei* were kept at 19 ± 1°C for the whole duration of the sporogonic cycle.

The possibilities to investigate host–parasite interactions have been greatly increased with the development of genetic modification technologies, that permit disruption and modification of genes ("reverse genetics") and for the introduction of transgenes (Joiner & Roos, 2002). In particular, the green fluorescent protein (GFP) is a valuable reporter protein since it can easily be detected in individual living cells by fluorescence microscopy.

Mosquitoes: For the *in-vivo* transmission blocking studies with the murine malaria parasite *Anopheles stephensi* mosquitoes were used as experimental vectors. The mosquito colony was maintained at a temperature of 30 °C (± 2°C), 12 h light/12 h dark cycle and 75 to 85% relative humidity in the insectary of the University of Camerino, Italy. Experiments were conducted with four- to five-day-old female mosquitoes that were transferred to a 19° C chamber (temperature required for the development of *P. berghei* in the mosquitoes) 24 h prior to administration of infectious blood-meals.

Cell lines for cytotoxicity assessment

Five cell lines were used to carry out the *in-vitro* cytotoxicity evaluations: two normal cell lines, namely *NHF-A12*-human dermal fibroblast and *EA.hy*926-endothelial cells, the two cancer cell lines *A375*-melanoma and *MDA-MB231*-breast cancer cells and one insect cell line*, Sf9* (*Spodoptera frugiperda* cells).

***In vitro* antiplasmodial activity against *P. falciparum* asexual blood stages.**

Maintenance of the *P. falciparum* strains *W2* and *3D7*

Two different strains of *P. falciparum*, *W2* (chloroquine resistant) and *3D7* (chloroquine sensitive), were used in this study. The parasites were cultured according to the method described by Trager and Jensen (Trager & Jensen, 1976) with slight modifications (D'Alessandro et al., 2016). The *Plasmodium* blood stages were cultured in human type A-positive or type O-positive erythrocytes at 5% hematocrit and at 37°C in a standard gas mixture consisting of 1% O2, 5% CO2, 94% N2. Medium was made up of RPMI-1640 with the addition of 1% AlbuMax, 0.01% hypoxantine, 20mM Hepes and 2mM L-glutamine. For routine parasite growth, the parasitemia was maintained within 1% and 5%, and evaluated as the number of infected RBCs with respect to the total number of red blood cells, counted in Giemsa stained blood smears.

Drugs sensitivity pLDH assay

For the drug sensitivity assay, the plant extracts were dissolved in DMSO and then diluted with medium to achieve the required test concentrations (final DMSO concentration <1%, which is non-toxic to the parasite). The plant extracts were placed in 96-well flat-bottom microplates in duplicate and seven twofold serial dilutions were made directly in the plate in a volume of 100µl. Asynchronous cultures with parasitemia of 1–1.5% and 2% hematocrit (1% final) were aliquoted into the plates and incubated for 72h, in a final volume of 200 µl/well. Chloroquine (CQ) was used as reference compound.

Parasite growth was determined by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method (Makler et al., 1993). The pLDH activity is distinguishable from the host LDH using the 3-acetyl pyridine adenine dinucleotide (APAD) as co-factor. Briefly, at the end of the incubation, the cultures were carefully re-suspended, and aliquots of 20 µL were removed and added to 100 µL of the Malstat reagent in a 96-well microplate. The Malstat reagent is made of 0.125% Triton X-100, 130 mM L-lactate, 30 mM Tris buffer and 0.62 µM APAD. Twenty-five µL of 1.9 µM NBT (Nitro Blue Tetrazolium) and 0.24 µM PES (phenazine ethyl sulphate) were added to the Malstat reagent. NBT is reduced to blue formazan and is spectrophotometrically (OD650 nm) read as a measure of pLDH activity and thus of parasite viability. Antimalarial activity was determined and expressed as the 50% inhibitory concentrations (IC50, concentration of drug required to inhibit 50% parasite growth).

***In vitro* activity against *Plasmodium falciparum stage V* gametocytes.**

***P. falciparum* gametocyte induction**

The transgenic *P. falciparum 3D7* strain *3D7elo1-pfs16-CBG99* expressing the Pyrophorus plagiophthalamus CBG99 luciferase under a gametocyte specific promoter was used for the drug sensitivity assay on gametocytes. Parasites were cultured and gametocytes obtained as described by

D'Alessandro et al. (D'Alessandro et al., 2013). Late-stage gametocytes were exposed to plant extracts at day 11 after N-acetylglucosamine (NAG) addition. Gametocyte stages were counted in Giemsa stained smears and cultures used for the gametocyte drug susceptibility assay when the percentage of stage V gametocytes was higher than 90%.

Gametocyte drug susceptibility assay

Plant extracts were prepared by serial dilution, in a 96-well plate, in complete medium as described for asexual parasites. Methylene blue was used as reference drug. After 72 h incubation, luciferase activity was taken as measure of gametocytes viability, as previously described by D'Alessandro et al. (D'Alessandro et al., 2016). Briefly, plant extract-treated gametocytes at 2% haematocrit were transferred to 96-well black microplates and D-luciferin (1 mM in citrate buffer 0.1 M, pH 5.5) was added at a 1:1 volume ratio. Luminescence measurements were performed after 10 min with 500 ms integration time using a Sinergy 4 (Biotek) microplate reader. The IC50 was calculated for the most active plant extracts as described for asexual parasites.

Ookinete Development Assay (ODA).

***Plasmodium* strain**

The *P. berghei CTRPp.GFP* strain, expressing *GFP* exclusively at early sporogonic stages (ESS), namely in zygotes and ookinetes, was used for the

assessment of crude plant extracts' activity against the development of ESS *in vitro*. The rodent malaria parasite strain was maintained in the Parasitology laboratory at the University of Camerino (Italy) by mouse to mouse acyclic and mouse to mosquito to mouse cyclic passages. BALB/c mice and *Anopheles stephensi* mosquitoes were employed as vertebrate and vector host respectively.

Vertebrate host for *P. berghei CTRPp.GFP* gametocyte production

Three- to four- week-old BALB/c mice were used as gametocyte donors for the *in-vitro* experiments. The mice were reared in the animal house (24 °C, 14 h light/10 h dark cycle and 70% relative humidity) of the University of Camerino, fed on standard mice pellets (Mucedola s.r.l., Milano, Italy) and provided with tap water ad-libitum. Experimental animal rearing and handling were in compliance with the Italian Legislative Decree on the "use and protection of laboratory animals" (D. Lgs. 116 of 10/27/92) and in full adherence with the European Directive (86/609) of 24/11/1986 (license no. 125/94A, issued by the Italian Ministry of Health). The experiments were in accordance with the protocols approved by the Animal Ethics committee of the University of Camerino.

Evaluation of effects on early sporogonic stages (ESS) *in-vitro:* ookinete development assay (ODA)

The impact of crude plant extracts on the development of ESS was evaluated in the ookinete development assay according to the method described by

Delves et al., (2012) with slight modifications (Tapanelli et al., 2016). Parasite infected BALB/c mice were used as a source of gametocytes for the *in-vitro* assay and obtained by the following procedure: To stimulate erythropoiesis, mice were treated with phenylhydrazine (120 mg/kg i.p.) four days prior to infection with *P. berghei CTRPp.GFP* through i.p. injection of 10^7 infected RBCs. Gametocytemia was checked four days post-infection by microscopic examination of thin blood films and the maturity of microgametocytes verified by testing their capacity to generate flagellated microgametes in the exflagellation assay. In brief, a drop of tail blood from a gametocytemic mouse was diluted at a ratio of about 1:25 in exflagellation medium (RPMI 1640 containing 25 mM HEPES, 25 mM sodium bicarbonate, 50 mg/L hypoxanthine, 100 µM xanthurenic acid, pH 8,3). Then 8 µL of the diluted blood sample was incubated in a hand-made coverslip/slide chamber consisting of a slide as a base, 2 cover slips placed on it laterally as spacers, and a third one placed on the top to close the chamber. Then, all sides of the cover slip were sealed with a mixture of Vaseline and Tween-80 (approximately 1:2 ratios). After 20 min incubation at 19 ± 1 ^{0}C, the slides were examined for exflagellation centers under the microscope (400X magnification). Mice with abundant exflagellation centers (more than 3 per 1000 red blood cells) were selected and used as blood donors for the ookinete development assay. This assay allows to assess effects of plant extracts on the sexual parasite stages developing in the vector, namely male and female gametes, zygotes and ookinetes, simulating *in-vitro* the physicochemical

conditions of the mosquito midgut environment: 80 µL of ookinete medium [exflagellation medium with 20% heat inactivated foetal bovine serum (FBS) and 1% penicillin (10000 U/mL) / streptomycin (10000 µg/mL) adjusted to pH 7.4] was added to the wells of a 96-well microplate (Nunc, Denmark). Dimethyl Sulfoxide (DMSO) was used as solvent for plant extracts. Each plant extract was dissolved at a stock concentration of 30 mg/ml and serial dilutions were performed to obtain a panel of concentrations. Ten microliter (10µl) samples of diluted plant extracts were then added to the microplate wells containing 80 µL of medium to obtain the desired test concentrations (1.56 - 100 µg/ml). DMSO at 0.2% was used as negative control. Then, 10 µL aliquots of blood obtained by cardiac puncture from gametocytemic mice were transferred to the microplate wells containing test extracts or solvent controls and mixed swiftly. The plates were then incubated at 19 °C for 24 h. At the end of incubation, well contents were mixed and 5µl cell suspension from each well was withdrawn and diluted with PBS (pH=7.4) at about a ratio of 1:25 to 1:50 in a separate 96-well microplate. This dilution step allowed to obtain - after cell settlement - a monolayer of blood cells and parasites, a condition that was required for an accurate microscopic examination. *GFP* expressing zygotes and ookinetes were visualized using a Zeiss fluorescent microscope (400 X magnifications) and quantified with the help of an ocular grid. Each extract was examined in 3 replicate wells and 6 wells were reserved for DMSO controls. Experiments were repeated at least

twice with different donor mice. The percent inhibition of ESS development induced by the molecules was calculated as follows:

$$\%\ of\ ESS\ inhibition = 1 - \left(\frac{n°ESS\ per\ test\ compound\ wells}{n°\ ESS\ per\ control\ wells}\right) * 100$$

Distinct counts of zygotes and retort to elongated ookinete forms were conducted based on the cell shape: round forms were counted as zygotes while elongated forms, including retorts , were considered as developing ookinetes.

In vitro exflagellation assay

Plant extracts found active in the ODA were further examined to assess whether the observed impact on ESS counts was due to extract activity on male gametogenesis. The exflagellation assay was carried out as previously described by Abay et al., 2015 (Abay et al., 2015). Gametocytemic mice were prepared as for the ODA (illustrated above).

On day four post-infection, 5 µl of tail blood were re-suspended in 140 µl of exflagellation medium (pH 8.3, details mentioned in ODA section above) in an eppendorf tube containing plant extract. Extracts were tested at a single dose, i.e. the concentration found to inhibit 50% of ESS counts in the ODA. Extracts were dissolved in DMSO to a maximum assay DMSO concentration of 0.2%. Slides were incubated for 20 minutes at 19 ± 1°C and read under a light microscope (ZEISS Axio Observer.Z1) at 400X magnification, equipped

with a 10x10 micrometer grid on the eyepiece. Exflagellation centers – visible as vibrating agglomerations of RBCs around motile microgametes detaching from the microgametocyte residual cell (Figure 9) - were counted against RBCs in grid fields across the diameter of the spread blood drop, reaching about 38±3 grid field counts. The number of exflagellation centers per 10000 RBCs was recorded in the drops of three chamber preparations derived from consecutive Eppendorf incubations of extract with gametocytemic blood using the same mouse. DMSO solvent was used as a negative control and NeemAzal® technical grade (Trifolio-M GmbH, Lahnau, Germany) containing 50% Azadirachtin A was chosen as positive reference using the product at its IC_{50} dose of 12.4µg/ml [95%CI, 11.0 - 14.0] (Dahiya et al., 2016). Reduction in exflagellation centers by each plant extract was recorded in triplicate chambers and repeated twice with different gametocytemic mice.

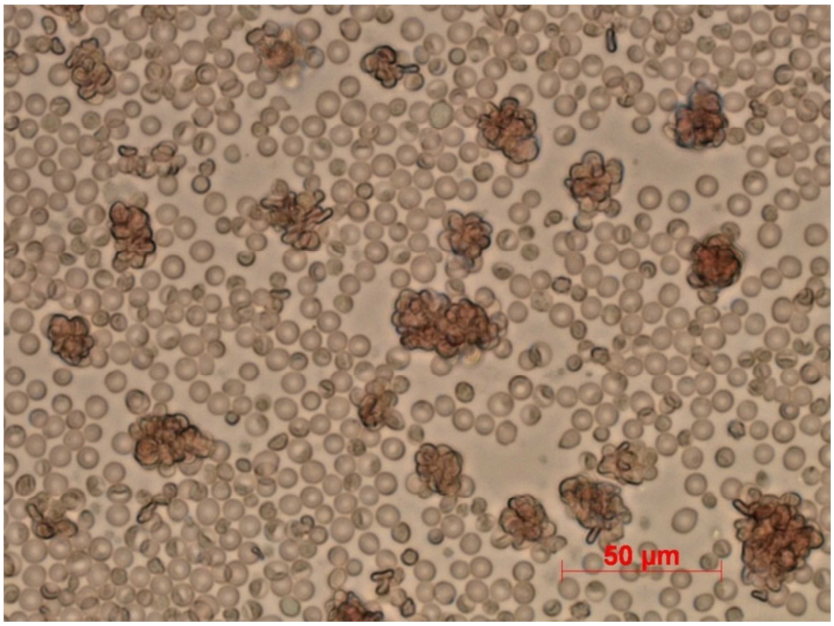


Figure 9 Exflagellation centers after incubation of gametocytaemic blood sample in exflagellation medium for 20 minutes.

Assessment of transmission blocking activity in vivo by the Direct Feeding Assay (DFA)

Preparation of infectious mice: Initially, four mice were infected with *PbGFPcon* using infected blood from capillaries stored in liquid nitrogen. Four to five days later, mice with parasitemia of about 5-7% were used to infect experimental mice with a standardized number of 10^6 *PbGFPcon* infected RBCs per mouse. Female BALB/c mice weighting 18 - 22g were used. On day 3 after infection, thin blood films were prepared from the tail tips of mice and parasitemia determined. Mice with a parasitemia in the range of 2-5% and presence of male and female gametocytes were selected for the DFA experiments on the following day. Three mice were allocated to each treatment and control group (Table 3). DFA experiments have been conducted with the plant extracts having demonstrated activity in the *in vitro* ODA assay, namely with the stem bark methanol extract of *P. americana* and *D. edulis*. Plant extracts were dissolved in DMSO (100 mg/ml stock solution) and then further diluted with PBS (pH 6.5) containing Tween 80 at 7.5%. Gametocytemic mice were treated intraperitoneally with a dosage of 150 mg/kg of extract. Control mice were treated with diluent (PBS pH 6.5 containing 7.5% Tween 80 and 14% DMSO).

