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Introduction

Le paludisme ou malaria est une érythropathie causée par des parasites du genre *Plasmodium*. Avec le SIDA et la tuberculose, c'est actuellement l'une des causes majeures de morbidité et de mortalité dans le monde. Cent cinq pays sont concernés, 48% de la population mondiale est susceptible d'être touchée.

Le paludisme est endémique des régions tropicales ou subtropicales, englobant essentiellement des pays à faible Produit Intérieur Brut (34).

Cette maladie sévit principalement :

- en Afrique sub-saharienne (90% de la population atteinte),
- sur une grande partie du sous-continent indien, en Asie du Sud et en Océanie. Les pays les plus touchés dans cette région sont : l'Inde, l'Afghanistan, le Sri Lanka, la Thaïlande, l'Indonésie, le Vietnam et le Cambodge,
- dans les états d'Amérique centrale, à Haïti, en République Dominicaine, ainsi que dans les pays d'Amérique du Sud. Le bassin amazonien brésilien recense le plus grand nombre de cas pour cette région.

Il faut également noter que la répartition mondiale varie en fonction de l'espèce plasmodiale en cause (Tableau 1).

Tableau 1 : répartition géographique des 5 espèces plasmodiales humaines

Espèces plamodiales	Zones géographiques concernées
Plasmodium falciparum	Afrique sub-saharienne Asie - Océanie Amérique Centrale et Sud
Plasmodium vivax	Afrique (peu représenté) Asie Amériques du Sud et Centrale
Plasmodium malariae *Plasmodium ovale*	Afrique Sporadique en Amazonie, en Océanie et en Asie
Plasmodium knowlesi	Asie du Sud-Est

I. Rappels sur l'écologie du parasite

Les *Plasmodiums* sont des protozoaires appartenant à l'embranchement des Sporozoaires et à l'ordre des Haemosporideae. Trois espèces sont exclusivement humaines : *P. falciparum*, *P. vivax* et *P. ovale*. Les espèces *P. malariae* et *P. knowlesi* sont humaines et simiennes.

L'écologie de *P. falciparum* a été la plus étudiée. C'est un parasite dixène qui se développe en suivant un cycle complexe (Annexe 1). L'homme, hôte intermédiaire, héberge la multiplication asexuée ou schizogonique du parasite. Le moustique du genre *Anopheles* est l'hôte définitif chez lequel s'effectue la multiplication sexuée ou sporogonique.

La phase asexuée érythrocytaire consiste à l'infestation des globules rouges par des mérozoïtes libérés après la multiplication asexuée hépatique. Les mérozoïtes effectuent un cycle du parasite appelé cycle asexué érythrocytaire. L'éclatement des globules rouges pour libérer de nouveaux mérozoïtes déclenche les accès de fièvre caractéristique de la maladie.

Dans le cadre de notre travail, l'activité des molécules antipaludiques est étudiée uniquement sur les phases asexuées qui sont soit maintenues en culture *in vitro* ou *in vivo* (modèles murins).

II. Rappels sur la pathologie

II. 1. Symptômes

La période d'incubation, délai entre l'infection et l'apparition des manifestations cliniques, dure en moyenne 12 jours mais peut varier entre 9 jours et 12 mois.

La phase hépatique est asymptomatique, les signes cliniques sont liés à la phase de schizogonie érythrocytaire. Les manifestations cliniques dépendent de l'espèce plasmodiale en cause, de l'immunité de l'hôte, de la parasitémie et de divers autres facteurs peu ou mal connus.

II. 2. Paludisme non compliqué

C'est la forme la plus fréquente. La fièvre est toujours présente et souvent associée à divers symptômes : syndrome pseudo-grippal (asthénie, algies multiples, céphalées) et digestif (vomissements et parfois diarrhées).

La primo-invasion apparaît chez des sujets non immunisés c'est-à-dire des enfants de 4 mois à 6 ans en zone d'endémie et des adultes non immunisés. L'accès palustre ou accès à fièvre périodique, est la forme classique chez les sujets adultes immuns.

Cette forme peut évoluer avec des phases de rémission et recrudescence. Chez les sujets faiblement immunisés, il existe à tout moment un risque de passage à la forme grave ou compliquée.

II. 3. Paludisme grave ou compliqué

Le paludisme grave est très majoritairement provoqué par *Plasmodium falciparum*. Si cette forme peut être retrouvée à tout âge, quelque soit le sexe, elle est plus souvent rencontrée chez les enfants de quatre mois à six ans.

Le paludisme grave peut apparaître de façon brutale, parfois foudroyante, avec une fièvre, des convulsions et un coma qui constituent la triade symptomatique caractéristique. L'évolution de cette forme dépend de la rapidité et de la qualité de la prise en charge. En l'absence de traitement, la mort survient en deux à trois jours.

Les critères de gravité actualisés par l'OMS en 2000 sont répertoriés sur le Tableau 2

Tableau 2 : critères de diagnostic du paludisme grave ou compliqué (69)

Prostration : extrême faiblesse ou, chez l'enfant : « impossibilité de tenir assis pour un enfant en âge de le faire, ou de boire pour un enfant trop jeune pour tenir debout »
Troubles de la conscience : Score de Glasgow modifié < 10 chez l'adulte et l'enfant de plus de 5 ans ; Score de Blantyre < 3 chez le petit enfant
Détresse respiratoire : définition clinique
Œdème pulmonaire : définition radiologique
Convulsions répétées : ≥ 2 par 24 h, malgré le traitement de l'hyperthermie
Collapsus circulatoire : PAS < 80 mmHg chez l'adulte en présence de signes périphériques d'insuffisance circulatoire ; PAS < 50mm Hg chez l'enfant
Saignement anormal : définition clinique
Ictère : définition clinique ou bilirubine totale > 50 µmol /l
Hémoglobinurie macroscopique : urines rouge foncées ou noires
Anémie profonde : Hémoglobine < 7 g/dl chez l'adulte ou hématocrite < 20% Hémoglobine < 5 g/dl chez l'enfant ou hématocrite < 15%
Hypoglycémie : Glycémie < 2,2 mmol/l
Acidose : pH < 7,35 ou bicarbonates < 15 mmol/l
Hyperlactatémie : lactates plasmatiques > 5 mmol/l
Hyperparasitémie : notamment > 4% chez le non immun
Insuffisance rénale : créatininémie > 265 µmol/l après réhydratation ou diurèse < 400 ml/24 h chez l'adulte (< 12 ml/kg/24 h chez l'enfant)

III. Molécules antipaludiques : efficacité et contrôle

Un traitement antipaludique doit être efficace, accessible, bien toléré et peu onéreux car les populations majoritairement concernées ont des faibles revenus avec un accès au soin limité.

III. 1. Cibles plasmodiales

Le parasite *Plasmodium* dispose pour son développement intra érythrocytaire d'un métabolisme et de moyens de défenses spécifiques qui constituent autant de cibles aux antipaludiques. Nous distinguerons :

- La vacuole digestive du parasite qui est le siège de la digestion de l'hémoglobine, de la cristallisation de l'hème et où des moyens de défense spécifiques contre le stress oxydatif sont retrouvés.
- Un cytoplasme comportant le cytosol et deux organites essentiels, les mitochondries et l'apicoplaste. Ils sont nécessaires à la biosynthèse des acides nucléiques.
- Une membrane plasmique, constituée de phospholipides, des canaux calciques et parasitophores, siège du trafic nutritionnel.

Les antipaludiques actuels peuvent être classés selon leur mode d'action en schizonticides actifs sur la phase asexuée érythrocytaire, et gamétocyticides actifs sur la phase sexuée érythrocytaire. Les amino-8-quinoléines (tafénoquine et primaquine) sont des molécules actives sur la phase hépatique du parasite. Du fait de leur index thérapeutique faible, leur usage exige une surveillance clinique rapprochée. Ils ne seront pas décrits dans ce travail.

III. 2. Molécules antipaludiques

III. 2.1. Les schizonticides

Ce groupe comprend les dérivés quinoléiques et les dérivés de l'artémisinine.

Les dérivés quinoléiques comprennent les amino-4-quinoléines (chloroquine, amodiaquine) et les amino-alcools (méfloquine, halofantrine, luméfantrine). Ces molécules interfèrent avec la digestion de l'hémoglobine dans la vacuole nutritive en inhibant la formation de l'hémozoïne.

Les dérivés de l'artémisinine (artésunate, artéméther, etc..). Cette nouvelle classe d'antipaludiques de type peroxyde interfère aussi dans la digestion de l'hémoglobine, par libération de radicaux libres, toxiques pour le parasite. Les dérivés de l'artémisinine ont une action gamétocytocide, qui réduit la transmission et limite les risques de voir émerger des résistances.

III. 2.2. Les inhibiteurs des acides nucléiques ou antimétaboliques

Ils bloquent la division du noyau de l'hématozoaire. Ce groupe comprend les antifolates, les naphtoquinones et les antibiotiques.

Les antifolates sont répartis en deux familles, les antifoliques (sulfamides, dont la sulfadoxine et sulfone) et les antifoliniques (proguanil et pyriméthamine). Ils agissent au niveau de la voie de synthèse des folates, qui sont essentiels à la biosynthèse des acides nucléiques du parasite. Les antifoliques inhibent la dihydroptéroate synthétase (DHPS) qui produit l'acide folique et les antifoliniques la dihydrofolate réductase (DHFR), qui produit l'acide folinique.

Les naphtoquinones : l'atovaquone est un inhibiteur puissant des fonctions mitochondriales en bloquant la chaîne de transfert d'électrons au niveau de son enzyme-clé, la dihydroorotate déshydrogénase. Elle a peu d'impact thérapeutique lorsqu'elle est utilisée seule. En combinaison avec un métabolite (proguanil), une intéressante synergie d'action s'observe grâce à une inhibition séquentielle de la synthèse des pyrimidines.

Les antibiotiques : les tétracyclines (doxycycline), les macrolides (érythromycine, azythromycine, clindamycine) peuvent inhiber la synthèse protéique par inhibition de certaines fonctions de l'apicoplaste.