Mosquito infection: Extracts were administrated to the selected mice 1h before mosquito infection. About 30 min after the treatment mice were

anaesthetized with a 1:1 mixture of xylazine (2%; Bayer) and acepromazine (10mg/mL; Fatro Spa) and placed for 30 to 45 minutes on cages containing each about 50 female *An. stephensi* mosquitoes (3-5 days old) for blood feeding. Three mouse/mosquito cage replicates were prepared for each treatment group. *P. berghei* mosquito infection was performed in a climate chamber at 19 ± 1°C and 70-80% relative humidity. Unfed mosquitoes were removed 24 h after the blood meal and fed females provided with 8% sugar solution supplemented with 0.05% para-amino benzoic acid (PABA; Sigma-Aldrich, USA) to support oocyst development (Ramakrishnan et al., 2012)

Assessment of transmission blocking effects: On day six (seven) after mosquito infection, 30 females were dissected per mosquito cage (3 x 30 = 90 per treatment group) and mid guts examined to assess the prevalence and density of oocysts under the fluorescent microscope (400×).

MTT cytotoxicity assay

Cells from the *Sf9* insect cell line (*Spodoptera frugiperda* cell line) were cultured in Grace's insect essential medium with 100 IU/ml penicillin, 100 µg/ml streptomycin and supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (PAA Laboratories GmbH, Pasching, Austria). *MDA-MB231* cells (human breast adenocarcinoma cell line), A375 cells (human malignant melanoma cell line) and *EA.hy926* cells (human endothelial cell) were cultured in Dulbecco's modified Eagle's medium with 2 Mm L-glutamine, 100

IU/ml penicillin, 100 µg/ml streptomycin, and supplemented with 10% HI-FBS. *NHF-A12*-human dermal fibroblasts cells (gently provided by Prof. Nabissi Ivan, from the School of Pharmacy - University of Camerino) were cultured in Dulbecco's modified Eagle's medium with 2 Mm L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1mM of sodium pyruvate and supplemented with 10% HI-FBS

Sf9 insect cells were cultured in an incubator at 27°C, in a non-humidified atmosphere without CO_2 and cells of the human cell lines were cultured in an incubator at 37 °C, in a humidified atmosphere with of 5% CO_2. The MTT assay was used as a relative measure of cell viability. The assays were carried out as described by Quassinti et al. (2013) (Quassinti et al., 2013). Briefly, cells were seeded at the density of 2 x 10^4 cells/ml (1 x 10^5 cells/ml in case of *Sf9 i*nsect cell line) into 96-well microtiter tissue culture plates (Falcon®). After 24 h, samples were exposed to different concentrations of test extracts (1.17–150 µg/ml) in a final volume of 100 µl of culture medium. After 72h of incubation (24h, 48h and 6 days for *Sf9* cell line), each well received 10 µl of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml in phosphate-buffered saline) and the plates were incubated for a further 4 h at 37 °C (27°C for *Sf*9 insect cell line). The intracellular reduction of tetrazolium salts by the enzyme succinate dehydrogenase causes the formation of blue formazin crystals at the bottom of the well. At the end of the incubation time, the supernatant was removed and 100ul of dimethylsulfoxide (DMSO; Sigma-Aldrich) were added per well. The plate

were stirred for about 15 min in order to solubilize the crystal formed. The extent of MTT reduction was measured spectrophotometrically at 540 nm using a OMEGA plate reader from BMG Labtech (Durham, NC, USA). Cell viability was calculated as a percentage ratio of the absorbance of the sample to the vehicle (DMSO, 0.25%). Every concentration was repeated in quadruplet. The concentration of plant extracts which inhibited cell growth by 50% was expressed (IC_{50}), calculated with GraphPad Prism 5 computer program (GraphPad Software, S. Diego, CA, USA).

6.5 Data elaboration and analysis

Excel 2010 spreadsheet and GraphPad Prism 5 statistical software were used for data analysis. In order to analyze ethnobotanical information, Microsoft Excel package 2010 was used where descriptive analysis methods were employed. The information obtained through the ethnobotanical interviews was analyzed and frequency of answers expressed as a percentages. Normally distributed data, such as ESS values and their % inhibition were expressed and the half inhibitory concentrations (IC_{50}) of active extracts were calculated by non-linear regression analysis using variable sloop ($\pm CI_{95\%}$). Gametocytocidal and asexual blood stages activity of plant extracts was graphically expressed by log-dose response % of inhibition (± S.D) and the half inhibitory concentrations (IC_{50}) of active extracts were calculated by non-linear regression analysis using variable sloop (±S.D). Similarly, the IC50 on cytotoxic effect of plant extracts were calculated by non-linear

regression analysis. Oocyst densities were expressed as geometric means of 30 midgut counts per mosquito cage ± CI95% and arithmetic means calculated from the 3 replicate means.

7 Results

7.1 Ethnobotanical *survey*

Characteristics of respondents

A total of 12 traditional practitioners (TPs) were interviewed exerting their professional activity in 6 communities of the Dschang district (Baleveng, Bafou , Foto , Foreke , Fongo-tongo , Santchou) (Table 2). The TPs had an elevated average age of 53 years (range 35 to 70 years). All the 12 interviewees had experience with treating pregnant women. In one case the interview was held with a TP couple, husband and wife.

The educational background was characterized by 64% of TPs with either no formal education or primary school level, 18% had secondary education and also 18% had at university training background.

Table 2 Traditional Practitioners (TPs) respondent's details.

Age (years)	**Number of TPs**
0-25	0
26-35	01
36-45	01
46-55	05
>55	05
Gender	
Male	11
Female	01

Villages	
Baleveng	02
Bafou	02
Foto	02
Foreke	02
Fongo-tongo	02
Santchou	02
Educational status	
No formal education	02
Primary level	06
Secondary level	02
Tertiary level (university)	02

Moreover, 38 women (interviewed in 8 discussion groups consisting of 4 to 6 women) from four local associations were involved in the study (Table 3). The women were aged between 22 and 56 years old. With regard to their level of education, 21% of women had received no formal education, 32% had a primary or secondary level of education and 15% have had the opportunity to get university training (Table 4). Most of these women (71%) practice agriculture and commerce as an income generating activity. Overall, the women involved in the study are all mothers of at least one child.

Table 3 Information on the discussion groups and the women associations involved

Number of group discussions	Number of women in each group	Local name of association (name in English)
1	05	"Femmes du Pipad" (Integrated Program for the Promotion of Self Development)
2	04	Women for better health
3	05	"Femmes en Politique de la ville de Dschang" (Women in Politics in Dschang city)
4	05	Women for better health
5	06	"Femmes du Pipad" (Integrated Program for the Promotion of Self Development)
6	04	"Femmes en Politique de la ville de Dschang" (Women in Politics in Dschang city)
7	05	"Femmes de l'Association des jeunes du quartier Nzenmeh-Foreke" (Nzenmeh-Foreke Youth Association)
8	04	"Femmes de l'Association des jeunes du quartier Nzenmeh-Foreke" (Nzenmeh-Foreke Youth Association)

Table 4 Information on the 38 women participating to the group discussions

		Number of women
Age (years)	0 - 25	03
	26 - 35	11
	36 - 45	16
	46 - 55	07
	> 55	01
Educational status	No formal education	08
	Primary level	12
	Secondary level	12
	Tertiary level	06
Occupation/ profession	Housekeepers /farmers	15
	tradeswoman	12
	Teacher/secretary in public administration	06
	University student	05

Knowledge on malaria and malaria in pregnancy

All interviewed TPs were aware of the severity of malaria in pregnancy and the particular risk of treatment with medicinal plants in gestation. All respondents declared that they execute anti-malarial treatments in pregnant women and claimed that traditional medicine provides the necessary knowledge for malaria management in pregnancy.

Most of the respondents (TPs and women included in the group discussions) identified fever (increased body temperature) as the main symptom associated with malaria. All traditional healers claimed to be able to distinguish fever due to malaria from fevers of other origin.

Each TP and participants of each women group were able to cite at least three of the following symptoms of malaria (Table 5).

Table 5 Symptoms of malaria cited by TPs and participants of women groups

Symptoms of malaria cited	**Number of citations by TPs [1] (n=11)**	**Number of citations by women groups (n=8)**
Increased body temperature	11/11	8/8
General fatigue	9/11	8/8
Aches (joint paint, etc…)	11/11	7/8
Chills	9/11	8/8
Sweat	7/11	5/8
Dry skin (pale face)	4/11	1/8
Headaches	4/11	7/8
Nausea	7/11	5/8

Vomiting	7/11	5/8

[1] the TP couple is considered as one responder

Women participating in the group discussions appeared to have a good knowledge on the medicinal use of plants in the community to treat malaria during pregnancy. They stated that they have acquired the knowledge from a family member (e.g. grand-parents), friends and neighbors or from traditional healers in some cases (even though, they expressed the belief that TPs prefer to keep their knowledge secret).

Table 6 list of medicinal plants reported to be used in the community for the prevention and or treatment of malaria during pregnancy.

N°	Scientific name of plant	Local name (Yemba/French)	Number of citation by TPs	Number of citation by group of women
1	*Cymbopongon citratus*	Fibergrass/ citronelle	10/11	8/8
2	*Picralima nitida*	Quinqueliba	9/11	8/8
3	*Dacryodes edulis*	Acup	8/11	5/8
4	*Ocimum gratissimum*	Cotemanjo	6/11	7/8
5	*Voacanga africana*	Atieuh-nieh	6/11	0/8
6	*Eucalyptus globulus*	Acaltusi	6/11	6/8
7	*Persea americana*	Apiah	6/11	5/8
8	*Mangifera indica*	Mango	5/11	6/8
9	*Senna alata*	Meskayo	4/11	4/8
10	*Aloe barbadensis*	Lelang/aloe	4/11	8/8
11	*Solanocia mannii*	Kepan	2/11	0/8
12	*Bidens pilosa*	Tse-tse neck	1/11	6/8
13	*Carica papaya*	Popoya/papaya	1/11	4/8
14	*Psidium guajava*	Goyave	1/11	0/8
15	*Citrus limon*	Citron	1/11	3/8
16	*Lantana camara*	Leukeuh deuh	1/11	0/8
17	*Aframomum melegueta*	Sock	0/11	1/8

18	*Vernonia guinensis*	Ginseng	0/11	2/8
19	*Emilia cocinea*	nvenglapin	0/11	1/8

Overall, this study allowed to identify 19 medicinal plants used for the prevention and treatment of malaria in pregnant women in the Menoua division (Table 6). On the specific question "which are the plants that you use cure malarial fevers in pregnant women", 10 medicinal plants have been cited (Table 7). These ten plants make part of the 19 plants indicated (Table 6), responding to the more general question on antimalarial plant use in pregnancy.

Table 7 Medicinal plants used for the management of malarial fever in women during pregnancy

Scientific name	**Number of citation by TPs**	**Number of citation by women groups**
Cymbopongon citratus	11/11	8/8
Aloe barbadensis	8/11	6/8
Ocimum gratissimum	6/11	4/8
Cassia alata	5/11	5/8
Citrus sinensis	5/11	4/8
Dacryodes edulis	4/11	7/8
Mangifera indica	4/11	3/8
Carica papaya	3/11	7/8
Picralima nitida	3/11	6/8
Bidens pilosa	0/11	3/8

TPs stated to use the same plants for women in pregnancy as they use also for men or non-pregnant women. However, some TPs indicated to have a preference for one plant respect to another according to the manifestations of the pregnant patient. Only *Bidens pilosa* was not mentioned by any TP but in three of the women groups. TPs stated to treat pregnant women whatever the

period of pregnancy. However, they were aware and expressed on possible adverse outcomes of herbal treatments and as a precaution measure they apply reduced doses.

As it emerges from table 6 and 7 the frequency of plant citations by TPs seems not to match the number of citations given by the women groups for the respective plants. Considering TPs as holders of traditional medicine knowledge, we referred to TPs citation frequency to choose a tens of the most frequently employed plants for performing antimalarial efficacy studies. Thus, medicinal plants cited at least twice by TPs (Table 6) were selected. These 11 plant species are belonging to 9 families (Table 8). The *Asteraceae* and *Apocynaceae* family are represented by 2 plant species each and the remaining seven plants belong to the families of *Fabaceae*, *Poaceae*, *Burseraceae*, *Anarcardiaceae*, *Aloeaceae*, *Lamiaceae* or *Lauraceae*.

Table 8 Ethnobotanical information on the 11 selected, commonly used medicinal plants

Scientific name and family	**Vernacular name**	**Voucher number**	**Part used**	**Way of preparation , route of administration and posology**
Picralima nitida (Apocynaceae)	quinqueliba	25247/HNC	Seed	An Infusion is made with seeds from 3 or 4 fruits in 1 liter of water and a cup is administered orally three times a day. A decoction can be prepared also in some cases.
Cymbopongon citratus (Poaceae)	fibergrass/ citronelle	18628/SRC	Whole plant	A decoction is made with a handful of the whole plant or leaves only in 1 liter water. The obtained solution is given at a dose of 1 cup three times a day. If necessary even 4 times is advised.
Mangifera indica (Anarcardiaceae)	mango	60447/HNC	Leaves	A decoction is made with powdered dried leaves (or by using fresh young leaves) and water. the solution obtained is filtered and one cup is given three times a day
Ocimum gratissimum (Lamiaceae)	cotemanjo	44996/HNC	Leaves	A decoction is made with a handful of fresh leaves in 1 liter of water. 1 cup is given twice a day. Steam bath is also advised.
Persea americana (Lauraceae)	apiah	33945/HNC	Stem bark	A decoction is made with 3 tablespoons of powdered dried stem bark in 1 liter of water. Half a cup is given twice a day.
Solanocia mannii (Asteraceae)	kepan	22051/SRF Cam	Leaves	A decoction is made with 10 to 15 leaves in 0.5 liter of water. Half a cup 2 - 3 times per day. The solution is given with a full stomach to avoid vomiting because of bitter taste.
Dacryodes edulis (Burseraceae)	acup	64988/HNC	Leaves/ stem bark	A decoction is made with powdered dried leaves (or fresh leaves) and water. In case of stem bark 3 tablespoon of powder is used for 1 liter of water. The solution obtained is filtered and one cup is given three times a day.
Senna alata (Fabaceae)	meskayo	45146/HNC	leaves	A decoction is made with powdered dried leaves (young leaves) in water. 1 cup is given twice a day.
Aloe barbadensis (Aloeaceae)	aloe	Not received yet	Gel from leaves	3 tablespoons of gel are collected from leaves and put in 1 liter of water or 3 leaves are put directly in 1.5 liter of water for 24 hours. Then 1 to 2 cups are given early in the morning, once a day.

Voacanga africana (Apocynaceae)	atieuh-nieh	9227/SRF Cam	Leaves	A decoction is made with powdered dried leaves in water. Half a cup is given twice a day.
Eucalyptus globulus (Asteraceae)	acaltusi	4077/SRFC	Leaves	A decoction is made with a handful of fresh leaves or 5 table spoons of powdered dried leaves in1 liter of water. 1 cup is given twice a day, combined with steam bath if fever is high.

The table illustrates the herbal medicines commonly used by traditional healers and women in Menoua West Cameroon for prevention and cure of malaria during pregnancy and their mode of preparation and administration as stated by the interviewees.

Two plants, namely *Solanocia mannii* and *Voacanga africana*) were mentioned by TPs but not in any of the women discussion groups for the treatment of malaria in women during pregnancy.

In this study, it emerged that leaves were the most used plant part. Decoction was cited as the main mode of preparation by traditional healers and women and oral administration was the common way of treatment.

Five medicinal plants. i.e. *Picralima nitida*, *Ocimum gratissimum*, *Dacryodes edulis*, *Cymbopongon citratus* and *Aloe barbadensis* were cited to be used not only for the treatment of malaria in pregnant women but also for prevention (Table 9).

Table 9 Medicinal plants used for prevention of malaria in women during pregnancy.

Plant names	Part used	N. of citations by TPs	N. of citations by women groups
Picralima nitida	Fruit/seed	10/11	8/8
Ocimum gratissimum	Leaves	6	1
Dacryodes edulis	Leaves	5	6
Cymbopongon citratus	Whole plant	8	8
Aloe barbadensis	Gel	4	8

Table 10 Combination of plants mentioned by TPs and in women groups as commonly used for the treatment of malarial fever in women during pregnancy.

Plant combinations (part used)	**N. of citations by TPs**	**N.of citations by the women groups**	**Way of preparation , route of administration and posology**
Mangifera indica (Leaves) + *Cymbopongon citratus* (Whole plant)	3/11	3/8	A handful of leaf of each plant is mixed and boiled for 45 minutes to 1 hour in 1 liter of water. The solution is allowed to cool, filtered and approximately 250 ml (1 glass) of the solution is orally given twice a day during 2 days.
Dacryodes edulis (Leaves)+ *Cymbopongon citratus* (whole plant) + *Mangifera indica* (leaves)	5/11	6/08	Same as described above. The preparation is advised to be taken during 3 days in case of high fever and headache.
Mangifera indica (leaves)+ *Cymbopongon citratus* (whole plant)+ *Eucalytus globulus* (leaves)	4/11	3/08	A handful of *Mangifera indica* leaves and *Cymbopongon citratus* whole plant plus 3 tablespoons of *Eucalyptus globulus* powder are added in 1.5 liter of water. The solution is administered through steam bath for 15-20 min (until the patient starts swelling). Also, half a cup of the solution is administered orally two times a day for 3 days.
Eucalyptus globulus (leaves)+ *Cymbopongon citratus* (leaves)+ *Dacryodes edulis* (stem bark and leaves)	2/11	5/08	3 tablespoons of powdered stem bark of *D. edulis* with crushed leaves of the others plants are mixed in 1.5 liter of water and boiled for 45 minutes. The filtrate (250ml) of the decoction is given orally (half a cup two times a day) combined with steam bath.

Combinations of plants used to treat malaria: way of preparation and mode of administration

Preparation of antimalarial medicines and administration

This study revealed, that TPs and women caretakers use various methods for the preparation of herbal medicines. The most common way of preparation is decoction (>90%), i.e. by boiling the plant material in water before use. Cold maceration was cited as preparation method for *Aloe barbadensis* gel based remedy and infusion for *Picralima nitida*, a remedy based on the fruit's seeds.

Most of the herbal preparations are given orally, in some cases combined with steam bathing. The majority of the herbal remedies are administered twice a day, once in the morning and then in the evening. On average, herbal remedies preparations are conserved for about one to two weeks, preferably in containers with a cap.

TPs reported that as a general rule, lower doses are given to pregnant women considering their particular physiological status. However, TPs expressed to be aware that sometimes they don't know whether a presenting woman is pregnant unless her state becomes visible.

Not any adverse effects of the cited plants were mentioned either in women group discussions or by TPs. Only the bitter taste was mentioned as a problem for intake with some preparations such as *Picralima nitida* seed infusion and *Aloe barbadensis* gel maceration. However, honey or milk is recommended after administration of some herbal remedies, to mask the bitter taste that remains in the mouth.

7.2 Plant extracts

Methanol and water extracts have been prepared from 10 out of the 11 mostly cited antimalarial plants. Unfortunately, we did not achieve in procuring material from *Aloe barbadensis*. A total of 22 extracts were obtained (Table 11), with a yield ranging from 0.06 to 0.16 %w/w. Each extract was prepared with 50g of powdered plant material in 500 ml of solvent.

Table 11 Methanol and water extracts obtained

Scientific name of the plant (Family)	**Site of collection**	**Part used**	**Solvent used**	**Yield (%w/w)**	**Extract Code**
Persea americana (**Lauraceae**)	Baleveng	stem bark	methanol	0.14	E1
			water	0.11	E2
Dacryodes edulis (**Burseraceae**)	Baleveng	leaves	methanol	0.13	E3
			water	0.11	E4
		stem bark	methanol	0.09	E5
			water	0.12	E6
Ocimum gratissimum (**Lamiaceae**)	Foreke	leaves	methanol	0.12	E7
			water	0.11	E8
Solanocia mannii (**Asteraceae**)	Baleveng	leaves	methanol	0.12	E9
			water	0.13	E10
Eucalyptus globulus (**Myrtaceae**)	Baleveng	leaves	methanol	0.12	E11
			water	0.11	E12
Cymbopongon citratus (**Poaceae**)	Foto	leaves	methanol	0.12	E13
			water	0.13	E14
Voacanga africana (**Apocynaceae**)	Foto	leaves	methanol	0.11	E15
			water	0.16	E16
Mangifera indica (**Anarcardiaceae**)	Foto	leaves	methanol	0.16	E17
			water	0.06	E18
Senna alata (**Fabaceae**)	Foreke	leaves	methanol	0.09	E19
			water	0.12	E20
Picralima nitida (**Apocynaceae**)	Foreke	Seed	methanol	0.07	E21
			water	0.12	E22

7.3 In vitro activities of crude plant extracts against asexual stages of P. falciparum

Comparing the anti-plasmodial activity of methanol and aqueous extracts of the tested plants, consistently higher activity was found in the former. In 8 out of the 11 tested plant parts, IC_{50} values of the methanol preparation amounted to about one third to one half of that measured for the water extract, indicating a more efficient extraction of the bioactive components by the alcoholic solvent.

Extracts from 5 plants were found to reduce 50% of asexual blood stage development at concentrations < 20 µg/ml (Table 12). The methanol extract of *Dacryodes edulis* stem bark showed an IC50 value of 10,3 µg/ml tested on the CQ-sensitive (D10) strain and of 8,6 µg/ml on the CQ-resistant (W2) strain. *Eucalyptus globulus* water and methanol extract showed an IC50 value of 19,2 and 17,7 µg/ml on D10 strain, and 19.8 and 16,4 µg/ml on W2 strain respectively.

Table 12 I*n vitro* activity of investigated crude plant extracts against asexual blood stages of *W2*, CQ-R, and *3D7*, CQ-S *P. falciparum* strains and comparison with data in the literature.

Plant name and plant part	**Extract code (extrac-tion solvent)**	**Activity against asexual blood stages; IC_{50} in µg/ml, S.D**		**Activity reported in literature**	
		D10 strain (CQ-sensitive)	**W2 strain (CQ-resistant)**	**IC_{50}(µg/ml) against asexual stages**	**Reference**
Dacryodes edulis	E3 (meth.)	24.9 ± 5.8	20.9 ± 4.4	4.3 on *3D7* and 6.4 on *Dd2* strain - CH_2Cl_2 used as extraction solvent	Zofou et al., 2011
	E4 (water)	38.6 ± 13.3	30.0 ± 5.6		
E3, E4 leaves;	E5 (meth.)	**10.3 ± 2.8**	**8.6 ± 3.3**	6.4 on 3D7 and 8.2 on Dd2 strain -	Zofou et

E5, E6 stem bark	E6 (water)	17.9 ± 3.1	15.0 ± 1.3	CH2Cl2+MeOH+hexane extraction solvent mixture of leaves	al., 2011
Persea americana stem bark	E1 (meth.)	**19.8 ± 1.8**	**19.9 ± 7.3**	Not reported in the literature	
	E2 (water)	34.2 ± 9.3	60.5 ± 13.2		
Picralima nitida seeds	E21 (meth.)	27.2 ± 2.6	20.7 ± 3.6	10.9 on CQ-resistant *W2* strain-MeOH solvent	Bickii et al., 2007
	E22 (water)	73.3 ± 15.8	71.2 ± 12.9		
Ocimum gratissimum leaves	E7 (meth.)	36.3 ± 12.1	31.0 ± 5.2	81.4 and 778.9 on *3D7* strain for EtOH and H2O extracts respectively; 121.5 and 118.9 on *Dd2* strains for EtOH and H2O extracts, respectively.	Tarkang et al. 2014
	E8 (water)	52.2 ± 21.3	43.2 ± 6.3		
Solanocia mannii leaves	E9 (meth.)	54.1 ± 9.9	47.2 ± 4.1	Not reported in the literature	
	E10 (water)	> 100.0	> 100.0		
Eucalyptus globulus leaves	E11 (meth.)	**17.7 ± 3.1**	**16.4± 3.4**	16.8 *3D7* and 26.45 *Dd2* parasite strain - CH2Cl2+MeOH+hexane	Zofou et al. 2011
	E12 (water)	**19.2 ± 6.5**	**19.8 ± 0.9**		
Cymbopongon citratus leaves	E13 meth.)	25.3± 4.0	**12.2 ± 1.0**	28.8 and 723.3 on *3D7* parasite strain for EtOH and H2O extracts respectively; and 54.8 and 141.0 on *Dd2* parasite strain for EtOH and H2O extracts, respectively.	Tarkang et al. 2014
	E14 (water)	> 100.0	> 100.0		
Voacanga africana leaves	E15 (meth.)	**16.4 ± 4.3**	**3.9 ± 0**	Not reported in the literature	
	E16 (water)	> 100.0	> 100.0		
Mangifera indica leaves	E17 (meth.)	44.3 ± 2.3	95.2 ± 24.3	24.3 and 82.5 on *3D7* parasite strain for EtOH and H2O extracts respectively; and 16.3 and 40.0 on *Dd2* parasite strain for EtOH and H2O extracts, respectively.	Tarkang et al. 2014
	E18 (water)	54.6 ± 9.8	69.2 ± 8.8		
Senna alata leaves	E19 (meth.)	> 100.0	> 100.0	7.02 on*D10* CQ-sensitive CH2Cl2+MeOH solvent mixture	Yerbanga et al. 2016
	E20 (water)	> 100.0	> 100.0		
Chloroquine control		0.005	0.13		

Notes: *CH2Cl2: dichloromethane; MeOH: methanol; EtOH: ethanol; H2O: water; 3D7:P. falciparum CQ-sensitive; Dd2: multidrug resistant strains of P. falciparum;* S.D= standard deviation; The results are the mean IC_{50} of three independent experiments performed in duplicate on *D10* and *W2 P. falciparum* strains.

With *Cymbopongon citratus* methanol extract an IC_{50} value below 20 µg/ml was observed on W2 (12,2 µg/ml) but not on D10 strain (25,3 µg/ml). The level of activity of these three plants observed in our tests is comparable with that reported in literature (Table 12). The methanol extract from *Voacanga africana* leaves (E15) was the most active extract among the tested plants. An IC_{50} value of 3,9 µg/ml was measured against the *P. falciparum* CQ-resistant (W2) strain. Ours is the first *in vitro* study of this plant. Also on *Persea americana* and *Solanocia mannii* we haven't found any reports, however only the stem bark methanol extract of the former showed some activity on D10 (19,8 µg/ml) and W2 (19,9 µg/ml) strain.

7.4 In vitro activity of plant extracts against stage V gametocytes of P. falciparum

At a primary screening dose of 100 µg/ml methanol extracts of only 5 plants out of the 22 investigated extracts showed a greater than 50% reduction of stage V gametocytes' viability when tested in cultures with the *P. falciparum 3D7elo1-pfs16-CBG99* strain. Among the 5 tested extracts, *Persea americana* stem bark methanol extract resulted to be the most active reducing the viability of mature gametocytes by 94% at this concentration. Dose range assays revealed a very low activity of the 5 extracts against the sexual stages of *P. falciparum*. *Persea americana* confirmed to be the relatively more active plant reducing 50% of gametocyte viability at a

concentration of 34,7 µg/ml. The IC50 values of the other 4 plants exceed 50 µg/ml.

Table 13 *In-vitro* gametocytocidal activity of plant extracts on *Plasmodium falciparum* mature stage V gametocyte.