III. 2.3. Les associations d'antipaludiques

Les nouveaux antipaludiques qui ont fait l'objet de développements récents sont tous associés, au moins en bithérapie, et se démarquent de la plus ancienne des associations, la sulfadoxine-pyriméthamine capable de sélectionner rapidement des mutants résistants. Certaines associations sont fixes : l'atovaquone-proguanil, l'arthéméther-luméfantrine et la chlorproguanil-dapsone. D'autres associations sont libres, associant toujours un dérivé de l'artémisinine vu la rapidité d'action, l'impact sur la transmission et l'absence de chimiorésistance de *P. falciparum* : artésunate-méfloquine, artésunate-amodiaquine, artéméther-proguanil et artésunate-sulfadoxine-pyriméthamine.

En prophylaxie, les associations chloroquine-proguanil (Savarine®) et atovaquone-proguanil (Malarone®) sont recommandées selon les zones de chloroquino-résistance.

III. 3. Molécules antipaludiques issues des plantes médicinales

Deux antipaludiques extraits des plantes médicinales sont toujours parmi ceux les plus utilisées et les plus efficaces. Il s'agit de la quinine, qui demeure un antipaludique majeur préconisé dans le traitement des cas de paludisme grave ou d'urgence et l'artémisinine (qinghaosu) ainsi que ses dérivés (artéether, artésunate et artéméther) obtenus par hémisynthèse qui occupent actuellement une place primordiale lors de paludismes dits récessifs et neurologiques.

III. 3.1. Les extraits actifs de *Cinchona sp.* et la quinine

Cinchona sp est un arbre de la famille des rubiacées, originaire d'Amérique du Sud. L'écorce de cette plante était utilisée par les indiens d'Amérique du Sud en médecine traditionnelle. Ce sont les jésuites espagnols qui en ont répandu l'usage au XVIème siècle. En 1630, au Pérou, Don Francisco Lopez avait observé l'utilisation par les Indiens de décoctions d'écorce de l'arbre pour leurs vertus curatives des accès fébriles.

Aujourd'hui, cette écorce est toujours utilisée sous la forme de décoction, infusion ou macération aqueuse dans le traitement du paludisme en Amérique du Sud, et aussi dans plusieurs pays d'Afrique, ou les espèces rencontrées sont : *Cinchona officinalis*, *Cinchona ledgeriana* et *Cinchona succirubra*. *C. ledgeriana*, importé de Java (Indonésie), fournit un bon rendement d'extraction à la quinine (70). Les sachets d'extraits secs de son écorce sont vendus sous l'appellation « Totaquina » par l'Institut Malgache de Recherches appliquées.

L'isolement et la purification de la quinine ont été obtenus en 1820 par deux pharmaciens militaires français, Pelletier et Caventou. Sa synthèse artificielle a été mise au point en 2001, mais économiquement peu intéressante, la quinine est encore aujourd'hui extraite de l'espèce *Cinchona sp.* cultivée.

Une trentaine d'alcaloïdes ont été décrits à partir de l'écorce de *Cinchona,* dont plusieurs, actifs *in vitro* sur les souches de *P. falciparum*. Les quatre alcaloïdes les plus connus sont la quinine, la quinidine, la cinchonine et la cinchonidine. La combinaison des alcaloïdes (quinine, quinidine, cinchonine) est 2 à 10 fois plus efficace sur des souches quininorésistantes que chaque alcaloïde testé séparément (70).

La quinine comporte un cycle quinoléine et un radical méthanol (carbinol) en position 4, que l'on retrouve chez d'autres méthanolquinoléines de synthèse.

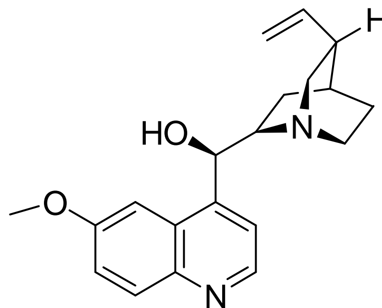

Figure 1 : structure chimique de la quinine

C'est un schizonticide sanguin à action rapide (10), ayant peu d'activité sur les sporozoïtes et les stades exo-érythrocytaires.

Son mécanisme d'action est proche de celui des amino-4-quinoléines. La quinine est une base faible qui se concentre dans les vacuoles digestives du parasite. Elle agit alors en se liant à l'hémine libre et donc en inhibant la polymérisation de l'hème (ferriprotoporphyrine IX). Le processus de détoxification par l'hémozoïne est bloqué, d'où l'accumulation de cette molécule, toxique pour *Plasmodium* (59).

La baisse de la quininosensibilité est connue dans l'Est africain, l'Asie du Sud-Est, le Brésil et l'Amazonie. Elle reste le plus souvent partielle et de faible niveau, et n'a pour l'instant que peu de conséquences cliniques perceptibles.

Les effets secondaires, dits « cinchonisme », suite au traitement par la quinine, sont importants et concentration-dépendants : acouphène, vertiges, céphalées. La conduite à tenir après intoxication consiste à une prise en charge en milieu hospitalier avec évacuation gastrique, lavage de l'estomac et administration de charbon activé.

III. 3.2. Les extraits actifs d'*Artemisia annua* et l'artémisinine (70)

L'espèce *Artemisia annua* L (Asteraceae) est native de la Chine et d'autres pays asiatiques (Japon, Corée et Thaïlande). C'est une herbe médicinale utilisée contre les fièvres, dont l'usage remonte à -168 avant Jésus-Christ.

La plante *Artemisia annua* est aujourd'hui cultivée dans plusieurs pays endémiques du paludisme, où les espèces ont été génétiquement modifiées pour obtenir un bon rendement à l'artémisinine. Il s'agit du Congo (41), du Brésil (39) et de l'Inde (42).

Différentes préparations sont recommandées. Mueller (40) préconise une infusion de 5g de feuilles séchées dans un litre d'eau bouillante, pour un bon rendement d'extraction de l'artémisinine.

Les études en Chine ont montré que le taux de recrudescence pouvait être réduit si les extraits de la plante *Artemisia annua* étaient combinés aux racines de deux autres plantes : *Astragalus membranaceus* (Leguminaceae) et *Codonopsis pilosa* (Campanulaceae).

La molécule a été isolée pour la première fois en 1972 par des chercheurs chinois, dans l'extrait à l'éther à froid (47). Le rendement d'extraction de l'artémisinine à partir de l'armoise peut varier d'un facteur 10 ou 20 selon la région de culture, ce qui rend son approvisionnement aléatoire. Par ailleurs, la synthèse totale de l'artémisinine est trop coûteuse pour être envisagée de façon industrielle.

L'artémisinine est l'une des 29 sesquiterpènes extraites d'*A. annua*, qui contient aussi une trentaine de flavonoïdes, aussi actifs *in vitro* pour quelques uns et pouvant potentialiser l'activité de l'artémisinine.

L'artémisinine est un sesquiterpène lactone contenant un groupe endopéroxide, qui semble être responsable de l'activité antipaludique de la plante.

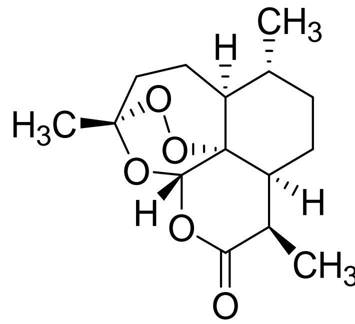

Figure 2 : structure chimique de l'artémisinine

L'artémisinine, comme ses dérivés, est un schizonticide sanguin (25) (24), inactifs sur les hypnozoïtes.

Elle agit par formation d'un radical alkyl, après ouverture du pont peroxyde en contact avec du FeII à l'intérieur des vacuoles digestives du parasite. Ce radical peut alkyler la molécule d'hème et inhiber ainsi le processus de détoxification, entraînant des altérations cytologiques notables (54, 68). De plus, l'ouverture du pont endopéroxyde entraîne une libération d'oxygène et de radicaux libres qui provoquent un blocage de la réplication de l'ADN et de la synthèse protéique, ainsi qu'une perturbation des membranes par alkylation des protéines.

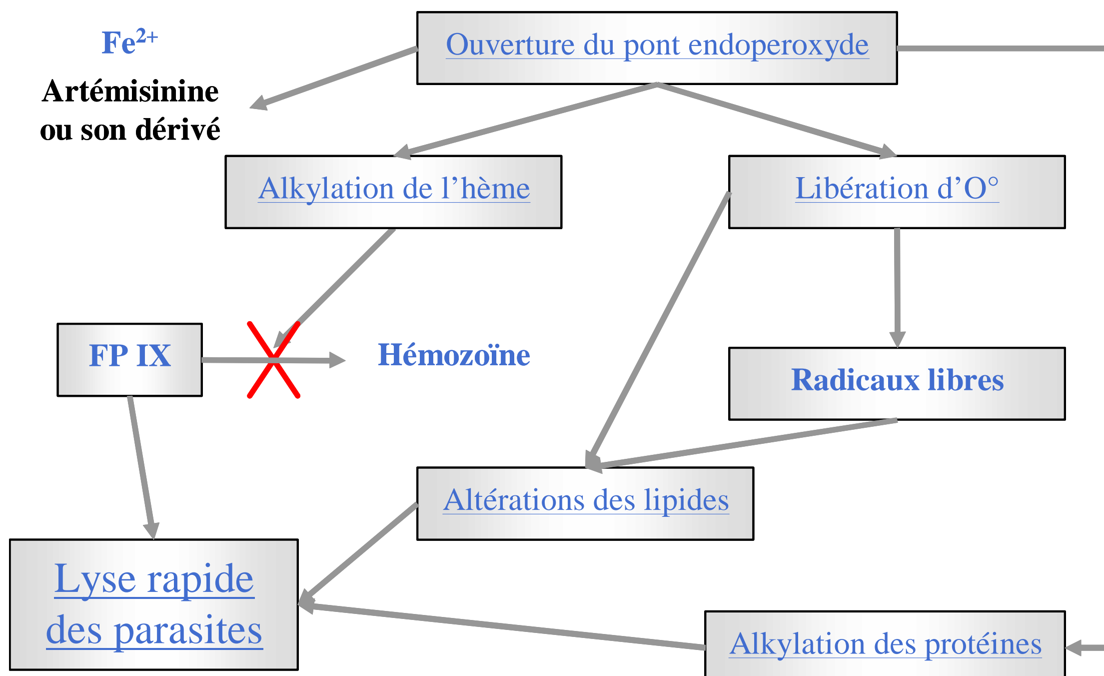

Figure 3 : mécanisme d'action possible de l'artémisinine et des ses dérivés

C'est le motif endopéroxyde qui leur confère l'activité antipaludique. Car les mêmes molécules délestées de ce motif perdent toute activité. Notons également que les globules rouges parasités concentrent cent fois plus l'artémisinine et ses dérivés que les globules rouges sains, ce qui rend ces molécules peu toxiques et hautement actives. L'artémisinine, comme ses dérivés, est hydrolysée en dihydroartémisinine.