Plants name and plant part	Extract code (extraction solvent)	Activity against asexual blood stages; IC_{50} in µg/ml; S.D		Activity against stage V gametocytes of *P. falciparum 3D7elo1-pfs16-CBG99 strain*	
		D 10 strain (CQ-sensitive)	W2 strain (CQ-resistant)	% of development at 100 µg/ml compared to controls; S.D [a]	IC_{50} (µg/ml) ; S.D [b]
Dacryodes edulis	E3 (meth.)	24.9 ± 5.8	20.9 ± 4.4	**42**	**84.7 ± 15**
	E4 (water)	38.6 ± 13.3	30.0 ± 5.6	60 ± 22	
E3, E4 leaves;	E5 (meth.)	**10.3 ± 2.8**	**8.6 ± 3.3**	79 ± 11	
E5, E6 stem bark	E6 (water)	17.9 ± 3.1	15.0 ± 1.3	63 ±16	
Persea americana stem bark	E1 (meth.)	**19.8 ± 1.8**	**19.9 ± 7.3**	**6**	**34.7 ± 5**
	E2 (water)	34.2 ± 9.3	60.5 ± 13.2	69 ± 21	
Picralima nitida	E21 (meth.)	27.2 ± 2.6	20.7 ± 3.6	**33**	**80.9 ± 17**
seeds	E22 (water)	73.3 ± 15.8	71.2 ± 12.9	84 ± 15	
Ocimum gratissimum leaves	E7 (meth.)	36.3 ± 12.1	31.0 ± 5.2	54 ± 25	
	E8 (water)	52.2 ± 21.3	43.2 ± 6.3	77 ± 6	
Solanocia mannii	E9 (meth.)	54.1 ± 9.9	47.2 ± 4.1	66 ± 23	
leaves	E10 (water)	> 100.0	> 100.0	86 ± 10	
Eucalyptus globulus leaves	E11 (meth.)	**17.7 ± 3.1**	**16.4± 3.4**	**27**	**67.9 ± 15**
	E12 (water)	**19.2 ± 6.5**	**19.8 ± 0.9**	65 ± 1	
Cymbopongon citratus leaves	E13 (meth.)	25.3± 4.0	**12.2 ± 1.0**	60 ± 11	
	E14 (water)	> 100.0	> 100.0	81 ± 15	
Voacanga africana	E15 (meth.)	**16.4 ± 4.3**	**3.9 ± 0**	**47**	**74.2 ± 23**
	E16 (water)	> 100.0	> 100.0	90 ± 11	

leaves					
Mangifera indica	E17 (meth.)	44.3 ± 2.3	95.2 ± 24.3	76 ± 10	
leaves	E18 (water)	54.6 ± 9.8	69.2 ± 8.8	77 ± 2	
Senna alata	E19 (meth.)	> 100.0	> 100.0	103 ± 6	
leaves	E20 (water)	> 100.0	> 100.0	107 ± 11	
CQ		0.0055	0.13		
MB					0.023 ± 0.009

Note: Data are the mean of two or three different experiments in duplicate wells; CQ: chloroquine; MB: methylene blue; S.D= standard deviation.

(a) The results are percentage of viable gametocytes compared to untreated control wells of two or three experiments performed in duplicate/triplicate.

(b) The results are the mean IC50 of three independent experiments performed in duplicate.

7.5 Effects of plant extracts on early sporogonic stages of P. berghei

At the initial screening dosage of 50 µg/ml, inhibitory effects on early sporogonic development were observed with water extracts of *Persea americana* stem bark and *Dacryodes edulis* stem bark (Figure 13 A). At this high dosage, the number of early sporogonic stages (ESS), i.e. the total counts of fluorescent zygotes, retort forms (early ookinete stage) and elongated ookinetes, was reduced by about 60% with *Dacryodes edulis* stem bark and by about 80% with *Persea americana* stem bark extract. None of the other water extract preparations reduced ESS by more than 40%.

The methanol extracts from the same plants and the same plant part, i.e. stem bark of *Dacryodes edulis* and *Persea americana* were also the most

active inhibiting ESS development by about 80% and almost 100% respectively (Figure 13 B). The methanol extracts of *Dacryodes edulis* leaves reduced early sporogonic development by 70% and that of *Ocimum gratissimum* leaves 60%. The majority of the water and methanol extracts reduced with similarly ESS counts and numbers of developing ookinetes, indicating activity on various stages of early sporogonic development. However, in the case of the methanol extracts from *Picralima nitida* seeds, *Voacanga africana* leaves and *Eucalyptus globulus* leaves a slightly stronger impact was noted on OM (ookinete maturation) counts than on ESS counts (early sporogonic stages), indicating relatively more activity on the zygote to ookinete development than on processes of gamete formation and fecundation, the sexual processes preceding zygote formation in the mosquito midgut.

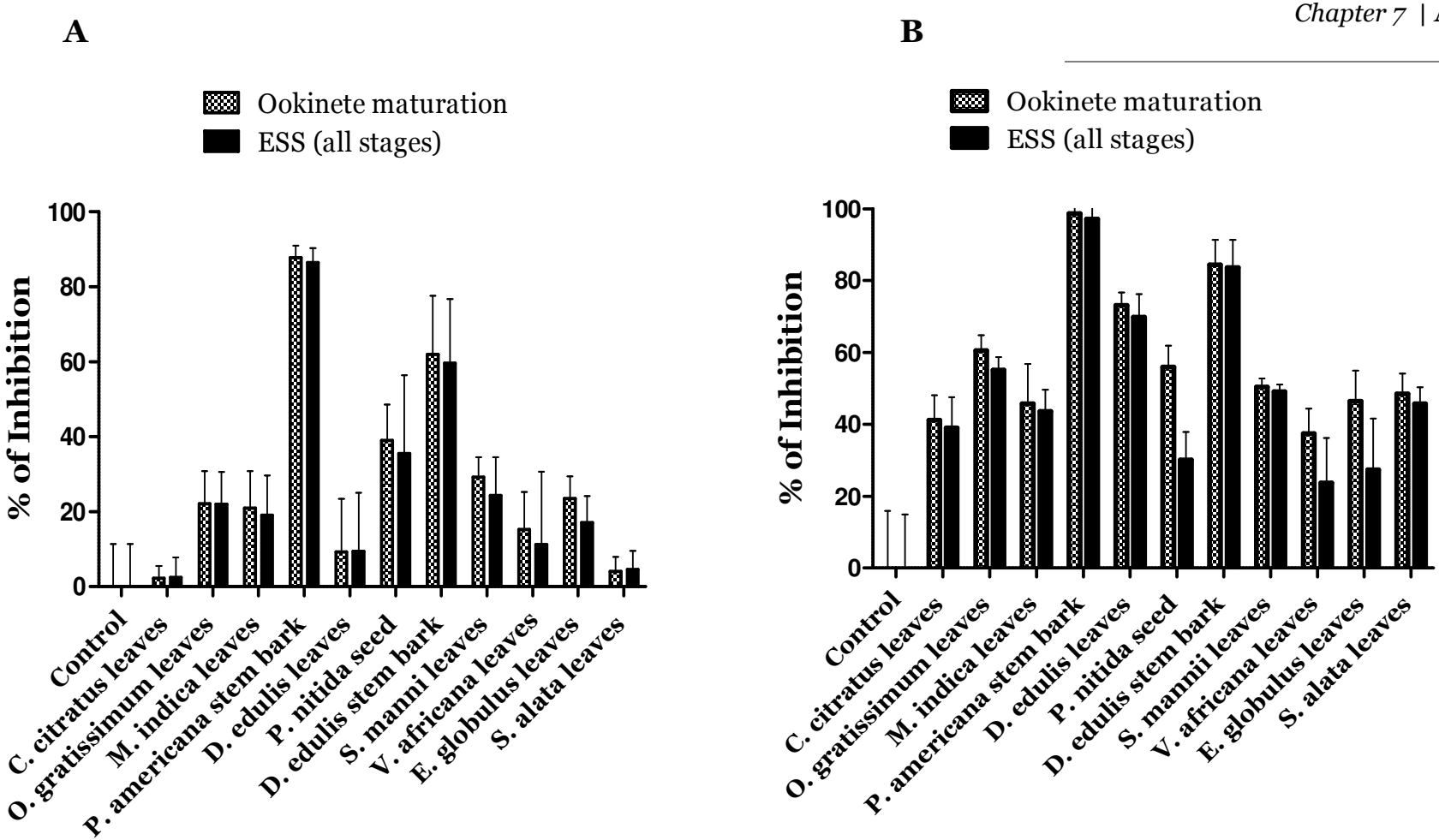


Figure 10 A and B Effects of the water (**A**) and methanol (**B**) crude extracts on early sporogonic development at 50 µg/ml against *P. berghei CTRPp.GFP*. Black bars: impact (% inhibition) on the formation of early sporogonic stages, counting all forms (zygotes, retort forms and fully elongated ookinetes); Grey bars: impact (% inhibition) on the formation of ookinetes counting elongated forms (retorts, stumpy and fully elongated ookinetes). Vertical bars depict standard deviation (SD).

Based on the primary screening results, the most active preparations, i.e. the methanol extract of *Persea americana* stem bark and *Dacryodes edulis* stem bark were selected for further examination. Tested at various concentrations in the range of 1.56 to 100 µg/ml, both plant extracts displayed a dose-dependent inhibitory activity (Figure 14 A and B). Also both, showed a slightly stronger effect on ookinete development (OM counts) than on total ESS counts (Table 14).

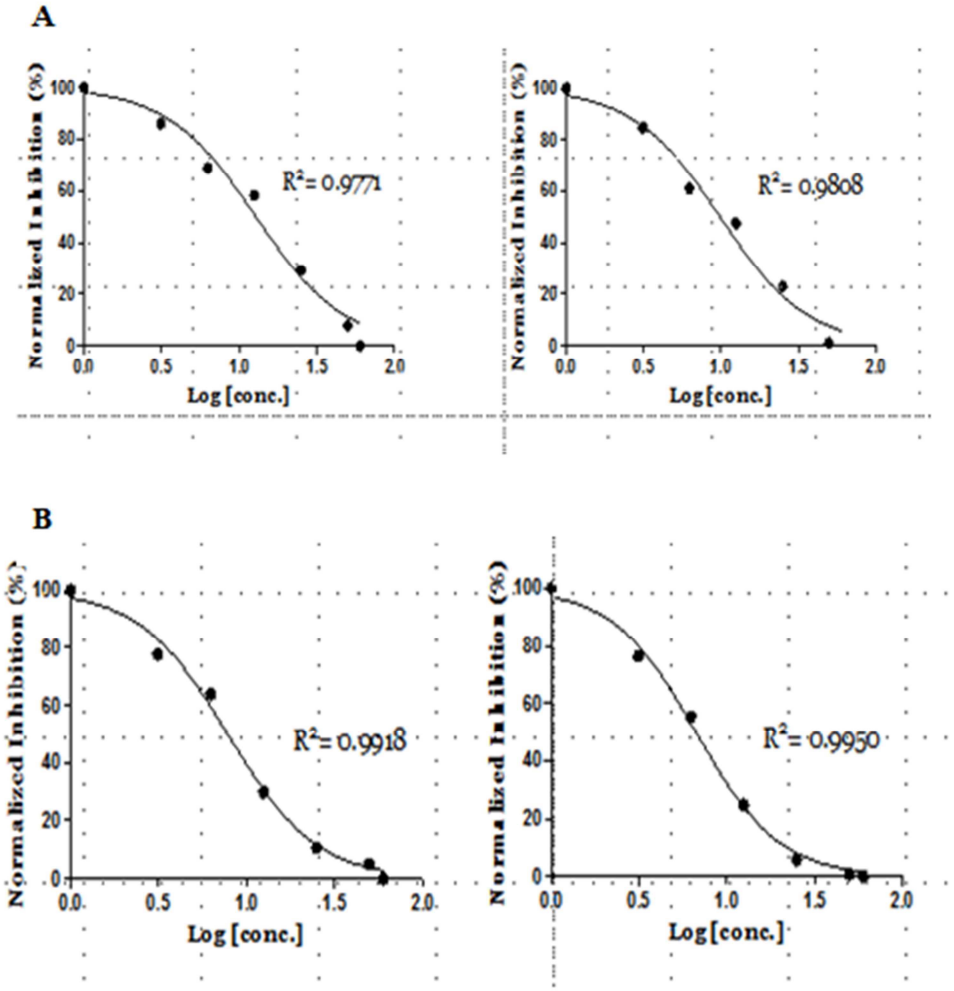


Figure 11 A and B: Dose dependent inhibitory activity of *D. edulis and P. americana* stem bark methanol extract on early sporogonic stages (ESS; left side) and on ookinete maturation

(OM; retort forms and elongated ookinetes; right side. **A**: *Dacryodes edulis* stem bark methanol extract; **B**: *Persea americana* stem bark methanol extract.

Table 14 IC_{50} values of the 2 most active extracts on early sporogonic stages and ookinete maturation.

Extract name	IC_{50} (µg/ml) [95% C.I.]	
	ESS[1] inhibition	OM[2] inhibition
Persea americana stem bark methanol extract	7.83 [7.33 - 8.36]	6.67 [6.33 - 7.01]
Dacryodes edulis stem bark methanol extract	12.81 [11.45 - 14.34]	9.88 [8.89 - 10.99]

1 Early sporogonic stages
2 OM: Ookinete maturation

The *Persea americana* extract reduced 50% of ESS counts at a concentration of 7.83 µg/ml and of OM counts at 6.67 µg/ml. For *Dacryodes edulis* IC50 values determined regarding ESS counts amounted 12.81 µg/ml and OM counts 9.88 µg/ml (Table 14).

7.6 Activity of methanol extracts of P. americana and D. edulis on male gametogenesis of P. berghei CTRPgfp

The active extracts, namely *Persea americana* and *Dacryodes edulis* stem bark methanol extracts were tested in the exflagellation assay to assess

whether the effect observed on ESS development in the ODA was due to a specific action on microgametogenesis.

Each extract was tested at the concentration that was found to reduce 50% of ESS counts in the ODA. *Persea americana* tested at 8 µg/ml was found to inhibit about 50% of microgamete formation, estimated as numbers of exflagellation centers. A similar inhibition of microgametogenesis was observed with *Dacryodes edulis* at 13 µg/ml, indicating that the impact on ESS counts recorded in the ODA is to a large extend explainable by the presence of extract components interfering with microgametogenesis. (Figure 15 A).

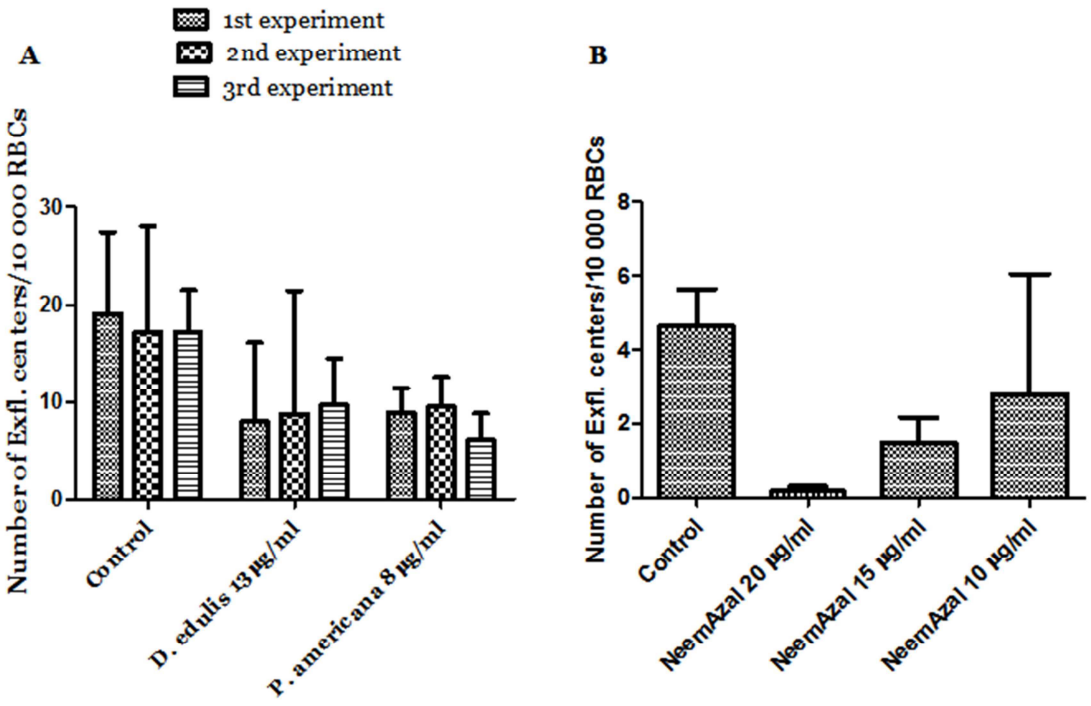


Figure 12 A and **B: A:** Activity of plant preparations on the formation of exflagellation centers at doses corresponding to IC_{50} values estimated in the ODA. **A:** *Persea americana* and *Dacryodes edilis* methanol extracts; **B:** NeemAzal® tested as a positive reference; negative control: DMSO 0.1% used to dissolve the extracts. Vertical bar represent the standard deviation (SD). Three consecutive experiments with different gametocytemic mice were conducted and three chamber replicates performed in each.

For comparison, exflagellation tests were also performed with NeemAzal®, a commercial azadirachtin A rich neem kernel product, extensively studied in our group and known to interfere with early sporogonic development (Dahiya et al., 2016a) and micro-gametogenesis (Billker et al., 2002). Its IC50 value in the ODA amounts to about 12 µg/ml. Tested in the exflagellation assay at 10, 15 and 20 µg/ml, the dose of 10 µg/ml appears sufficient to reduce about half of micro-gametogenesis, suggesting – as in the case of the 2 plant

extracts – that micro-gametogenesis is probably the main target of NeemAzal's effects on early sporogonic development.

7.7 Assessment of *in-vivo* transmission blocking activity of *P. americana* and *D. edulis* stem bark methanol extracts

Experiments in the Direct Feeding Assay (DFA) were performed to explore the transmission blocking characteristics of stem bark methanol extract from *P. americana* and *D. edulis in vivo*. The two extracts, i.p. administrated to gametocytemic mice at 150mg/kg were found to reduce by about ¾ the sporogonic development in the mosquito host after infective blood feeds on treated mice. Taking as experimental readout numbers of oocysts developing on mosquito mid guts, a reduction in oocyst density by 73% (95% CI: 55.7–90.5%) and 74% (95% CI: 55.4 - 102.8) was recorded with *Persea americana* and *Dacryoles edulis* respectively (Table 16). In mosquito groups fed on extract treated mice, a mean number of 89 and 87 oocysts were counted in the *Persea americana* and *Dacryodes edulis* treatment groups compared to 331 in control mosquitoes. Plant extract treatment did not affect the prevalence of infected mosquitoes. Infection rates ranged from 93 – 100% in control mosquitoes, from 83 – 97% in *Persea americana* treated and amounted to 93 and 97% in the two replicate cages of *Dacryoles edulis* treated mosquitoes (one mouse died).

Table 15 *In vivo* transmission blocking effects of methanol stem bark extracts from *P. americana* and *D. edulis* measured in the DFA using *Anopheles stephensi* mosquitoes.

Group	Mouse ID *	Prevalence of infected mosquitoes (infected / total examined)	Oocyst density by mouse replicate (95% CI)	Oocyst density by treatment group (95% CI)	(%) Oocyst reduction (95% CI)
P. americana stem bark - methanol extract	1	93.3 (28/30)	94.1 (72 - 116.2)	89 (72 - 106)	73 (55.7 - 90.5)
	2	83.3 (25/30)	56.9 (40.1 - 73.7)		
	3	96.7 (29/30)	115.9 (102.5 - 129.3)		
D. edulis stem bark - methanol extract	1	93.3 (28/30)	63.4 (34.5 - 92.3)	87 (58 - 116)	74 (55.4 - 102.8)
	2	96.7 (29/30)	110.4 (80.9 - 140.0)		
	3**				
Solvent control	1	100 (30/30)	401.0 (360.2 - 441.8)	331 (278 - 385)	
	2	93.3 (28/30)	279.8 (214.1 - 345.4)		
	3	96.7 (29/30)	312.4 (258.4 - 366.4)		

The oocyst densities (geometric mean) were calculated on oocyst positive mosquitoes only.
* Each number represents one gametocytaemic treatment or control mouse used for the infection of a separate batch of mosquitoes (30 females per mouse).
** mouse died shortly after treatment

7.8 Cytotoxicity of plant extracts

All the 22 plant extracts were screened for cytotoxic effects on normal fibroblast cells (Table 16). Each extract was tested at 8 different concentrations ranging from 150 – 1,17 µg/ml.

Seven (methanol extract of *D. edulis* stem bark and leaves, water extract of *P. americana*, water extract of *P. nitida*, water extract *S. mannii*, and water and methanol extract of *S. alata*) out of the 22 extracts resulted slightly toxic on the fibroblast cell line only at the highest test concentration of 150 µg/ml. Thus, we were unable to determine the IC_{50}.

For *Eucalyptus globulus* and *Voacanga africana* leaves methanol extract, having revealed slight activity at primary screening, IC50 values were determined and resulted 85.2 µg/ml (95% CI: 78.5 - 92.8) and 88.1 µg/ml (79.3 - 98.7), respectively.

In order to confirm results obtained with the normal fibroblast cell line, *Eucalyptus globulus*, *Voacanga africana* and the plant extract having revealed activity against early sporogonic stages were also tested on the normal endothelial cell line *EA.hy926*. Whereas *E. globulus* and *V. africana* resulted nontoxic (no activity at 120 and 150 µg/ml respectively) *Persea americana* and *Dacryodes edulis* reduced cell viability by 50% at a concentration of 81.17 µg/ml (95% CI 71.99 - 91.95) and 70.05 µg/ml (95% CI 60.37 - 82.05) respectively. Thus, although no cytotoxic effects were found on normal fibroblasts at the highest dosage of 150 µg/ml, the two extracts were found to possess a slight cytotoxic effect on the *EA.hy26* mammalian cell line. The selectivity index (SI: ratio of mammalian cell viability to ESS development inhibition) determined for *Persea americana* amounts 10.36 and that for *Dacryodes edulis* 5.53 (Table 17).

Interestingly, almost all the 22 extracts have shown to have some anti-tumor effects reducing cell viability of melanoma cells and / or human breast cancer cells (Table 18). Prominent activity was found with the methanol extract of *Dacryodes edulis* stem bark with which an IC50 value of 6.23 µg/ml (95% CI, 5.65 - 6.89) was recorded on the melanoma cell line and with *Picralima nitida* that gave an IC50 value of 10.47 µg/ml (95% CI, 9.692 - 11.31) on the same cell line.

Table 16 Activity of the water and methanol extracts on human breast cancer, human melanoma cancer, and normal endothelial and fibroblast cell lines.

		Cell line, IC_{50} µg/ml (95% CI)		
Plants name and plant part	**Extract code (extraction solvent)**	***A375*** Human breast cancer cell line	***MDA-MB231*** Human melanoma cell line	***NHF-A12 Fibroblast*** Normal cell line
Dacryodes edulis E3, E4 leaves; E5, E6 stem bark	E3 (methanol)	42.52 (37.09 - 48.76)	13.18 (12.13 - 14.32)	>150
	E4 (water)	55.31 (48.59 - 62.96)	17.81 (14.69 - 21.59)	>150
	E5 (methanol)	29.13 (24.73 - 34.31)	6.23 (5.646 - 6.888)	>150
	E6 (water)	79.13 (70.06 - 89.37)	57.18 (52.31 - 62.52)	>150
Persea americana stem bark	E1 (methanol)	43.64 (38.74 - 49.17)	17.67 (15.62 - 20.00)	>150
	E2 (water)	55.58 (41.73 - 74.03)	87.05 (71.73 - 105.60)	>150
Picralima nitida seeds	E21 (methanol)	41.78 (33.61 - 51.95)	10.47 (9.692 - 11.31)	>150
	E22 (water)	16.50 (6.004 - 45.36)	36.46 (32.97 - 40.32)	>150
Ocimum gratissimum leaves	E7 (methanol)	19.23 (9.654 - 38.29	27.26 (15.42 - 48.18)	>150
	E8 (water)	31.11 (22.69 - 42.66)	28.88 (24.42 - 34.16)	>150
Solanocia mannii leaves	E9 (methanol)	54.59 (40.62 - 73.37)	18.77 (9.813 - 35.90)	>150
	E10 (water)	22.82 (16.64 - 31.31)	33.66 (24.52 - 46.19)	>150
Eucalyptus globulus leaves	E11 (methanol)	19.89 (18.38 - 21.52)	>100	85.2 (78.5 - 92.8)
	E12 (water)	43.09 (38.59 - 48.12)	17.58 (15.36 - 20.13)	>150
Cymbopongon citratus leaves	E13 (methanol)	24.22 (16.76 - 33.77)	18.98 (14.91 - 29.90)	>150
	E14 (water)	34.22 (26.76 - 43.77)	22.98 (18.93 - 27.90)	>150
Voacanga africana leaves	E15 (methanol)	29.52 (27.47 - 31.72)	72.98 (68.95 - 77.26)	88.1 (79.3 - 98.7)
	E16 (water)	38.05 (26.07 - 55.54)	>100	>150
Mangifera indica leaves	E17 (methanol)	77.98 (70.32 - 86.46)	50.20 (45.27 - 55.67)	>150
	E18 (water)	42.48 (22.62 - 79.77)	32.12 (26.71 - 38.64)	>150
Senna alata leaves	E19 (methanol)	44.72 (88.75 - 49.22)	22.30 (19.43 - 38.06)	>150
	E20 (water)	34.72 (28.55 - 42.22)	25.00 (17.33 - 36.06)	>150

Table 17 Selectivity index (SI) of *Persea americana* and *Dacryodes edulis* extracts

	IC50 value in µg/ml		
Plant extracts	Activity on endothelial cell viability	Activity on early sporogonic stage development	Selectivity Index (SI)
Persea americana stem bark methanol extract	81.17	7.83	10.36
Dacryodes edulis stem bark methanol extract	70.08	12.81	5.53

SI: Selectivity Index value= IC50 on mammalian cell line viability/IC50 on ESS development

In addition, to explore whether the extracts possess mosquitocidal potential, when taken up by the blood feeding female, we assessed cytotoxicity on an insect cell line. Effects of stem bark methanol extracts from *Persea americana* and *Dacryodes edulis* were estimated using the normal insect cell line Sf9 (*Spodoptera frugiperda*) and evaluation of toxicity was performed after different times of incubation (24, 48 hours, 6 days). NeemAzal®, known from previous studies (Dembo et al., 2015) to affect insect fitness was also included in the tests as a positive reference.

Table 18 cytotoxic activity of stem bark methanol extract from *P. americana* and *D. edulis* and of NeemAzal on the Sf9 insect cell line after 24hrs, 48hrs and 6 days of incubation.

Plants extracts	***Activity on Sf9* insect cell viability, IC_{50} µg/ml (95% CI)**		
	24hrs	**48hrs**	**6 days**
Persea americana stem bark methanol extract	21.80 (15.45 - 30.75)	18.20 (21.33 - 24.23)	15.61 (13.39 - 18.20)
Dacryodes edulis stem bark	53.79 (42.40 - 68.24)	24.60 (21.20 - 28.55)	11.98 (9.909 - 14.47)
NeemAzal®	4.51 (1.739 - 11.70)	4.35 (3.59 - 6.44)	6.39 (5.45 - 7.50)

IC50 values of *Dacryodes edulis* decrease by half comparing the extracts activity after 24 hrs, 48 hours and 6 days of incubation, indicating an evident increase in activity with incubation time. In wells incubated for 6 days 11,98 µg/ml of *D. edulis* extract are enough to kill 50 % of the insect cells. A similar IC50 value of 15,61 was determined for *Persea americana* after 6 days of incubation. NeemAzal® showed a cytotoxic effect on the Sf9 insect cell line twice as potent as the two plant extracts and effectively killed insect cells already after 24 hrs of incubation. IC50 values determined for NeemAzal® at the 3 time points were in the range of 4,35 – 6,39 µg/ml.

8 Discussion

In the present Ph.D. study, a gender oriented ethnobotanical survey was conducted with the objective to identify and validate medicinal plants used by TPs and women to prevent and treat malaria in pregnancy, focusing on the Menoua district (West Region of Cameroon) as study area. This study aims to fill at least part of the gap by documenting medicinal plants used by TPs and women in the here mentioned community for the management of malaria during pregnancy, a topic that has been scarcely investigated, although we know pregnant women represent a considerable proportion in those communities and would need more attention due to their particular condition. Furthermore, to validate their use as anti-plasmodial agents, the identified plants were screened for their *in-vitro* activity against *Plasmodium falciparum* asexual blood-stages and gametocytes and against sporogonic stages of *Plasmodium berghei in-vitro* and *in-vivo,* in an attempt to formulate an improved traditional remedy for women in pregnancy, targeting multiple stages of the *Plasmodium* life cycle.

8.1 Medicinal plants identification and ethnobotanical information collected

In our ethnobotanical study, a total of 50 respondents (Table 2 and 3) were involved comprising 12 traditional healers (one woman with her husband) and 38 women (in 8 discussion groups consisting of 4 to 6 women). The

educational background consisted of 64% and 52.6% of the participants with no formal or primary education only, regarding TPs and women, respectively. Moreover, the median age of TP respondents was 56 years and that of the women participating to the group discussion ranged from 22 to 56 years, with a majority of women having more than 36 years of age. Similarly, in a study conducted by Yemele et al., 2015 in the same division of the country (Yemele et al., 2015), among the totally 203 persons interviewed a relatively smaller proportion of TPs (12%) were involved than women (87%). This "disparity" is related to the fact that traditional healers are present in very low numbers in the study area and difficult to retrieve. However, the relative low number of TPs involved in the survey may be also due to the fact that, although all the interviewers were native or able to speak the local language, TPs still are reluctant to unveil their traditional medicine knowledge. Some have claimed that their earnings are so scarce (many people rely on self-treatment purchasing the plants on local markets) that they need to get compensated by whatever monetary mechanism for sharing their knowledge.