L'efficacité de l'artémisinine et de ses dérivés est encore très bonne sur toutes les espèces de *Plasmodium* résistantes ou non à la chloroquine, à l'association sulfadoxine-pyriméthamine et de sensibilité diminuée à la quinine. Ils sont plus actifs sur les souches chloroquinorésistantes que sur les souches chloroquinosensibles.

En raison de la faible biodisponibilité, la faible concentration de dihydroartémisinine et la courte demie vie de cette molécule (2 heures environ) (22), il n'y a plus de doute sur la recrudescence du paludisme après utilisation de l'artémisinine en monothérapie. Il est donc indispensable d'associer systématiquement un autre antipaludique à l'artémisinine ou à ses dérivés afin d'éviter l'apparition de résistances (66).

Le développement d'analogues synthétiques contenant la base endopéroxyde de l'artémisinine a été nécessaire pour améliorer les propriétés pharmacocinétiques de cette molécule.

Trois dérivés principaux sont produits à partir de l'artémisinine : l'artéméther, l'artésunate et l'artééther. Leur efficacité dans la réduction de la charge parasitaire est supérieure à celle des autres antimalariques. Mais leur demi-vie courte est aussi responsable de nombreux cas de recrudescence s'ils ne sont pas utilisés en association.

L'**artésunate** (Arsumax®) est un dérivé hémisuccinate hydrosoluble de l'artémisinine.

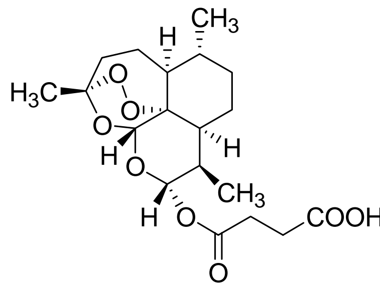

Figure 4 : structure chimique de l'artésunate

Son association à la méfloquine reste très active contre toutes les souches de *Plasmodium falciparum*, même les plus résistantes (35). Son activité prooxydante a été démontrée par **Arreesrisom** et coll (2).

L'**artéméther** (Paluther®) est un dérivé liposoluble de l'artémisinine (7).

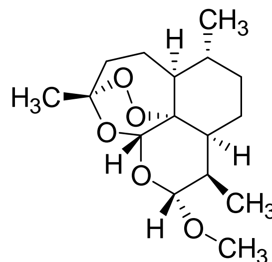

Figure 5 : structure chimique de l'artéméther

De nos jours, l'utilisation de ce dérivé en association est fréquente (26). En effet, il est plus efficace que la quinine, même dans le traitement du paludisme grave (27).

Il est indiqué dans le traitement de l'accès palustre grave à *Plasmodium falciparum* multirésistant (27).

L'**artééther** est un dérivé éthyléther de l'artémisinine. Il a des propriétés semblables à celles de l'artéméther et est conseillé dans le traitement du paludisme grave à *P. falciparum*.

IV. Résistance aux antipaludiques et contexte actuel

La résistance aux antipaludiques dans le monde est un phénomène qui s'aggrave depuis 40 ans selon plusieurs dimensions :

- extension géographique, à partir de l'Amérique du Sud et du Sud-Est Asiatique,
- augmentation continue des pourcentages de souches résistantes,
- augmentation du niveau de résistance des souches,
- poly-chimiorésistance.

La véritable menace en terme de santé publique est apparue au début des années 1960 avec la résistance à la chloroquine,, antipaludique le plus utilisé et le moins onéreux (12).

Cette résistance, qui avait épargné l'Afrique pendant 20 ans a fait son apparition au début des années 80, elle s'est ensuite répandue extrêmement rapidement. Face à cette situation, il a fallu recourir à d'autres antipaludiques (association sulfadoxine-pyriméthamine et méfloquine). De nos jours, l'arsenal thérapeutique pour lutter contre le paludisme en Afrique est limité. La chloroquine en monothérapie est toujours utilisée en traitement curatif, malgré l'inefficacité démontrée. Parce que l'efficacité thérapeutique de ces molécules a été démontrée, l'OMS recommande d'utiliser (20):

- les dérivés de l'artémisinine en association ou la quinine, pour les traitements curatifs,
- la chloroquine-proguanil, la méfloquine ou l'atovaquone-proguanil, pour la chimioprophylaxie.

L'évaluation permanente de l'efficacité des antipaludiques est nécessaire pour assurer aux populations cibles des médicaments efficaces et bien tolérés, afin de mieux lutter contre ce parasite qui a montré sa capacité d'adaptation (53).

IV. 1. Efficacité des antipaludiques

Le dernier rapport mondial sur le paludisme (OMS, 2008) a montré que l'évolution récente des résistances aux antipaludiques se répartie inégalement selon les pays et même à l'intérieur des pays. C'est le cas de la chloroquine, la sulfadoxine-pyriméthamine et la méfloquine en Afrique. En Asie du Sud-Est, la résistance à la méfloquine est telle que de nouveaux protocoles thérapeutiques à base d'association de dérivés de l'artémisinine sont préconisés. Il est dorénavant indispensable d'évaluer l'étendue des résistances dans les régions endémiques du paludisme (37).

Plusieurs critères, dont les données cliniques et biologiques, peuvent être utilisés pour évaluer l'efficacité des antipaludiques.

Des études et rapports de différentes régions du continent africain indiquent que l'efficacité des antipaludiques peut varier d'une région à l'autre (3ème Conférence panafricaine sur le paludisme, 1998). Notamment pour la chloroquine, dont la résistance est répartie très inégalement à travers le continent africain, même pour des régions proches.

Des facteurs, pouvant altérer l'efficacité des antipaludiques sont identifiés et discutés. Il s'agit de la pression sélective des traitements et de l'usage irrationnel des antipaludiques.

En zone d'endémie, la résistance aux antipaludiques représente un problème de santé publique complexe à résoudre. Le recours à de nouveaux schémas thérapeutiques sans contrôle approprié peut provoquer l'apparition de nouvelles résistances, ce qui peut mettre en péril la santé tant au niveau de l'individu qu'au niveau de la collectivité.

Un système standardisé de tests *in vivo* pour évaluer la réponse de *P. falciparum* à la chloroquine a été mis au point pour la première fois en 1965. Les modifications apportées ultérieurement ont permis de choisir entre une période d'observation raccourcie, de sept jours (le « test standard OMS ») et une période d'observation plus longue, de 28 jours (le « test prolongé »), selon qu'il était possible ou non d'inclure des cas de réinfestations. Du fait de l'accent croissant mis sur la réponse clinique et l'immunité acquise sous jacente en fonction des niveaux de transmission dans les régions données, la tendance a évoluée vers le « test prolongé » permettant de déceler des modifications de l'état clinique et des paramètres hématologiques (au moins 14 jours) (67).

L'OMS propose de nouveaux protocoles sur la surveillance de la résistance aux antipaludiques avec les recommandations suivantes (67) :

- une classification des réponses thérapeutiques modifiée pour parvenir à une classification identique dans les régions à transmission intense et dans celle à transmission palustre faible à modérée,
- une révision des méthodes analytiques et statistiques,
- une meilleure définition des contrôles de qualité appliqués à la surveillance,
- une recherche opérationnelle appliquée à la mise au point de nouveaux outils tels que les tests *in vitro* et les marqueurs moléculaires existants standardisés.

Dans ce contexte, une étude a été menée entre 2003 et 2007 au laboratoire de Parasitologie du CHU de Toulouse (Annexe 2) sur l'évaluation des niveaux de résistance aux divers antipaludiques en Afrique. Cette étude a consisté, à effectuer des tests *in vitro* de chimiosensibilité aux antipaludiques les plus courants et à rechercher la présence des marqueurs moléculaires de résistance. Les isolats de *Plasmodium falciparum*, proviennent de patients diagnostiqués au laboratoire, ayant effectué un cours séjour en Afrique sub-saharienne. Des informations ont été recueillies auprès des patients sur le lieu et la durée du séjour en Afrique, la prise effective de chimioprophylaxie et sur leur ethnie.

Soixante dix isolats de *P. falciparum* sont répertoriés dans cette étude et font l'objet des tests de chimiosensibilité *in vitro.* La chloroquine, l'artésunate, l'atovaquone, la quinine, la méfloquine et l'artémisinine ont été testés en prenant pour seuils de résistance 100 nM (chloroquine), 10.5 nM (artésunate), 6 nM (atovaquone), 800 nM (quinine) et 30 nM (méfloquine). Le seuil de résistance pour l'artémisinine n'est pas défini à ce jour. Par la suite, des marqueurs moléculaires de résistance ont été recherchés, tels que la mutation K76T sur le gène *PfCRT*, la mutation N86Y sur le gène *PfMDR1* et l'amplification du gène *PfMDR1*.

Tous les patients de l'étude ont été traités et suivis dans le Service de Maladies Infectieuses et Tropicales du CHU de Toulouse. Ils ont reçu un traitement standard à base de quinine par voie orale ou par voie intraveineuse.