It emerged also from this study that older aged persons, i.e. over 46 years (10 TPs and 08 women in this study), had a profounder knowledge on medicinal plants and their uses than the younger study participants. This is due to their long direct contact with plant resources and their use in over years in patients. On the other hand, most young people have little interest in traditional medicine in general and there appears to be a risk of knowledge loss if nothing is done to motivate TPs to share them with the scientific

community. Indeed, most often young men and women to whom traditional knowledge on medicinal plants effects should been transmitted are not interested and prefer to move to cities in search for other work opportunities and where they find more easier conditions of live. In addition, young people are getting exposed to modern medicine through the public education system, acquiring knowledge on health problems and confidence on resolving them by modern medicine approaches. Therefore the educated generations are little interested in learning and practicing ethnobotanical wisdom that would impart them indigenous knowledge. Differences in medicinal plants knowledge according to age but also by ethnical group was reported in a study conducted in Ethiopia (Chekole et al., 2015). The respondents were in majority belonging to the Ateso group (68%) who had a stronger background in knowledge on medicinal plants than Bagwere people (32%). In that study, most respondents were educated up to the level of secondary education (98%).

TPs in our study area, although they have a lower level of education compared with that of the women involved, participate to the monthly recycling courses organized by the Dschang Health District in collaboration with REPLAMET, the TPs organization. This might also explain their ability to recognize the signs and symptoms of fevers due to malaria referring to the terminology used by modern medicine (Table 5). Similarly, a high acquaintance has been noted among women during the group discussions,

probably because of health training received on major health issues at the local associations. For example, members of the women association of PIPAD participated to the awareness raising campaigns on sexual transmitted diseases and family planning, promoted by the Dschang Health District.

Indigenous people from different localities have their own specific knowledge of plant use and management. It has been suggested that the varied employment of medicinal plants is one of the most significant ways in which humans directly reap the benefits provided by biodiversity. Treating malaria during pregnancy is a well-established practice in the study area. During our field survey, nineteen plant species (Table 6) were identified as being used by the community as antimalarial plants in pregnant women. All the medicinal plants were reported in local language (Yemba) or French. Eleven out of the 19 medicinal plants were considered as commonly used in the community for the treatment of malaria (Table 8). This might be linked to their perceived broad beneficial effects, i.e. not only for the treatment of malaria but also for the management of various other ailments (Focho et al., 2009). *Cymbopongon citratus* was the most cited plant as in the case of the study of Yemele et al., 2015 carried out in the same communities (Yemele et al., 2015). The 11 commonly used medicinal plants are belonging to the family of *Apocynaceae* (2 species), *Asteraceae (2 species), Poaceae (1 species), Anarcardiaceae (1 species)*, *Lamiaceae (1 species)*, *Lauraceae (1 species)*, *Burseraceae (1 species)*, *Fabaceae* (1 species) and *Aloeaceae* (1 species).

The study conducted by Norden et al., 2013 in Mali identified 48 medicinal plants used traditionally to cure malarial fever in pregnant women (Nordeng et al., 2013). The much higher number of plants identified in the Malian study compared to ours might reflect a truly higher amount of plants used in the Malian traditional medicine or might be due to the different ethnobotanical methodology employed (medicinal plants were presented to the respondents visually and by mentioning the local names of the plants).

Antimalarial plants belonging to the family of Asteraceae have been identified also by other studies carried out in different localities of Cameroon (Telefo et al., 2011; Tsobou et al., 2016; Yemele et al., 2015). Species of this plant family are among the most frequent ones, growing widely in the country (Dibong et al., 2011; Focho et al., 2009). The common utilization of plants belonging to the family of Asteraceae and Apocynaceae (in different areas of Cameroon) for the treatment of malaria in women during pregnancy, is supported by the presence of bioactive secondary metabolites that are effective against the malaria parasite (Kumari P. et al., 2017; Tsobou et al., 2016). This wide use may also be attributed to a high degree of sharing the traditional knowledge on malaria treatment in pregnancy by people living in the study area and neighboring areas (Telefo et al., 2011) related also to the perceived high effectiveness of the medicinal plants as reported by the study participants.

Apart from treating malaria during pregnancy with these 19 medicinal plants, five of them have also been mentioned to be employed for the prevention of malaria in pregnancy (Table 9). *C. citratus* and *P. nitida* were cited by all women groups and almost all TPs, as plants used for the management of malaria (prevention and treatment). Also in the study of Yemele et al. 2015, *C. citratus* yielded a high frequency of citation, probably also because this plant is readily available in the villages and easily cultivable even at homes. Interestingly, Focho et al., 2009, reported that *Cymbopogon citratus* is also used for the treatment of swelling of the legs, a typical ailment of women in advanced pregnancy (Yemele et al., 2015).

Different parts of the plants were stated to be used including leaves, seeds and stem bark, among which leaves resulted to be the most used plant part for the preparation of remedies in the study area. This result is comparable to findings published by Tsobou et al., 2009, and Yemele et al., 2015. In fact, leaves are known to accumulate plants secondary metabolites such as alkaloids, tannins and inulins, which are bioactive components responsible for many medicinal properties (Focho et al., 2009). Moreover, utilization of leaves and stem bark is advantageous for the survival of plants since their harvest does not induce the irreversible destruction of them as it is the case when roots or whole plants are being used (Telefo et al., 2012).

Combination of 2 and more plant species was also mentioned by the interviewees to be practiced for the preparation of remedies. TPs and caretakers of the study area prepare remedies for malaria treatment, either from single plants or plant parts, or by mixing several of them (not more than three plants usually). Four different combinations were reported by participants of this study based on the combinations of four different plants species, namely *Mangifera indica*, *Cymbopongon citratus, Dacryodes edulis* and *Eucalyptus globulus* (Table 10). The most cited combination by both TPs (5/11) and women group respondents (6/8) is the infusion based on *Cymbopongon citratus* and *Dacryodes edulis* leaves, an antimalarial preparation well known in all the western region of Cameroon (Olivier et al., 2016). Traditional remedies often consist of a combination of several plants, being aware that specific plants used in the combination have therapeutic effects acting against signs and symptoms such as fever, body pain, vomiting and others, harbor components that are effective against the parasite, either directly or by exerting immunomodulatory effects. The different constituents of the mixture can exert an additive or synergistic action against the various signs and symptoms of malaria. However, TPs expressed awareness on the fact that particular plant combinations may also provoke adverse or toxic effects and thus, if taken during pregnancy such effects may affect the unborn child. TPs reported that as a general rule, lowered doses are given to pregnant women considering their particular physiological status. In a study in Mali conducted by Nordeng et al., 2013, it was reported that, TPs generally

recommend pregnant women to avoid medicinal plants with bitter taste like for example the stem and root bark of *Khaya senegalensis* and *Opilia amentacea* (Nordeng et al., 2013).

According to our results, medicinal plants were prepared in different ways including maceration, decoction and infusion. Decoction in water is mostly used and then the remedy administered orally. The dominant use of medicinal plant decoctions for malaria treatment is probably related to the empirically experienced effectiveness of this type of preparation over many generations. The frequent use of this preparation method may be also due to the fact that boiling the ingredients kills pathogenic microbes possibly present on the plant material (Salhi et al., 2010; Ugulu et al., 2009). This mode of preparation and oral intake is the mostly used procedure in traditional medicine in general. Similar results were obtained in other ethnobotanical surveys carried out in Cameroon (Focho et al., 2009; Telefo et al., 2012, 2011). The choice of oral administration over possible alternatives may be related to the use of water as solvent that is commonly believed to constitute an appropriate vehicle to transport remedies' active principles.

A lack of consistency regarding the dosages be given was observed among the respondents during interviews with both TPs and women groups. In our study, the highest dose recommended for administration was 2 glasses per day over a period of 3 days. This data is in agreement with commonly used remedy administration posology. Inhalation through steam bath administration was also mentioned. Nordeng et al., 2013 considered the

dermal way (by washing the body of pregnant women with plant remedy preparations) of administration as the most safe way of herbal remedy treatment for pregnant women (Nordeng et al., 2013).

Honey or milk was recommended to be taken after administration of some herbal remedies to mask the bitter taste that remains in the mouth. Tsobou et al., 2016, reported different additives such as limestone, citrus lemon or palm oil in order to improve the taste, flavor and general palatability of certain orally administered herbal preparations (Tsobou et al., 2016). This means that since traditional medicines occasionally have sour or bitter tastes, in most of the cases the additives would reduce such unpleasant tastes.

The high level of consensus among the respondents about the employment of medicinal plants for the prevention and treatment of malaria during pregnancy in the study area, suggests that the use of these plants nowadays is still a widespread practice in this community.

Literature review on the medicinal plants identified here as commonly used against malaria during pregnancy, shows that most contain numerous and diversified phytochemical compounds. Given the fact that not any or just few toxicological information is available for many of the recorded medicinal plants, attention should be paid on their indication to be given to women in pregnancy, notably those plants requesting high dosages and several days' treatment. Indeed, there is evidence that plant extracts such as stem and roots of *Khaya senegalensis*, *Nauclea latifolius*, and *Opilia amentacea*, bar

of *Adansonia digitata*, root of *Detarium microcarpum* and leaves and roots of *Ximenia americana* can be toxic for pregnant women. Toxicity has been related to metabolites such as methyl salicylate, present in the root of *Securidaca longipedunculata* and cyanogenic compounds, present in *Ximenia americana* (Dapar et al., 2007; Le et al., 2012; Nordeng et al., 2013). As our goal is to formulate safe and effective antimalarial herbal preparations for pregnant women, an investigation of the biological and cytotoxic activities has been carried out on ten out of the eleven commonly used medicinal plants.

8.2 *In vitro* and *in vivo* activities of crude plant extracts targeting multiple stages of *Plasmodium*

Activity on asexual blood stages of *Plasmodium falciparum*

When comparing the *in vitro* antiplasmodial activity of methanol and aqueous extracts of the tested plants against asexual blood stages of *P. falciparum* (stages responsible for the clinical symptoms of the disease), consistently higher activity was found in the methanol preparations. In 8 out of the 11 tested extracts (10 plants, 11 plant parts), IC50 values of methanol preparation amounted to about one third to one half of those measured for the water extracts, indicating a more efficient extraction of the bioactive components by the alcoholic solvent. Indeed, methanol being a solvent more polar than water is capable of extracting more bioactive compounds owing to this chemical property. Phytochemical studies on these plant

extracts report the presence of many classes of chemical compounds including terpenoids, alkaloids, flavonoids and saponins known to be likely to exert antimalarial effects (Focho et al., 2009; Telefo et al., 2011; Zofou et al., 2013). In addition, extracts from 5 plants were found to reduce 50% of asexual blood stage viability at concentrations lower than 20 µg/ml (Table 12). Particularly, the methanol extract of *Dacryodes edulis* stem bark showed an IC50 value of 10.3 tested on the CQ-sensitive (D10) strain and of 8.6 µg/ml on the CQ-resistant (W2) strain. *Eucalyptus globulus* water and methanol extract showed an IC50 value of 19.2 and 17.7 µg/ml on D10 strain, and of 19.8 and 16.4 µg/ml on W2 strain, respectively. With *Cymbopongon citratus* methanol extract an IC50 value below 20 µg/ml was observed on W2 (12.2 µg/ml) but not on D10 strain (25.3 µg/ml). The level of activity of these three medicinal plants observed in our tests is comparable with that reported by studies in the literature (Table 12). Differences in terms of level of activity can be explained by the choice of the solvent for extraction and the parasite strain used for the drug sensitivity assay. The importance of the method of extraction, in particular temperature at extraction and type of solvent is critical with consequences on the composition of the extracted secondary metabolites and their activity as illustrated by the example of artemisinin and its story of discovery. Examining ancient Chinese medicine reports on the malaria healing effect of Qinghao (Chinese name of the plant *Artemisia annua*), Nobel Prize holder Youyou Tu found a document in which soaking Qinghao with cold water was mentioned. This gave Youyou Tu the hint that the effective compound might be labile at high temperature. So, she modified the extraction methods and used ether to extract under room temperature instead of using boiling water as it is the common preparation method of most traditional Chinese medicines. This extract showed

prominent antimalarial activity, both in animal models as well as in patients. In 1972, the pure substance was obtained and named Qinghaosu, which literally means the essence of Qinghao. In Western medicine, the compound was commonly called artemisinin (Tu, 2011; Zhang, 2011). Our results showed that the methanol extract from *Voacanga africana* leaves was the most active among the tested plants on asexual blood stages. An IC50 value of 3.9 µg/ml was measured against the *P. falciparum* CQ-resistant (*W2*) strain. Ours is the first *in vitro* study of this plant. Also, on *Persea americana* stem bark and *Solanocia mannii* leaves we haven't found any reports, however only the stem bark methanol extract of the former showed some activity on *D10* (19.8 µg/ml) and *W2* (19.9 µg/ml) strain.

Gametocytocidal activity of water and methanol plant extracts

While extensive studies have been carried out to assess plant extracts' activity against the asexual blood stages of the parasite, comparatively less screenings have been undertaken to evaluate effects *in vitro* against the gametocyte stages. Human-to-mosquito transmission blocking entails targeting the sexual stages of the parasite, i.e. gametocytes circulating in the vertebrate host. Micro- and macrogametocytes represent the stages responsible for the transmission of the disease from the human host to the mosquito vector (Baker, 2010). In the current study, at primary screening at an extract concentration of 100 µg/ml, methanol extracts of only 5 plants showed a reduction greater than 50% of stage V gametocyte viability when tested in cultures with *P. falciparum 3D7elo1-pfs16-CBG99* strain. Among the 5 tested extracts, *Persea americana* stem bark methanol extract resulted to be the most active reducing the viability of mature gametocytes by 94% at this

concentration. The dose range assays revealed low activity of the 5 extracts against the sexual stages of *P. falciparum*. However, *P. americana* confirmed to be the relatively more active plant, reducing 50% of gametocyte viability at a concentration of 34.7 µg/ml. A drawback of the pLDH assay is its inability to interrogate viability of male and female gametocyte separately, the cultures containing both, microgametocytes and macrogametocytes (Robert E. Sinden, Blagborough, et al., 2012). Hence, the methanol extract from *P. americana* stem bark may be active on only one of the two sexes but with a stronger effect than what resulted from the overall gametocyte readout. Thus, *P. americana* stem bark methanol extract, should not be discarded as a gametocytocidal candidate for employment in a combination formulation of a herbal preparation with transmission blocking potential, possibly to be used also for prevention and treatment in pregnant women.