Sur le plan clinique, 62 cas de paludisme non compliqué et 8 cas de paludisme compliqué sont rapportés, ces derniers ne concernant que des sujets caucasiens. Trois patients sont décédés.

Dans les cas de paludisme compliqué, la parasitémie est significativement plus élevée que dans les cas de paludisme non compliqué.

Alors que les mutations T76 sur le gène *PfCRT* et Y86 sur le gène *PfMDR1* sont retrouvées chez 62.5% et 42% des isolats respectivement, nous ne notons pas d'évolution significative de la proportion de souches résistantes entre 2003 et 2007.

Nous confirmons qu'il existe une corrélation entre :

- la présence de la mutation K76T sur le gène *PfCRT* et la résistance à la chloroquine,
- la présence de la mutation N86Y sur le gène *PfMDR1* et la baisse de sensibilité à la méfloquine
- l'amplification du gène *PfMDR1* et la baisse de sensibilité à la méfloquine.

Cependant, aucun lien n'a pu être associé avec les autres molécules étudiées.

Cette étude est l'une des premières qui observe une amplification du gène *PfMDR1* et une baisse de sensibilité à la méfloquine pour les isolats de *P. falciparum* d'Afrique. Ceci a déjà été suggéré uniquement pour les isolats de *P. falciparum* d'Asie et d'Amérique du Sud (50).

Cette étude présente cependant des limites telles que le faible nombre d'isolats étudiés et la difficulté de les cultiver *in vitro* notamment lorsque les patients ont reçu un traitement avant le prélèvement d'échantillon biologique. Par ailleurs, certains points de mutation restent à explorer. Des critères de baisse de sensibilité *in vitro* vis à vis des antipaludiques doivent être redéfinis, en tenant compte de l'échec clinique.

Les résultats de ces travaux (Annexe 2) sont soumis pour publication dans « Malaria Journal »

IV. 2. Contexte et traitements alternatifs antipaludiques

IV. 2.1. Contexte épidémiologique

Les dernières estimations de l'OMS (2008) donnent un taux de mortalité lié au paludisme à 881 000 (610000-1212000) en 2006, dont 91% en Afrique et 85% chez les enfants de moins de cinq ans. Ces nouvelles estimations s'appuient sur des données issues de la surveillance systématique (dans une centaine de pays d'endémie) et d'enquêtes auprès des ménages (dans quelques 25 pays, principalement en Afrique), ainsi que sur l'amélioration des analyses statistiques. Ceux-ci mettent en lumière des réelles avancées sur la réduction de l'incidence de la pathologie (37).

En 2005, pour progresser plus rapidement dans le combat contre le paludisme, l'OMS a fixé pour cible une couverture mondiale d'au moins 80% de la population en zone d'endémie pour quatre interventions :

- moustiquaires imprégnées d'insecticide pour les personnes à risque ;
- médicaments appropriés pour les cas de paludisme probables ou confirmés ;
- pulvérisation intra domiciliaire à effet rémanent pour les ménages à risque ;
- traitement préventif intermittent pendant la grossesse.

L'OMS a en outre précisé que, grâce à ces interventions, la morbidité et la mortalité palustres par habitant devraient diminuer d'au moins 50% entre 2000 et 2010 et d'au moins 75% entre 2005 et 2015.

Une prise de conscience accrue est ainsi observée au niveau des instances éthiques des pays du Nord. L'approche du contrôle du paludisme tend à devenir plus globale et à ne plus reposer entièrement sur une innovation technique donnée (vaccin efficace, bien toléré et peu cher). Cela se traduit par la constitution d'alliances ou de consortiums internationaux associant différents intervenants : gouvernements, ONG, organisations internationales, industries, fondations. L'exemple de récentes initiatives : Roll Back Malaria, Medicine for Malaria Venture et le fond global pour lutter contre le paludisme, le sida et la tuberculose visent entre autre à rendre plus accessibles les traitements aux populations.

L'élimination permanente de la transmission du paludisme en zone d'endémie devra attendre la mise au point de nouvelles stratégies thérapeutiques ou de vaccins efficaces.

Selon l'OMS, des quinze molécules existantes dans le traitement du paludisme, deux sont des produits naturels (quinine et quinidine), cinq des hémisynthèses à partir des produits naturels (artéméther et artésunate issus de l'artémisinine, atovaquone du lapachol, doxycycline et tétracycline de l'espèce *Streptomyces sp*), quatre partagent le noyau quinoléine de la quinine (amodiaquine, chloroquine, méfloquine et primaquine) et enfin quatre molécules synthétiques sont sans lien avec des molécules naturelles (benflumetol, proguanil, pyriméthamine et sulfadoxine) (9).

Partant de ce constat, notre travail a pour but de contribuer à la diversification de l'arsenal thérapeutique antipaludique en ayant recours aux produits d'origines naturelles.

Il faut noter que l'artémisinine et la quinine ont été des molécules largement utilisées sous la forme de produits naturels dans le traitement des fièvres palustres pendant plusieurs siècles. En zone d'endémie aujourd'hui, des populations ont toujours recours aux traitements traditionnels à base de plantes bien souvent en premières lignes thérapeutiques (70).

IV. 2.2. Les traitements alternatifs antipaludiques

Le paludisme est une pathologie bien connue des populations. Des enquêtes ont montré que les populations alternent entre les médicaments antipaludiques prescrits et les traitements traditionnels antipaludiques. C'est le cas de cette étude menée en Guyane française ou il apparaît que, malgré la facilité d'accès et la gratuité des médicaments, les populations préfèrent se traiter à la fois avec des médicaments et des plantes. Le recours aux traitements traditionnels ne dépend pas seulement des facteurs socio-économiques comme souvent très suggéré, mais aussi de la certitude de guérison pour une partie d'entre eux (57).

Actuellement, il est estimé que 80% de la population mondiale se soignent en faisant appel aux ressources des flores et des pharmacopées traditionnelles; parfois par choix, mais trop souvent faute d'avoir accès aux avantages de la médecine scientifique (5).

Dans les pays du Sud où la situation sanitaire est souvent déplorable, le système de soins traditionnels joue un rôle important. Cependant, les connaissances d'une riche pharmacopée végétale et de savoir-faire ancestraux sont en train de s'éroder, parallèlement à la dégradation de la diversité végétale.

Pour conserver les cultures, les savoirs ancestraux et les plantes de cette pharmacopée traditionnelle, notre travail à pour but de valoriser ces connaissances et de les dynamiser en

rationalisant ces pratiques. Ceci dans un souci d'autonomie des communautés et dans le cadre d'un développement durable et intégré.

IV. 2.3. Ethnopharmacologie et nouveaux antipaludiques (18)

L'ethnopharmacologie se définit comme l'étude scientifique interdisciplinaire de l'ensemble des matières d'origine végétale, animale ou minérale, et des savoirs ou des pratiques s'y rattachant, que les cultures vernaculaires mettent en œuvre pour modifier les états des organismes vivants, à des fins thérapeutiques, curatives, préventives, ou diagnostiques. L'intérêt considérable pour l'ethnopharmacologie ces dernières années s'expliquent par trois faits majeurs :

- La pertinence fréquente des indications thérapeutiques de remèdes vernaculaires qui a frappé les scientifiques, médecins, pharmacologues et ethnologues occidentaux,
- Les contraintes économiques : Comment trouver de nouvelles substances naturelles intéressantes dans un milieu naturel dont on sait que, pour les seuls végétaux, il contient plusieurs dizaines de milliers d'espèces, dont chacune peut produire plusieurs dizaines, voire centaines, de molécules différentes ? Comment décider du choix des espèces à étudier, sachant le coût de telles recherches ? A ces questions, l'ethnopharmacologie apporte une réponse originale, en amont de la mise au point de nouveaux produits.
- La prise de conscience au niveau mondial, à la fois de l'ampleur des problèmes sanitaires demeurés sans solution, et même de leur aggravation.

L'ethnopharmacologie peut ainsi intervenir à deux niveaux différents en y apportant des contributions originales. Dans le domaine de la connaissance, elle associe de façon nouvelle les compétences propres à diverses disciplines jusque-là éloignées les unes des autres, et ouvre la voie à l'échange de savoirs scientifiques et non scientifiques. D'un point de vue plus pratique, elle peut contribuer à l'avènement de solutions alternatives aux problèmes sanitaires des pays les plus démunis.

L'ethnologie est indiscutablement une discipline qui agit en amont. Son apport se situe principalement à deux niveaux :

- au niveau ethnographique, sa mission est de donner la description des usages médicinaux, de façon aussi détaillée et fidèle que possible,

- au niveau ethnologique, elle contribue à la mise en relation des usages entre eux, afin de restituer les principes d'organisations des pharmacopées, et plus largement, des systèmes thérapeutiques étudiés (48).

Les travaux de laboratoire, guidés par les usages « vernaculaires » relevés sur le terrain par l'ethnologue, tentent dans un premier temps de constater le bien-fondé de l'usage en démontrant les effets biologiques par des techniques pharmacologiques. Dans un deuxième temps, le pharmacologue s'intéresse à la plante en procédant à l'extraction et au fractionnement des extraits actifs, dans le but d'obtenir une ou des molécules actives, éventuelles candidates pour le développement de nouvelles thérapeutiques.

Il convient de noter que la recherche pharmacologique développe ses stratégies de découverte à partir de sources très diverses, dont les savoirs « vernaculaires » ne représentent qu'une partie limitée.

En général, la recherche de nouvelles molécules actives se fait selon 3 voies :

- la sélection au hasard pour les analyses chimiques (random selection screening),
- ou l'utilisation d'informations provenant des médecines « vernaculaires » (traditional medicine),
- ou l'exploitation des données expérimentales publiées qui indiquent une activité pour les extraits de plante.

La comparaison du rapport coûts-efficacité de deux de ces approches, montre que le nombre d'espèces végétales présentant, *in vitro* ou *in vivo*, une activité cytotoxique ou antitumorale est multiplié par deux, lorsque les plantes sont sélectionnées sur la base de leur utilisation en médecine traditionnelle pour l'indication recherchée, en comparaison avec les résultats du screening au hasard (18).

En ce qui concerne le paludisme, où l'urgence de la diversification de l'arsenal thérapeutique est très forte, l'approche ethnopharmacologique semble être une voie alternative très intéressante. Le fait que deux antipaludiques majeurs, la quinine et l'artémisinine, soient extraits de plantes utilisées dans les médecines traditionnelles contre le paludisme renforce cette idée. Dans plusieurs pays en zone d'endémie, particulièrement en Afrique, l'instabilité des schémas thérapeutiques antipaludiques, le coût et la toxicité des médicaments antipaludiques, ont renforcé l'intérêt des populations locales pour les plantes médicinales (5).

Les premières expérimentations sur le fractionnement chimique bio dirigé des extraits actifs de plantes pour la recherche de nouveaux antipaludiques ont été publiées par O'Neill et coll (44). Par la suite, plusieurs articles dans la même thématique ont été publiés. En effet, les nouveaux outils (culture continue de *P. falciparum*, évaluation de l'activité antipaludique par micro méthode radioactive, protéomique et génomique de *Plasmodium falciparum*) ont amené des équipes de recherche à évaluer l'efficacité antipaludique des produits naturels.

Dans cette étude, notre but est dans un premier temps, de rechercher de nouvelles molécules antipaludiques à partir d'extraits de plantes identifiées lors d'études ethnopharmacologiques en Afrique de l'Ouest, et dans un deuxième temps, d'explorer les propriétés antipaludiques des molécules les plus intéressantes par diverses méthodologies en laboratoire.

IV. 2.4. Revue Bibliographique des extraits aux propriétés antipaludiques : Publication 2

La recherche de nouvelles cibles thérapeutiques par la méthode ethnopharmacologique est une voie intéressante, notamment pour la recherche de nouveaux antipaludiques. Nous avons donc essayé ici de répertorier les substances d'origine végétale issues de pharmacopées traditionnelles d'Afrique de l'Ouest qui ont fait l'objet d'études.

Nous nous sommes intéressés à l'Afrique de l'Ouest parce que nous avons différentes collaborations scientifiques dans cette zone géographique :

- au Niger (Professeurs Ikhirit et Khalid de la faculté des sciences de l'Université A. Moumouni de Niamey),
- au Sénégal (Dr Rita Nongonierma de la Faculté des sciences de l'Université Cheick Anta Diop de Dakar (Sénégal),
- et au Burkina Faso (Pharmacie traditionnelle de la Comoé).

Cette région d'Afrique occupe en superficie le 1/5 du continent Africain (6 140 000 km^2). Cette zone riche en biodiversité végétale, englobe des pays aux situations socio-économiques très précaires. Ce sont des régions situées dans des environnements tropicaux, subtropicaux, ou sahéliens, avec des végétations variant de savanes arbustives aux zones semi-arides. Avec 300 millions d'habitants, l'Afrique de l'Ouest représente une des régions d'Afrique où les niveaux de transmission du paludisme sont les plus élevés. La pharmacopée traditionnelle antipaludique très répandue comprend une grande variété de plantes médicinales.

Les plantes, ayant fait l'objet des tests d'activités antipaludiques *in vitro* sur les souches de laboratoire ainsi qu'*in vivo* sur des modèles murins de paludisme ont été recherchées dans Pubmed.

Entre 1997 et 2007, des tests d'activités antipaludiques *in vitro* ont été menés sur 610 extraits de plantes issues de 16 pays d'Afrique de l'Ouest. 94 extraits ont présenté une très bonne activité *in vitro* sur des souches de *Plasmodium falciparum* de laboratoire avec une CI_{50} des extraits bruts inférieure à 5µg/ml.

Les résultats ont montré que seuls les extraits bruts issus de quatre familles de plantes (Combretaceae, Rubiaceae, Meliaceae et Euphorbiaceae), principalement représentés par quatre genres (*Cochlospermum sp, Cryptolepsis sp, Guiera sp* et *Azadiracta sp*), sont

prometteurs, du fait de leur bon index de sélectivité *in vitro* et de leur bon index thérapeutique *in vivo*. Par ailleurs, les molécules qui en sont issues (cryptolepine, acide ellagique, harman, tetrahydroharman et gedunin) semblent aussi avoir un potentiel antipaludique. Très peu d'activités *in vivo* sur des modèles murins de paludisme ont été publiées, comparées aux tests *in vitro*.

Dans le cadre de cette revue bibliographique exhaustive, la sélection des plantes médicinales par la méthode ethnopharmacologique donne des résultats intéressants, car 15 % des plantes répertoriés montrent une efficacité *in vitro* sur souches de laboratoire. Plusieurs familles de plantes de l'Afrique de l'Ouest (Euphorbiaceae, Meliaceae et Combretaceae), qui présentent une efficacité antipaludique, sont retrouvées dans d'autres régions endémiques d'Asie et d'Amérique du Sud.

Cependant, pour 85% des plantes sélectionnées par la méthode ethnopharmacologique (donc qui semblent présenter une efficacité clinique), l'efficacité antipaludique n'a pas pu être mise en évidence *in vitro*. Plusieurs raisons peuvent être évoquées :

- problème de reproductibilité de la méthode traditionnelle en laboratoire
- dégradation possible du ou des principes actifs au cours de l'extraction
- efficacité dépendante des associations de plantes
- pas d'action directe sur le parasite (symptômes, immunomodulation)…

Ces limites dans la stratégie ethnopharmacologique montrent l'intérêt d'améliorer le concept, et l'obligation de standardiser les procédés, que ce soit dans la sélection des plantes médicinales antipaludiques, ou dans les stratégies d'explorations pharmacologiques.

Ce travail est détaillée dans la revue intitulée : « Are West African plants a source of future antimalarial drugs ? » publiée dans « Journal of Ethnopharmacology » 114 (2007) 130-140.

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Review

Are West African plants a source of future antimalarial drugs?

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Abstract

Ethnopharmacology is a very interesting resource in which new therapies may be discovered. In the case of malaria, two major antimalarial drugs widely used today came originally from indigenous medical systems, that is quinine and artemisinin, from Peruvian and Chinese ancestral treatments, respectively. There is an urgent need for the discovery of new drugs due to the critical epidemiological situation of this disease. New inexpensive therapies that are simple to use and that will limit the cost of drug research are good justifications for this ethnopharmacological approach.

Therefore, the aim of this review is to empirically analyse plants that are used for antimalarial treatment in West Africa, and to determine those with real promising antimalarial activity. The major leads such as those extracted from *Cochlospermum*, *Cryptolepsis*, *Guiera* and *Azadirachta* have been highlighted. Indeed, some extracts seem to be promising in future research, but development of new isolation and characterization techniques, for designing new derivatives with improved properties need to be discussed.
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Keywords: Traditional medicine; Antiplasmodial assays; West African plants

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1. Malaria and ethnopharmacology

One of the major threats concerning world public health is malaria. The mortality rate from malaria has been estimated to be approximately between 1.5 and 2.7 million per year, with more than 75% of these deaths occurring among African children (WHO, 2000).

The main reasons that explain this worsening situation are:

- Resistance to the current antimalarial drugs by *Plasmodium falciparum* (Trape et al., 2002).
- Lack of new therapeutic targets (Bathurst and Hentschel, 2006).
- Unavailability and unaffordability of antimalarial drugs (Benoit-Vical, 2005; Mutabingwa, 2005).

New drugs against malaria are thus urgently needed, but malaria is one of the diseases, that are commonly treated with natural products, mainly from plants.

Natural products and their derivatives have traditionally been the most common source of drugs, and still represent more

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than 30% of the current pharmaceutical market (Kirkpatrick, 2002). Of the 877 new small-molecule chemical entities introduced between 1981 and 2002, roughly half (49%) were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural product pharmacophores (Newman et al., 2003).

It has long been recognized that natural product structures have the characteristics of high chemical diversity, biochemical specificity, and other molecular properties that make them favourable structures for drug discovery, and which serve to differentiate them from libraries of synthetic and combinatorial compounds (Clardy and Walsh, 2004).

In the case of malaria, the new drug discovery approaches need to take into account some specific concerns, in particular, the requirement for new therapies to be inexpensive and simple to use, as well as the need to limit the cost of drug research. Among the currently ongoing efforts is the discovery of new antimalarial targets from natural products. The search for new bioactive plant products can follow two main routes: random or ethnobotanical and ecological research.

This present study is an analysis of ethnopharmacological publications describing research into antimalarial treatments.

2. Ethnobotany and ethnopharmacology research

These approaches can benefit from the knowledge of medicinal plants among natives of regions of endemic malaria, where the appreciation of the use of plant products to treat febrile illnesses has evolved over many generations.

The main goal of ethnopharmacology has been to discover novel compounds, derived from plants used in indigenous medical systems, and which can be utilised in the development of new pharmaceuticals. An aspect of major interest is that the medicines were used directly on humans (Verpoorte et al., 2005).

The first antimalarial drug provided by ancestral treatment was quinine, derived from the bark of the Peruvian *Cinchona* L. tree (Camacho et al., 2000). Despite the cost and adverse effects, a standard treatment for severe malaria in Africa and in Europe is still the intravenous administration of quinine (WHO, 2001). Still now, resistance against quinine in Africa is absent (Le Bras et al., 2006).

One of the new antimalarial molecules discovered by ethnopharmacological plant research was artemisinin. Initially, hot water extracts from *Artemisia annua* L were tested in mice infected with a rodent malaria parasite, but no activity was found. Fortunately researchers later tested ether extracts of the plant and these were found to be active. This lead in 1972 to the isolation of the active principle, initially named Qinghaosu (substance from Qing Hao) and later named artemisinin. The elucidation of the structure revealed that artemisinin was a sesquiterpene lactone that was highly unusual as it contained an endoperoxide moiety (Wright, 2005).

Clinical trials on a large number of patients showed that artemisinin was highly effective in clearing parasitaemia and reducing symptoms in patients with malaria, including some with chloroquine-resistant malaria and cerebral malaria (Woodrow et al., 2005).