Inhibition of early sporogonic development by water and methanol plant extracts

Methanol and aqueous extracts of ten anti-malarial plants identified in this study as the commonly used plants to prevent and cure malaria during pregnancy were screened *in vitro* in the *P. berghei* ookinete development assay (ODA), in order to identify extracts active against the sexual stages developing in the mosquito after feeding on an infectious human host. The ODA screening revealed methanol stem bark extracts of *Dacryodes edulis* and *Persea americana* as the most active plant preparations (Figure 13 A and B). Tested at a primary screening dose of 50 µg/mL both, methanol and aqueous extracts of these plants reduced early sporogonic development by more than 60%. At this high dosage, the number of ESS (the total

counts of zygotes, retort forms, early ookinete stages and elongated ookinetes) was reduced by about 80% for *D. edulis* stem bark and by 100% with *P. americana* stem bark extract, respectively with the methanol extracts. Dose range experiments yielded IC_{50} values in the range of 6 – 13 µg/mL for both plants (Table 14). The IC50 values against ESS estimated for both extracts are similar to those obtained with the standardized product NeemAzal® (Azadirachtin A enriched formulation) IC50= 12.4 µg/ml (Dahiya et al., 2016b).

In addition, at the primary screening concentration of 100 µg/mL the methanol extract of *Dacryodes edulis* leaves reduced early sporogonic stage development by 70% and that of *Ocimum gratissimum* leaves by 60%. The majority of water and methanol extracts reduced similarly ESS counts and numbers of developing ookinetes, indicating activity on various stages and processes of early sporogonic development. In the case of the methanol extracts from *Picralima nitida* seeds, *Voacanga africana* leaves and *Eucalyptus globulus* leaves a slightly stronger impact was noted on ookinete maturation (OM) counts than on ESS counts, indicating relatively more activity on the zygote to ookinete formation than on processes of gamete formation and fecundation, the sexual process preceding zygote formation in the mosquito midgut.

The fact that these extracts are interfering with at least two different stages or processes during early sporogonic development, increases the chance to obtain a total block of transmission, profiting also from the natural bottleneck of the parasite cycle represented by this phase in which a relatively low number of individual *Plasmodium* organisms are needed to be hit. (Robert E. Sinden, Carter, et al., 2012).

Our findings, provide evidence of plants as a valid source of compounds with effects on transmissible *Plasmodium* stages. Hence, we hope that these evidences may act as stimulus for further research in this field to provide transmission blocking compound candidates to the drug discovery&development pipeline. Moreover, the use of such plant extracts in standardized herbal remedies or phytomedicines, taken by entire populations for malaria treatment or prevention (including women in pregnancy) could be highly beneficial for malaria control.

Inhibition of microgamete formation by the methanol extract of *P. americana* and *D. edulis*

In order to explore stage specific effects of the two active extracts on microgametogenesis, they were screened in the microgamete exflagellation assay. Tests were performed with the *P. americana* and *D. edulis* extracts at their respective $IC_{50,}$ measured against ESS in the ookinete development assay (Figure 15 A and B). *P. americana* tested at 8 µg/ml was found to inhibit about 50% of microgamete formation and similarly, *Dacryodes edulis* decreased the number of exflagellation centers by half at 13 µg/ml. This finding indicates the presence of bioactive constituents having an effect on microgamete formation in both the plants.

For comparison, Abay et al., 2015 reported that the ethanol extract of *Vernonia amygdalina* leaves at 50 µg/ml (dosage found to inhibit ESS by 80

- 95%), was found to be inactive on exflagellation (Abay et al., 2015). NeemAzal® used here as reference at different concentrations (10, 15 and 20 µg/ml), confirmed the inhibitory effect found by Dahiya et al., 2016 (Dahiya et al., 2016a). In the case of NeemAzal®, its action on microgamete formation has been attributed to its major component azadirachtin A. *In vitro* studies conducted by Jones and colleagues showed that azadirachtin A interferes with the exflagellation process of *P. falciparum* and *P. berghei* microgametocytes, inducing an interruption of the endomitotic divisions and the formation of rigid extensions on axonemes, preventing their motility (Jones et al., 1994). In a subsequent study, it was demonstrated by Billker et al., 2002 that azadirachtin A disrupts cytoplasmic and axonemal microtubule organization, possibly by compromising the functionality of the microtubule-organizing centers (MTOC), including spindle plaques (Billker, 2002).

These results are interesting, it appears that both, *Persea americana* and *Dacryodes edulis* stem bark extracts exhibit some activity against gametocytes as well as on microgametogenesis, thus are active on transmissible stages in both, the vertebrate and the mosquito host. Given this multi-stage transmission blocking effects of the two plants, they merit attention to be included in an antimalarial herbal remedy aimed not only to protect the person who takes it but if taken by a large proportion of people may also contribute to lower locally intensity of malaria transmission.

In-vivo transmission blocking effect of methanol stem bark extracts from *P. americana* and *D. edulis*

A Direct Feeding Assay (DFA) conducted with the *Plasmodium berghei GFPcon* parasite strain and *Anopheles stephensi* mosquitoes revealed *in vivo* transmission-blocking activity of both stem bark methanol extracts of *Persea americana* and *Dacryodes edulis*. Taking as experimental readout numbers of oocysts developing on mosquito midguts, a reduction in oocyst density by 73% and 74% was recorded with *P. americana* and *D. edulis* respectively (Table 16). In mosquito groups fed on extract treated mice (150 mg/kg), a mean number of 89 and 87 oocysts were counted in the *Persea americana* and *Dacryodes edulis* treatment group compared to 331 in control mosquitoes. For comparison, experiments conducted by our group with NeemAzal® given i.p at 150 mg / kg, 1hr before feeding, blocked oocyst development completely (Lucantoni et al., 2010). The stronger transmission blocking effect of NeemAzal® compared to the crude plant extracts can be explained by the fact that 50% of the NeemAzal® product is constituted by azadirachtin A, the active compound whereas in the crude extracts the concentration of the active compounds is most likely less than 5%.

Cytotoxicity assessment of water and methanol medicinal plant extracts

Our results show that seven out of the 22 extracts tested, namely the methanol extract of *D. edulis* stem bark and leaves, the water extract of *P. americana*, water extract of *S. manni*, and water and methanol extract of *P.*

nitida, reduced viability of normal *NHF-A12*-human fibroblast cells by less than 20% at the highest test concentration of 150 µg/ml. Based on this criterion, the IC50 of 20 out of 22 extracts was estimated to be greater than 150 µg/ml (Table 16), and thus the extracts considered non-toxic. For two extracts, *Eucalyptus globulus* and *Voacanga africana* leaves methanol preparations, having revealed slight activity at primary screening, IC50 values were determined and resulted to be 85.2µg/ml and 88.1µg/ml, respectively. Cytotoxicity studies on *D. edulis* leaves and stem bark, *E. globulus* and *M. indica* reported in literature are in line with our results, confirming low or absence of toxicity on normal cells (Arrey Tarkang et al., 2014; Zofou et al., 2011). Investigations on *Senna alata* leaves have shown the plant to contain mutagens and to have an abortifacient potential in rats at the dosage range of 500 to 1000 mg/kg (Yakubu et al., 2010). In addition, *Picralima nitida* ethanol seed extract has shown to significantly ($P<0.05$) inhibit human T cell (Jurkat) proliferation in a concentration-dependent manner when activated by anti-CD3 antibody (Erharuyi et al., 2014).

Although no cytotoxic effects were found on normal fibroblasts at the highest test dosage of 150 µg/ml, two extracts exhibited (stem bark methanol extract of *P. americana* and *D. edulis*) a slight cytotoxic effect on the *EA.hy926* mammalian cell line. Considering the IC50 value of *P. americana* and *D. edulis,* found with both the extracts against asexual blood stages of *P. falciparum* D10 and W2 parasite strain, the selectivity index (SI: ratio of mammalian cell viability to asexual blood-stage inhibition) resulted 10,36

and 5.53 respectively (Table 18). An index of 10 or above is considered acceptable in the field of drug discovery&development. The level of SI obtained with the two extracts evidence specific activity of plants compounds against targets of early sporogonic development of the *Plasmodium* parasite.

Interestingly, almost all the 22 extracts tested have shown to have anti-tumor effects reducing cell viability of *A375*-melanoma cells and/or *MDA-MB231*-human breast cancer cells (Table19). Prominent activity was found with the methanol extract of *Dacryodes edulis* stem bark with which an IC50 value of 6.23 µg/ml was recorded on the melanoma cell line and with *Picralima nitida* that gave an IC50 value of 10.47 µg/ml on the same cell line. *In-vitro* anti-proliferative and apoptotic effects of the crude methanol extract and of chloroform fractions from *P. nitida* root bark, have been reported using the human breast cancer cell line (Erharuyi et al., 2014). The results of that study indicated a marked reduction in cell proliferation and increased apoptosis in *MCF-7* cells after extract treatment, effects that were highly significant with the chloroform fraction of the extract (Erharuyi et al., 2014).

In addition, the stem bark methanol extract of *P. americana* and *D. edulis* were found to possess cytotoxic effects on the insect cell line *Sf9* (*Spodoptera frugiperda*) at different times of incubation (24hrs, 48hrs and 6 days). These cytotoxic effects were found to be increased with incubation time from 24 hrs to 48 hrs to day 6. NeemAzal® used as a positive reference was found to display relevant insect cell cytotoxicity whatever the time point of incubation, showing an IC_{50} ranging from 4 to 6 µg/ml. NeemAzal® is known to

possess mosquitocidal activity (Dembo et al., 2015). Dembo et al.,2015 demonstrated the impact of the product on the feeding capacity of mosquitoes after repeated blood meals on mice treated intraperitoneally with NeemAzal® (at an azadirachtin A concentration of 105 and 150 mg/kg). Moreover, at these same concentrations, an effect on oviposition was observed, an effect that appeared partly to be the consequence of reduced blood intake (Dembo et al., 2015). In previous studies conducted by our group, whereby NeemAzal® was administered through membrane feeding, a significant reduction in numbers of eggs per microliter of blood was recorded in the 100 µg/ml group after the second blood meal, but no oogenesis inhibitory effect on oviposition was found in mosquitoes treated with NeemAzal® medicated sucrose solution (Lucantoni et al., 2010). The cytotoxic effect of NeemAzal® found here on the normal insect cell line SF9, is compatible with the findings regarding the product's capacity to interfere with various physiological parameters of the mosquito vector.

8.3 *General discussion and future prospective*

This study allowed to identify medicinal plants used to prevent and treat malaria during pregnancy and investigated the safety and biological effectiveness of water and methanol extracts obtained from 10 commonly used plants, in order to design evidence based, multi-stage antiplasmodial herbal formulations for this high risk group, to be employed in Cameroon

and other African endemic areas. It is reported that over 76% of patients with febrile illness, including malaria disease, resort to first treatments with herbal preparations at home before seeking help at modern medicine structures (Mazigo et al.,2011). The use of the here investigated medicinal plants, in particular, *Dacryodes edulis* leaves and stem bark, *Mangifera indica* leaves, *Eucalyptus globulus* leaves, *Cymbopogon citratus* leaves or whole plant and *Ocimum gratissimum* leaves for the cure of various ailments by the general population as well as pregnant women is documented for the area (Tarkang et al., 2014a; Tarkang et al., 2014; Yemele et al., 2015; Zofou et al., 2011). Thus, their wide use also as anti-malarial remedies during pregnancy is probably linked to the perceived safety and varied effectiveness of each, passed on from generation to another.

Thanks to the wide geographical distribution of these plant species in Cameroon and their ability to grow easily in different ecological settings, they can be considered universally available and a sustainable reservoir of bioactive natural components with antimalarial potential to be exploited in Cameroon but also in other countries for the management of malarial fever in the general population and in pregnancy.

Our results are in support of their use putting in evidence antiplasmodial activity in about half of the tested medicinal plants against one or different life cycle stages of the parasite. Thus the use of those medicinal plants for which evidence on their safety (not cytotoxic) and antiplasmodial efficacy is available should be encouraged, particularly in areas where women do not

have access to modern drugs or do prefer traditional remedies for cultural reasons.

The currently employed way of plant preparation includes decoction, infusion and maceration of different plant parts from single plants or in combination. However, these traditional ways of preparation entail a relatively short conservation time and limited palatability due to the bitter or otherwise unpleasant taste of many of the preparations. In order to facilitate remedy administration and acceptability of plant-based preparations at the community level a syrup based formulation is proposed, that might find compliance by TPs as well as pregnant women. Such a syrup preparation can easily be prepared by dissolving 67 g of sucrose into 33 g of water to make 100 g of syrup. After filtering, decoctions of the antimalarial plants can then be added to the concentrated sugar solution. This will mask the bitter taste of these preparations and at the same time increase the period of conservation while reducing the development of microbes (the high amount of sugar present in syrups avoids bacterial replication, acting as a preservative).

Designing a rational, improved antimalarial formulation, based on plant combinations (to be prepared in a basically equipped laboratory), plants and plant part extracts with the following antiplasmodial characteristics should be considered:

1. Plants (extracts) with consolidated evidence to be active *in vitro* on the asexual blood-stages of *Plasmodium*. Based on the results from our study and considering literature data the following indications can be deduced:

- First choice: leaves methanol extracts of *Voacanga africana*, *Eucalyptus globulus* and *Cymbopongon citratus*; stem bark methanol extract of *Dacryodes edulis*.

- Second choice: methanol extracts of *Persea americana* stem bark and *Dacryodes edulis* leaves; water extracts of *Eucalyptus globulus* and *Mangifera indica* leaves.

2: Plant extracts with activity against the transmissible stages of the human host, i.e. based on our results, plants with evidence on effects on late stage gametocytes of *Plasmodium*.

- First choice: stem bark methanol extract of *Persea americana*

- Second choice: methanol extracts of *Dacryodes edulis* leaves and *Ocimum gratissimum* leaves.

3: Plant extracts with evidence of activity on transmissible stages of the parasite in the mosquito vector.

- First choice: stem bark methanol extract of *Persea americana* and *Dacryodes edulis*. Evidence both *in vitro* and *in-vivo*.

- Second choice: methanol leaves extracts of *Dacryodes edulis* and *Ocimum gratissimum*. Evidence *in-vitro* only.

Importantly, the choice of combinations should be also in agreement with traditional medicine indications. In our ethnobotanical study four plant combinations were cited, each using not more than 3 medicinal plants, including *Dacryodes edulis* leaves and stem bark, *Cymbopogon citratus* leaves or whole plant, *Mangifera indica* and *Eucalyptus globulus* leaves.

Thus, based on antiplasmodial activity patterns and traditional medicine knowledge three improved antimalarial formulations might be hypothesized based on the following plant combinations:

1. Stem bark methanol extract of *Dacryodes edulis* for its activity against transmissible stages of the parasite (in the mosquito vector) + leaves methanol extract of *Cymbopongon citratus* for its additional or synergistic effects on asexual blood stages (responsible for clinical symptoms) of the parasite.

2. Methanol leaves extracts of *Eucalyptus globulus* and *Cymbopogon citratus*, both mainly targeting blood stages of the parasite + stem bark methanol extract of *Persea americana* targeting transmissible stages of the parasite in the human host and mosquito vector.

3. *Dacryodes edulis* stem bark methanol extract mainly against transmissible stages of the parasite in the mosquito vector + *Cymbopogon citratus* leaves

methanol extract and *Dacryodes edulis* leaves, both acting on asexual blood stages of the parasite.

Table 19: Summary of proposed plant combinations for pregnant women and targets of each plant extract on the *Plasmodium* life cycle used in combination.

		Selected plant extracts according to the activity against *Plasmodium* stages		
N°	**Formulation: Plant extracts used in the combination**	**Asexual blood stages**	**Gametocytocytes**	**Transmissible stages of the parasite (in vitro and in vivo)**
1	*D. edulis* (Stem bark Me-OH) + *C. citratus* (leaves Me-OH)	*C. citratus* (leaves Me-OH)		*D. edulis* (Stem bark Me-OH)
2	*E. globulus* (leaves Me-OH) + *C. citratus* (leaves Me-OH) + *P. americana* (stem bark Me-OH)	*E. globulus* (leaves Me-OH) and *C. citratus* (leaves Me-OH).	*P. americana* (stem bark Me-OH)	*P. americana* (stem bark Me-OH)

3	*D. edulis* (leaves Me-OH) + *D. edulis* (stem bark Me-OH) + *C. citratus* (leaves Me-OH)	*D. edulis* (leaves Me-OH) and *C. citratus* (leaves Me-OH)	*D. edulis* (stem bark Me-OH)

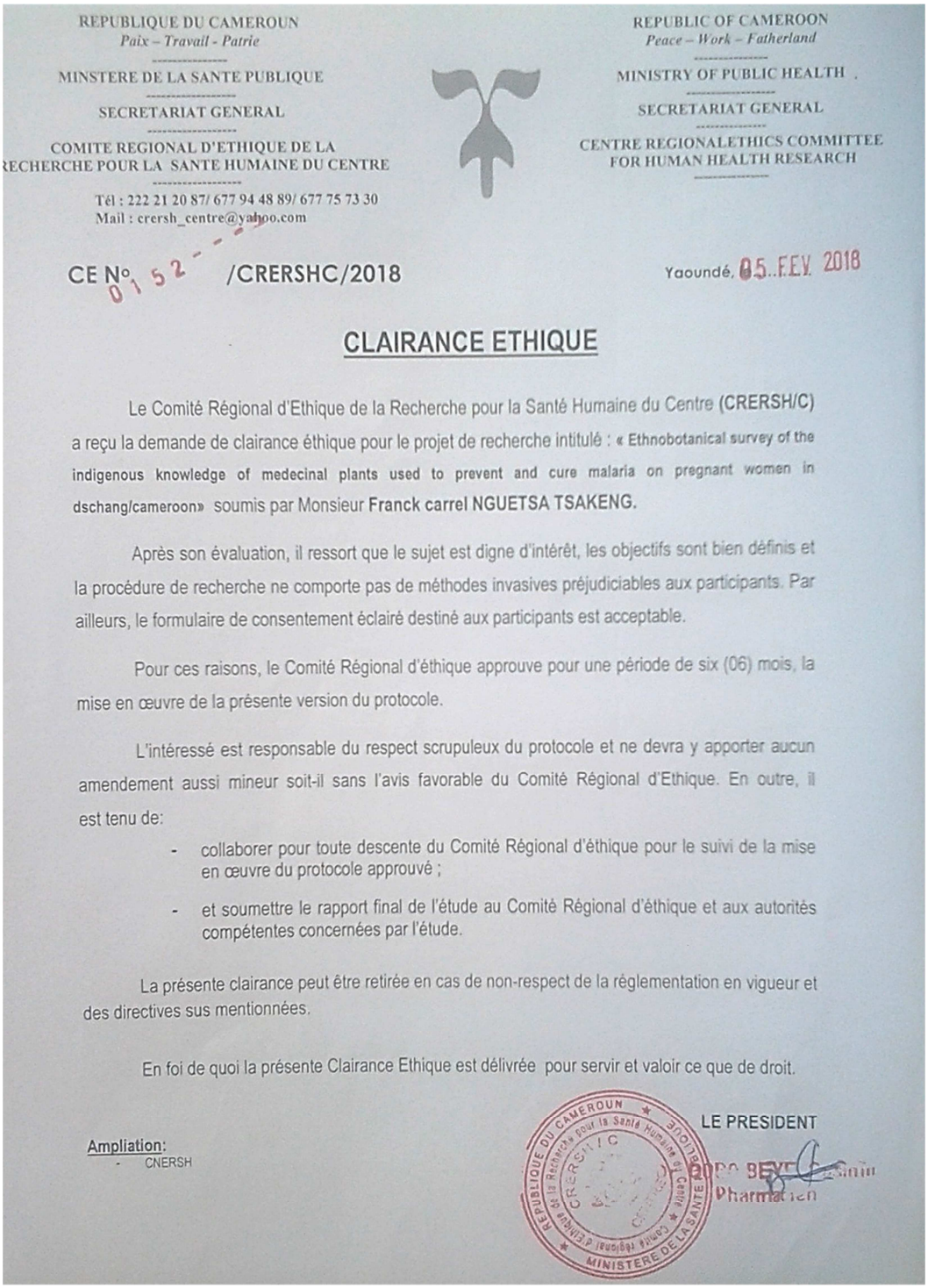
In summary, due to the difficulty of access to certain villages and the lack of financial means, our ethnobotanical study was limited just to 6 villages of the Menoua division. Hence, a study including a larger number of villages and more respondents from the community would allow to verify the results obtained and possibly allow to discover other plants and plant combinations used for the treatment of malaria in pregnancy.

However, our investigation has allowed to identify several medicinal plants used by women during pregnancy for the management of malaria, to reveal multi-stage effects on *Plasmodium* of some of the plants and has allowed to characterize their cytotoxicity as an indicator of safety. Bio-guided fractionation studies with the most active extracts (stem bark methanol extracts of *D. edulis* and *P. americana*) may allow to identify and isolate the compounds responsible for the transmission-blocking activity observed.

Also, further studies should be conducted with the proposed combinations to confirm their antiplasmodial activity *in vivo* on blood schizogony and

gametogenesis of *P. berghei* in mice and on sporogony in the *Anopheles* vector. Once validated also on *P. falciparum* field isolates for transmission blocking activity in the direct membrane feeding assay, health policy makers in Cameroon may be addressed for obtaining endorsement to the their production and employment as improved antimalarial remedy formulations aimed not only at preventing and curing malaria in pregnant women but also – if taken by a large proportion of the population - at decreasing the diffusion of this deadly parasitic disease in endemic areas.

<u>Annex 1</u>: Ethical clearance for authorization of the ethnobotanical study in Cameroon.



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SECRETARIAT GENERAL

CENTRE REGIONALETHICS COMMITTEE FOR HUMAN HEALTH RESEARCH

CE N° 0152 /CRERSHC/2018

Yaoundé, 05 FEV 2018

CLAIRANCE ETHIQUE

Le Comité Régional d'Ethique de la Recherche pour la Santé Humaine du Centre (CRERSH/C) a reçu la demande de clairance éthique pour le projet de recherche intitulé : « Ethnobotanical survey of the indigenous knowledge of medecinal plants used to prevent and cure malaria on pregnant women in dschang/cameroon» soumis par Monsieur Franck carrel NGUETSA TSAKENG.

Après son évaluation, il ressort que le sujet est digne d'intérêt, les objectifs sont bien définis et la procédure de recherche ne comporte pas de méthodes invasives préjudiciables aux participants. Par ailleurs, le formulaire de consentement éclairé destiné aux participants est acceptable.

Pour ces raisons, le Comité Régional d'éthique approuve pour une période de six (06) mois, la mise en œuvre de la présente version du protocole.

L'intéressé est responsable du respect scrupuleux du protocole et ne devra y apporter aucun amendement aussi mineur soit-il sans l'avis favorable du Comité Régional d'Ethique. En outre, il est tenu de:

- collaborer pour toute descente du Comité Régional d'éthique pour le suivi de la mise en œuvre du protocole approuvé ;
- et soumettre le rapport final de l'étude au Comité Régional d'éthique et aux autorités compétentes concernées par l'étude.

La présente clairance peut être retirée en cas de non-respect de la règlementation en vigueur et des directives sus mentionnées.

En foi de quoi la présente Clairance Ethique est délivrée pour servir et valoir ce que de droit.

LE PRESIDENT

Pharmacien

Ampliation:
- CNERSH

Annex 2: Questionnaire administered to traditional healers

Ethnobotanical study: questionnaire for collecting medicinal plant data used by traditional healers to prevent and cure malaria in women during pregnancy.

Informants' consent for the participation in the study:
Hello Mrs. / Mr. I am ..... a doctoral student and we are investigating to identify medicinal plants that are used in the community for the prevention and treatment of malaria in pregnant women. Indeed we wish this information for a doctoral research in order to improve the health of the women and in particular that of pregnant women in the Menoua division. The data collected here can be published in scientific journals. Thank you.

Are you available to participate in this study by sharing with us your knowledge?
☐ yes ☐ No

Informants' details/ Identification:

Participant code (name): (I.D. number):	
Age : _____ (years)	
Marital status: I__I	1= Married 2= single 3= Divorced 4= Widowed 5= Separate
Education/ Instruction level: I__I	1= No formal education 2= Primary level 3= High school (secondary level) 4= Tertiary level (University)
Location/ Village: I__I	

Which plants do you use to treat fever?

_____

_____

Do you make a difference between simple fever and fever due to malaria?

_____

_____

If the patient is a pregnant woman do you use the same plant to treat fever?

_____

_____

For the case of malaria associated with a pregnancy which plant do you use?

_____

_____

At how many months of pregnancy do you use these plants?

_____

_____

Do you recommend some of your plants to your patients to prevent malaria?

_____

_____

Do you use herbal combinations also for the treatment of malaria in pregnant women?

_____

_____

What quantity of each of the plants do you use?
(Do you measure it and how? Do you weigh the plant material?).....

Which specific part of the plant comes to be used? Or is it the whole plant?.....

How do you administer plant preparations to pregnant women? Posology? With what means? Days of perceived effectiveness?

_____

_____

_____

How do you prepare your herbal remedies (method)? Conservation method and duration of treatment?

_____

_____

_____

Do these plants have unwanted/unpleasant side effects? If yes, which?

_____
_____

Did you, Do you use plants for treating or preventing malaria in pregnant women? In which period of pregnancy is the plant used?

_____
_____
_____

Remarks:

Annex 3: Questionnaire administered to women groups

Informants' consent for the participation in the study:
Hello Mrs., I am ..... a doctoral student and we are investigating to identify medicinal plants that are used in the community for the prevention and treatment of malaria in pregnant women. Indeed we wish to obtain this information for a doctoral research in order to improve the health of the women and in particular that of pregnant women in the Menoua division. The data collected here can be published in scientific journals. Thank you for your participation.

Participant number of women: (Name of women)	**Name of the local association**:
Place of the interview:	
Age of each woman : _____ (years)	
Education Status/level of each: I__I	1= No formal education 2= Primary level 3= High school (secondary level) 4= Tertiary level (University)

Profession/Occupation of each: I__I	1= Housekeepers/farmers 2= Tradeswoman 3= Teacher/secretary in public administration 4= Student 5= Other.....

Do you have children? ☐ yes ☐ No

Do you know what is malaria and what are the signs and symptoms to recognize it?_____

_____

_____

What importance do you give to medicinal plant uses for the treatment of malaria during pregnancy?

_____

_____

Which medicinal plants did you take or were you advised to take as a traditional treatment for malaria during pregnancy?

_____

_____

_____

If you want to prevent malaria during pregnancy, are you using the same plants as for non-pregnant women?

_____

_____

Do you know any combinations of plants used to treat malaria during pregnancy? Which ones?

_____

_____

How these plants are they prepared and taken?

_____

_____

Did you notice some adverse effect?

_____

Ackknowledgments

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LIST OF PUBLICATIONS

Alain R. Tenoh, Yehenew Ebstie, D'Alessandro S., L. Quassinti, Parapini S., Bramucci M., Sanou M., Taramelli D., Habluetzel A.; ***Ethnobotanical study, antimalarial characterization and safety of medicinal plants used during pregnancy***. (Manuscript in preparation)

Yehenew Ebstie, **Alain R. Tenoh**, Annette Habluetzel; ***In vivo Plasmodium* transmission-blocking activity of *A.indica* A.Juss. seed kernel phases combined with artemisinin (post-treatment, combination treatment)**. (Manuscript in preparation)

Harouna Sore, Annalisa L., **Alain R. Tenoh**, Yehenew Ebstie, Adama Hilou, Taramelli D., Habluetzel A. and Taglialatela-Scafati O.; ***Plasmodium* stage-selective antimalarials from *Lophira lanceolata* stem barks;** Journal of Phytochemistry. (Accepted)

Yehenew A. Ebstie, **Alain R. Tenoh**, Annette Habluetzel; **A murine malaria protocol for characterizing transmission blocking benefits of antimalarial drug combinations**; The Malaria World Journal. (Under review)

Hamissi M., , D'Alessandro S., Sore Harouna, **Alain R. Tenoh**, Yehenew A. Ebstie, , Parapini S., Taramelli D., and Habluetzel A..; ***In vitro* multistage malaria transmission blocking activity of selected Malaria Box compounds**; Journal of Drug Design, Development and Therapy. (Accepted)

Carmina Sirignano, Alì Snene, **Alain R. Tenoh**, Ridha El Mokni, Daniela Rigano, Annette Habluetzel, Saoussen Hammami, Orazio Taglialatela-Scafati. **Daucovirgolides I-L, four congeners of the antimalarial daucovirgolide G from *Daucus virgatus***; Journal of Fitoterapia, 2019. (Accepted)

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Carmina S., Ali Snene, Daniella R., Tapanelli S., Carmen F., Paolo L., Ridha El M., Saoussen H., **Alain R. Tenoh**, Habluetzel A. and Taglialatela-Scafati O.; **Angeloylated Germacranolides from *Daucus virgatus* and their *Plasmodium* Transmission Blocking Activity**; Journal of Natural Products, 2017. (Accepted)