3. Situation in West Africa

West Africa occupies an area in excess of 6,140,000 km^2, or approximately one-fifth of Africa. The United Nations definition of Western Africa includes the following 16 countries: Benin, Burkina Faso, Cape Verde, Ivory Coast, Gambia, Ghana, Guinea, Guinea-Bissau, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone and Togo.

This area is one of the poor socio-political situations in the world, but West Africa remains rich in biological resources, both plants and animals. The natural environment in this area consists of subtropical and tropical regions with semi-arid and humid climates. Numerous varieties of medicinal plants growing there are widely used against many diseases such as malaria.

With a total population of nearly 300 million people, subsaharan West Africa represents the region with the largest population exposed to high levels of malaria transmission (Kleinschmidt et al., 2001).

The aim of this review is thus to analyse empirically the plants used for antimalarial treatment in West Africa and to determine those with real promising antimalarial activity.

4. Methods: search strategy and selection criteria

Many different studies have been carried out on African herbal treatments for malaria. A PubMed research using key words (malaria, Africa, ethnobotany, West Africa, antimalarial activity, plant extracts) showed the burden of this disease, the limitations of the current global strategy and the role of traditional medicine in each country studied.

The articles selected concern studies on ethnobotany, on toxicity and on *in vitro* or *in vivo* antimalarial activity of plants from West Africa, and references from relevant articles.

All publications here cited have used classical methodologies such as the continuous culture of *Plasmodium falciparum* strains (Trager and Jensen, 1976); the *in vitro* antimalarial activity tests using radio-isotopic methods (Desjardins et al., 1979) or the microscopic methods; the *in vivo* antiplasmodial assays with the reference for blood schizonticidal activity of plant extracts called the 4-day suppressive Peters' (1970) test using a rodent malaria model. The cytotoxicity concerned *in vitro* known cell-lines and the *in vivo* toxicity was carried out in murine model.

5. Results

Forty-eight ethnobotanical studies from 1987 to 2007 in 9 out of 16 West sub-Saharan Africa countries have been collected (Table 1). The countries were: Benin, Burkina Faso, Ghana, Ivory Coast, Mali, Niger, Nigeria, Sierra Leone, and Togo. The other seven countries (Cape Verde, Gambia, Guinea, Guinea-Bissau, Liberia, Mauritania, Senegal) did not have any ethnobotanical studies published in this data bank. Pharmacological studies have demonstrated some *in vitro* and/or *in vivo* activity and toxicity of extracts from up to 100 species (Table 1).

The Ivory Coast is the country where the most studies have been carried out, particularly *in vitro* and cytotoxicity tests. In the case of *in vivo* tests, the highest number of studies

Table 1
Antiplasmodial plants reported in different West Africa countries from 1987 to 2007

	Benin	Burkina Faso	Mali	Sierra Leone	Togo	Niger	Ghana	Ivory Coast	Nigeria	Total
Number of antiplasmodial plant studies	1	7	3	1	2	1	3	17	13	48
Number of antiplasmodial tests carried out	40	55	48	20	12	9	59	346	21	610
Number of *in vivo* antiplasmodial studies	0	1	0	0	0	1	1	0	10	13
Number of plants with *in vitro* antiplasmodial activity $IC_{50} < 5\ \mu g/ml$	12	14	5	0	3	0	0	60	0	94
Percentage of active ($IC_{50} < 5\ \mu g/ml$) extract of plants per country (%)	30	26	10	0	25	0	0	17	0	15
Number of plant species studied	8	13	10	2	10	3	55	106	32	109

was carried out in Nigeria (Table 1). Some genus of plants were tested against *Plasmodium falciparum* up to five times by one or several authors in the same country, for example *Alchornea* (four authors in Ivory Coast), or *Croton* (eight times by the same author: Weniger in Benin (Weniger et al., 2004)); whereas *Nauclea*, *Mitragyna*, *Pavetta*, *Acanthospermum* and *Cochlospermum* were studied by different authors from several West Africa countries (data not shown).

From the 610 *in vitro* antiplasmodial activity tests carried out in these countries, 94 (15%) showed very good antimalarial activity, with an IC_{50} below 5 μg/ml for the crude extracts.

Antimalarial plants showing high activity *in vitro* concerned the five countries Ivory Coast, Burkina Faso, Benin, Mali and Togo. Even though the Ivory Coast showed many reports of *in vitro* antimalarial tests and numerous good antimalarial plants, the best ratio between the number of plants tested and the number of plants with good activity was found in studies from Burkina Faso and Togo.

The plant families with interesting *in vitro* antimalarial activities and cytotoxicity studies on different *Plasmodium falciparum* strains and cell-lines respectively are described in Table 2 .

Combretaceae, Rubiaceae, Meliaceae and Euphorbiaceae are the families showing the highest number of plants with good *in vitro* antimalarial activity. The plants showing the best antiplasmodial properties, some with IC_{50} under 1 μg/ml for their crude extracts are: *Cochlospermum* sp., *Combretum micranthum*, *Croton lobatus*, *Enantia polycarpa*, *Guiera senegalensis*, *Nauclea latifolia*, *Sida acuta*, *Terminalia glaucescens* and *Ximenia americana*. To estimate the potential of molecules or extracts to inhibit parasite growth without toxicity, the selectivity index (SI) was introduced. The SI was defined as the ratio of the IC_{50} on a cell line to the IC_{50} on *Plasmodium falciparum* for the plant extracts. A high selectivity index (greater than 25) enabled us to select candidates for further investigations. These are *Anogeissus leiocarpus*, *Azadiracta indica*, *Cochlospermum tinctorium*, *Cochlospermum planchonii*, *Guiera senegalensis*, *Nauclea latifolia*, *Rauvolfia vomitoria*, *Terminalia schimperiana* and *Vernonia colorata*. In the case of the cytotoxicity of *Azadiracta indica* extracts, no data was found.

The review of *in vivo* antimalarial activity and toxicity studies produced few results. Nigeria was the country where the most *in vivo* antimalarial tests have been carried out (Table 1). Results using plant extracts or molecules from interesting West African plants, tested *in vivo* are shown in Table 3. Crude extracts from *Quassia amara* (Simaroubaceae), *Momordica balsamina* (Cucurbitaceae) and *Cassia singueana* (Fabaceae) showed the most promising *in vivo* activity with low toxicity in animals.

Some molecules have been isolated and identified from plant species with good antimalarial activity (Table 4). This table shows that gedunin and ellagic acid extracted from *Azadiracta indica* and *Alchornea cordifolia*, respectively, had a very interesting selectivity index. Both these molecules appear to be promising antimalarial candidates.

6. Discussion

Many countries in West Africa have switched their first-line antimalarial drugs, but clinical failure to the newer antimalarial drugs is also occurring at an alarming rate.

Because of the lack of safe and affordable antimalarial drugs, many people in areas where malaria is endemic use natural products (Benoit-Vical, 2005). It is estimated that 80% of the population of many developing countries still use plant-based traditional medicines (Willcox and Bodeker, 2000). A WHO report about traditional medicine showed that 60% of children with high fever resulting from malaria in countries of West Africa were treated with herbal medicine at home (WHO, 2003).

However, most plant extracts in these countries are still processed using old traditional methods that do not always ensure efficacy, stability and safety. One of the most difficult tasks in developing new antimalarial drugs from traditional medicine is the selection of the lead compounds or plants that have the best chance of yielding safe and efficacious antimalarial drugs or to be used in phytomedicine.

The publication of O'Neill et al. (1985) was among the first published that demonstrated the use of an in vitro assay using *Plasmodium falciparum* for the bioassay guided fractionation of plant extracts in the search for new antimalarial drugs. He determined the ratio of *in vitro* antimalarial activity and *in vitro* toxicity before any *in vivo* work. From this there followed many research papers.

These new tools have enabled many scientific teams to evaluate the efficacy of West African plants.

In our review, the Ivory Coast showed the most antimalarial studies because of a high level of exchanges and many collaborations with other more developed laboratories. Indeed for some countries the paucity of antimalarial studies resulted more often from a lack of material and financial support than poor ethnopharmacological practices.

Table 2
Plants showing high antimalarial activity with an IC_{50} inferior to 5 µg/ml and their cytotoxicity values

Families	Plant species	IC_{50} of cytotoxicity test (µg/ml) and (cell-lines used)	Extraction solvent: IC_{50} of antimalarial plants (µg/ml) and (strains of *Plasmodium falciparum* used)	Selectivity index: ratio cytotoxicity/activity	Authors
Annonaceae	*Enantia polycarpa* (Oliver)		Ethanol: 0.12 (K1)		Kamanzi Atindehou et al. (2004)
Apocynaceae	*Funtumia elastica* (Preuss)		Ethanol: 3.3 (FcB1)		Zirihi et al. (2005)
	Rauvolfia vomitoria Afzel	>250 (MRC-5)	Ethanol: 2.5 (FcB1)	>100	
Asteraceae	*Acanthospermum hispidum* (DC) Kuntze	22.7 (L-6)	Chloroform: 4.69 (D6)	4.84	Sanon et al. (2003b) and Zirihi et al. (2005)
	Erigeron floribundus (H.B. & K)	>50 (MRC-5)	Pentane: 4.0 (FcM29)	>12	Menan et al. (2006)
Bixaceae	*Cochlospermum planchonii* Hook	67.3 (L-6)	Methylene chloride: 4.4 (K1)	15.30	Togola et al. (2005), Benoit et al. (1995), Vonthron-Senecheau et al. (2003), Benoit-Vical et al. (1999)
			Ethanol: 2.3 (3D7)	29.26	
			Ethanol: 3.8 (FcB1)	17.71	
	Cochlospermum tinctorium A. Rich.	3.8 (K562)	Water: 0.93 (F32)	4.08	Ballin et al. (2002), Benoit-Vical et al. (1999)
			Water: 1.31 (FcB1)	2.90	
			Water: 1.35 (F32)	2.81	
			Ethanol: 2.3 (3D7)	1.65	
			Ethanol: 3.8 (FcB1)	1	
Combretaceae	*Anogeissus leiocarpus* Guill. and Perr.	71.9 (L-6)	Methanol: 2.6 (FcB1)	27.65	Okpekon et al. (2004), Vonthron-Senecheau et al. (2003)
			Methylene chloride: 2.6 (K1)	27.65	
	Combretum micranthum G. Don		Water: 0.8 (W2)		Benoit et al. (1996), Ancolio et al. (2002)
			Water: 1.18 (FcB1)		
			Water: 1.7 (F32)		
	Guiera senegalensis J.F. Gmel	>25 (Thp1)	Water: 0.79 (FcB1)	>25	
Combretaceae	*Terminalia glaucescens* Planch. Ex Benth	9.35 (Hela)	Pentane: 2.5 (FcM29)	3.74	Mustofa et al. (2000), Okpekon et al. (2004)
			Water: 2.34 (FcM29)	4	
			Water: 2.36 (Nigerian)	3.96	
			Methanol: 2 (Nigerian)	4.67	
			Ethanol: 0.4 (Nigerian)	23.38	
			Methylene chloride: 1.8 (FcB1)	5.19	
	Terminalia macroptera Guill. et Perr.		Water: 2 (W2)		Sanon et al. (2003a)
	Terminalia schimperiana Hochst.	165.8 (L-6)	Ethanol: 2 (K1)	82	Kamanzi Atindehou et al. (2004)
Composae	*Vernonia colorata* (Willd.)	140 (Human Melanoma)	Water: 2.35 (FcB1)	59	Benoit et al. (1996), Addae-Kyereme et al. (2001), Menan et al. (2006)
Euphorbiaceae	*Alchornea cordifolia* (Thonn and schumach) Mull. Arg	54.97 (Hela)	Ethanol: 2.3 (FcM29)	23.90	Mustofa et al. (2000)
			Ethanol: 3.06 (FcB1)	17.96	
			Ethanol: 3.15 (Nigerian)	17.45	
			Water: 3.5 (FcB1)	15.71	
			Water: 4.01 (FcB1)	13.71	
			Water: 2.47 (Nigerian)	22.26	
Euphorbiaceae	*Croton lobatus* L.		Methylene chloride: 2.8 (K1)		Weniger et al. (2004)
			Methylene chloride: 3.6 (K1)		
			Methylene chloride: 3.74 (3D7)		
			Methylene chloride: 4.42 (3D7)		
Euphorbiaceae	*Croton lobatus* L.		Methanol: 4.9 (3D7)		Weniger et al. (2004)
			Methanol: 0.38 (3D7)		

Table 2 (*Continued*)

Families	Plant species	IC_{50} of cytotoxicity test (μg/ml) and (cell-lines used)	Extraction solvent: IC_{50} of antimalarial plants (μg/ml) and (strains of *Plasmodium falciparum* used)	Selectivity index: ratio cytotoxicity/activity	Authors
	Gomphrena celosioides Mart.		Methanol: 4.26 (3D7)		
Fabaceae	*Erythrina senegalensis* DC.		Ethanol: 1.82 (K1)		Kamanzi Atindehou et al. (2004)
	Pterocarpus erinaceus Poir.		Chloroform: 1.93 (Human infected erythrocytes)		Karou et al. (2003)
Malvaceae	*Sida acuta* Burm.f.		Ethanol: 3.9 (FcM29)		Banzouzi et al. (2004), Karou et al. (2003)
			Water: 0.92 (FcM29)		
			Chloroform: 0.87 (FcM29)		
Maranthaceae	*Thalia geniculata* L.		Methanol: 2.8 (3D7)		Weniger et al. (2004)
Meliaceae	*Azadiracta indica* (A. Juss)		Ethanol: 2.4 (W2)	25.42	Benoit et al. (1996), MacKinnon et al. (1997), Kirira et al. (2006)
			Ethanol: 2.5 (D6)	24.40	
			Water: 4.17 (FcB1)	24.22	
	Cedrela odorata L.		Ethanol: 2.77 (W2)		MacKinnon et al. (1997)
	Trichilia emetica Vahl.	8.36 (L-6)	Ethanol: 3.9 (K1)	2.14	Togola et al. (2005), Kamanzi Atindehou et al. (2004)
			Methanol: 2.5 (Dd2)	3.34	
	Trichilia monadelpha (Thonn.)		Ethanol: 3.6 (K1)		Kamanzi Atindehou et al. (2004)
Mimosaceae	*Schrankia leptocarpa* DC. Status		Methylene chloride: 3.38 (K1)		Weniger et al. (2004)
Oleaceae	*Ximenia americana* L.		Water: 0.6 (F32)		Benoit et al. (1996)
			Water: 2.6 (F32)		
			Water: 1.05 (FcB1)		
			Water: 1.83 (FcB1)		
Papilioaceae	*Afrormosia laxioflora* (Benth) Harms.		Methylene chloride: 1.5 (FcB1)		Okpekon et al. (2004)
Piperaceae	*Pothomorphe umbellata* (L.) Miq		Ethanol: 3.74 (K1)		Kamanzi Atindehou et al. (2004)
Rubiaceae	*Cathium setosum* Hiern		Methylene chloride: 2.7 (3D7)		Weniger et al. (2004)
			Methylene chloride: 4.2 (K1)		
	Mitragyna inermis (Willd.) Ktze		Chloroform: 4.3 (W2)		Traore-Keita et al. (2000)
			Chloroform: 4.8 (3D7)		
	Morinda morindoides (Baker) Milne-Redh		Ethanol: 3.54 (K1)		Kamanzi Atindehou et al. (2004), Addae-Kyereme et al. (2001)
Rubiaceae	*Nauclea latifolia* (L.) Sm.	400 (Human melanoma)	Water: 0.6 (FcB1)	666.67	(Benoit-Vical et al., 1998); (Menan et al., 2006)
			Water: 0.7 (FcB1)	571.43	
			Water: 1.3 (FcB1)	307.70	
			Water: 1.7 (FcB1)	235.29	
			Water: 0.7 (Nigerian)	571.43	
			Water: 0.8 (Nigerian)	500	
	Pavetta crassipes K. Schum.		Chloroform: 1.02 (D6)		Sanon et al. (2003a)
			Chloroform: 1.23 (W2)		
Rutaceae	*Fagara macrophylla* (Oliv.)	28.5 (L-6)	Ethanol: 2.3 (FcB1)	12	Zirihi et al. (2005)
Verbenaceae	*Lippia multiflora*Mold.		Water: 1.1 (F32)		Benoit et al. (1996)
			Water: 2.3 (F32)		
			Water: 1.6 (FcB1)		
			Water: 2.2 (FcB1)		
Violaceae	*Hybanthus enneaspermus* (L.) F. Muell.		Methylene chloride: 2.57 (K1)		Weniger et al. (2004)

Table 3
In vivo antimalarial activity and toxicity of West Africa plants

Plants	Extract	*Plasmodium* species	*In vivo* activity (route)	*In vivo* toxicity	Extract plant country	Authors
Azadiracta indica (Meliaceae)	Aqueous extract	*Plasmodium yoelii nigeriensis*	68% suppression at 800 mg/kg/day (oral)	Non-toxic in murine model	Nigeria	Isah et al. (2003)
Cassia singueana Del. (*Fabaceae*)	Methanol extract	*Plasmodium berghei*	80% suppression at 200 mg/kg/day (sub-cutaneous)	Non-toxic in murine model	Nigeria	Adzu et al. (2003)
Momordica balsamina L. (Cucurbitaceae)	Aqueous extract	*Plasmodium vinckei petteri*	50% suppression at 100 mg/kg/day (oral)	Non-toxic in murine model	Niger	Benoit-Vical et al. (2006)
Quassia amara L. (Simaroubaceae)	Hexane extract	*Plasmodium berghei*	98% suppression at100 mg/kg/day (intraperitoneal)	Non-toxic in murine model	Nigeria	Ajaiyeoba et al. (1999)
Erythrina senegalensis (Papilionaceae)	Aqueous extract	*Plasmodium yoelii nigeriensis*	23% suppression at100 mg/kg/day	Non-toxic in murine model	Nigeria	Saidu et al. (2000)
Solanum erianthum D.Don. (Solanaceae)	Aqueous extract	*Plasmodium berghei*	no suppression (oral)	Not available	Nigeria	Makinde et al. (1987)
Striga hermonthica (Del.) (Scrophulariaceae)	Methanol extract	*Plasmodium berghei*	51.3% suppression at 400 mg/kg/day	Not available	Nigeria	Okpako and Ajaiyeoba (2004)
Tapinanthus sessilifolius (P. Beauv) van Tiegh (Loranthaceae)	Methanol extract	*Plasmodium berghei*	68.5% suppression at 400 mg/kg/day	Not available	Nigeria	Okpako and Ajaiyeoba (2004)
Picralima nitida Stapf (Apocynaceae)	Methanol extract	*Plasmodium berghei*	no suppression at 300 mg/kg/day (intraperitoneal)	Not available	Nigeria	Ezeamuzie et al. (1994)
Tithonia diversifolia (Hemsley) A. Gray. (Asteraceae)	Ethanol extract	*Plasmodium berghei*	82% suppression at 400 mg/kg/day	Not available	Nigeria	Elufioye and Agbedahunsi (2004)
Crossopteryx febrifuga (G. Don). Benth. (Rubiaceae)	Ethanol extract	*Plasmodium berghei*	71% suppression at 400 mg/kg/day	Not available	Nigeria	Elufioye and Agbedahunsi (2004)
Amaranthus spinosus L. (Amaranthaceae)	Aqueous extract	*Plasmodium berghei*	50% suppression at 789 mg/kg/day	Non-toxic in murine model	Burkina Faso	Hilou et al. (2006)
Boerhaavia erecta L. (Nyctagynaceae)	Aqueous extract	*Plasmodium berghei*	50% suppression at 564 mg/kg/day	Non-toxic in murine model	Burkina Faso	Hilou et al. (2006)
Cylicodiscus gabunensis (Taub.) Harms (Mimosaceae)	Ethanol extract	*Plasmodium berghei*	20–60 mg/kg/day showed significant schizonticidal activity	Low toxicity in murine model	Nigeria	Okokon et al. (2006)
Uvaria chamae P. Beauv. (Annonaceae)	Ethanol extract	*Plasmodium berghei*	300–900 mg/kg/day showed significant schizonticidal activity	Toxic in murine model	Nigeria	Okokon et al. (2006)
Hippocratea africana (Willd.) (Hippocrateaceae)	Ethanol extract	*Plasmodium berghei*	200–600 mg/kg/day showed promising schizonticidal activity	Toxic in murine model	Nigeria	Okokon et al. (2006)

Table 4
Antimalarial activity and cytotoxicity of molecules from West Africa plants

Plants species	Isolated molecules	Antimalarial activity, IC_{50} (μg/ml) and strains used	Cytotoxicity IC_{50} (μg/ml) and cell-lines used	Selectivity Index (Cytotoxicity/Activity)	Authors
Acanthospermum hispidum (Asteraceae)	Alkaloids	5.02 (W2)	60.2 (THP1)	12	Sanon et al. (2003b)
			79.8 (MHN)	15.90	
			100.8 (HTB66)	20.08	
	Alkaloids	4.69 (D6)	60.2 (THP1)	12.84	
			79.8 (MHN)	17.02	
			100.8 (HTB66)	21.50	
Alchornea cordifolia (Euphorbiaceae)	Ellagic acid	0.08 (FcM29)	6.2 (Hela)	77.5	Banzouzi et al. (2002)
	Ellagic acid	0.14 (Nigerian)	6.2 (Hela)	44.29	
Azadiracta indica (Meliaceae)	Gedunin	0.039 (D6)	2.3 (KB)	58.98	MacKinnon et al. (1997), Bray et al. (1990)
	Gedunin	0.020 (W2)	2.3 (KB)	115	
Cochlospermum tinctorium (Cochlospermaceae)	3-0-E-P-coumaroylalphitolic acid	2.3 μM (3D7)	43 μM (PHA)	18.70	Ballin et al. (2002)
		3.8 μM (Dd2)	43 μM (PHA)	11.32	
Cryptolepsis sanguinolenta (Periplocaceae)	Cryptolepine	0.44 (K1)	0.3 (B16 Melanoma)	0.68	Bonjean et al. (1998), Wright et al. (2001)
Guiera senegalensis (Combretaceae)	Harman	2.2 (D6)	49 (THP1)	22.27	Ancolio et al. (2002)
	Harman	1.3 (W2)	49 (THP1)	37.70	
	Tetrahydroharman	3.9 (D6)	90 (THP1)	23.08	
	Tetrahydroharman	1.4 (W2)	90 (THP1)	64.28	
Triphyophyllum peltatum (Dioncophylaceae)	Dioncophylline	0.063 (K1)	Not tested		François et al. (1994)
	Dioncopeltine	0.330 (K1)			
Pavetta crassipes (Rubiaceae)	Alkaloids	1.23 (W2)	46.1 (THP1)	37.48	Sanon et al. (2003a)
			62.7 (MHN)	50.98	
			79.9 (HTB66)	64.96	
	Alkaloids	1.02 (D6)	46.1 (THP1)	45.20	
			62.7 (MHN)	61.47	
			79.9 (HTB66)	78.33	
Strychnos usumbarensis	Alkaloids	0.1 μM (K1)	Not tested		Wright et al. (1991)

Only 15% of plants traditionally used showed good *in vitro* antimalarial activity. However, plants reported in this review that are not effective against the parasite itself, could act on the symptoms of malaria and/or enhance immunological responses.

Four plant families are widely used in the West Africa area as antimalarial drugs (Combreteceae, Euphorbiaceae, Meliaceae and Rubiaceae). The plant family Euphorbiaceae encompasses species such as *Jatropha* sp., *Ricinus* sp., etc., used against fever or malaria by the populations of the three continents Africa, South America and Asia. Whereas Meliaceae and Rubiaceae are used in both the continents of Africa and South America (Willcox and Bodeker, 2004).

Among the best plant extracts with high *in vitro* activity against *Plasmodium falciparum*, our review showed that polar extracts are the most effective on both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Polar solvents, particularly methyl or ethyl alcohol have the ability to extract a broad spectrum of chemical substances present in traditional mixtures as has been confirmed in screening programs searching for antimalarial compounds from Bolivian plants (Munoz et al., 2000).

Firstly, *in vitro* antimalarial activity with some IC_{50} values below 1 μg/ml lead to the selection of 9 plants. Among these, seven plants gave interesting SI, but only four of the seven plants contained molecules with interesting SI, that is *Cochlospermum tinctorium*, *Cryptolepis sanguinolenta*, *Azadirachta indica* and *Guiera senegalensis*. These four plants seem to be promising sources of antimalarial molecules.

Three West African plants (*Quassia amara*, *Momordica balsamina* and *Cassia singueana*) showed promising *in vivo* activity and low toxicity but without interesting *in vitro* SI.

Some plant extracts of our study, widely use as antimalarial treatment by population, had been explored to find new antimalarial molecules.

Azadirachta indica or Neem (Meliaceae), a West African plant, is widely used around the world for malaria treatment, and is also a well-established insect repellent with insecticide properties. Some *in vitro* studies against *Plasmodium falciparum* were interesting, with extracts from Sudan (El-Tahir et al., 1999), the Ivory Coast (Benoit et al., 1996), India (Dhar, 1998) and from Thailand (Rochanakij et al., 1985). Tests showed that the cytotoxicity of crude Neem extracts was lower than synthesized molecules (Badam et al., 1987). *In vivo* studies in mice with *Plasmodium berghei* have been uniformly disappointing (Ekanem, 1971; Obih and Makinde, 1985). Many reasons could explain these poor *in vivo* activities. It may be that mice do not metabolise Neem extracts in the same way as humans. Murine *Plasmodium* have different properties and sensitivities compared with human *Plasmodium falciparum* and the 4-day suppressive test may be not sufficient to evaluate plant extracts (Willcox and Bodeker, 2004).

Gedunin, isolated from active extracts of Neem, showed an interesting SI (MacKinnon et al., 1997) but did not inhibit *Plasmodium berghei* in the 4-day test in mice at the dose of 90 mg/kg/day (intraperitoneal route).

Cryptolepine, an indolisoquinoline antimalarial alkaloids from *Cryptolepis sanguinolenta* with IC_{50} value approximately half that of chloroquine (Kirby et al., 1995), was inactive in mouse against the *Plasmodium berghei* model (Federici et al., 2000), whereas Wright et al. (1996) showed 80% suppression of *Plasmodium berghei* in mice at a dose of 50 mg/kg/day.

Cochlospermum sp. is also a West African plant widely use in the treatment of fever or malaria and several studies in West Africa showed the interesting antimalarial activity *in vitro* of this plant (Table 2). No data were found concerning *in vivo* antimalarial activity but the SI for molecules from this plant (Table 4), could ascertained its promise as an antimalarial for the future.

The same situation applies to *Guiera senegalensis*, which is a West African plant largely used as an infusion by the population to treat fever or malaria. Antiplasmodial activity of the stems and leaves of *Guiera senegalensis* has been demonstrated (Benoit et al., 1996) and extracted molecules exhibited a significant antimalarial activity and low toxicity (Ancolio et al., 2002). Interesting SI of extracts and molecules of *Guiera senegalensis* (Fiot et al., 2006) demonstrated the additive or synergistic effects of molecules extracted from this plant with those from other plants (*Mitragyna inermis* and *Pavetta crassipes*) against *Plasmodium falciparum*. However, no data were found concerning *in vivo* antimalarial activity of *Guiera senegalensis*.

Actually, few *in vivo* antiplasmodial tests were reported in literature. The advanced technological infrastructures required to carry these tests explain the lack of *in vivo* antiplasmodial experiments. Moreover, whereas all the crude extracts tested *in vivo* are used daily for malaria treatment and show high *in vitro* activity, only a few extracts showed a good *in vivo* efficacy.

Ideally, extracts effective at the blood stage of the malaria parasite should have strong *in vitro* and *in vivo* antimalarial activities and should be devoid of cytotoxicity.

But our results firstly showed that the extracts effective *in vitro* are often without *in vivo* activity, and on the other hand, the extracts active *in vivo* do not show systematically interesting *in vitro* activity. Extrapolation of the data obtained from the pharmacological screening of plants in these models can be misleading. This point is important to note because it seems that Peters' test is not suitable for evaluating *in vivo* antimalarial activity. However, all of the clinically used antimalarial drugs are active in Peters' test and this test is regarded as a good screen for *in vivo* activity. No explanation can solve this problem. It could be voiced that natural crude extracts with their complex mixture of several compounds could explain their high *in vitro* activity thanks to potential synergism but fragile chemical structure could be damaged during *in vivo* assays.

Concerning West African plants, some authors, with socio-epidemiological situation of malaria have carried out "Clinical trials" (Willcox and Bodeker, 2004). Benoit-Vical et al. (2003) showed promising activity of *Cochlospermum planchonii* extracts in the treatment of uncomplicated malaria in Burkina-Faso, on population with the mean age 23 years (ranging from 12 to 45 years old).

However, clinical trials pose as well a problem about the relevance of the clinical data obtained. Indeed, trial patients are almost always adults who have developed some immunity to malaria whereas most of the deaths and severe disease due to malaria occurs in young children and in pregnant women who have low immunity. But studies with new drugs and in particular with traditional medicines on these two groups are not easily achieved.

It is clear that ethnopharmacology offers an interesting way to find new antimalarial molecules from West African plants, but some limitations are obvious, beginning with the lack of an adequate infrastructure in West Africa countries to enhance the selection of interesting plant species.

A second point concerns the need to assess the efficacy of herbal antimalarials as they are used in real life, for example the traditionally prepared decoctions. Bioassay-guided fractionations need to be planned to ensure that the activity of extracts or molecules will not be reduced or lost following isolation and purification.

New innovative approaches are used to assess efficacy of natural products: example of continuous flow enzymatic reactions that are capable of providing real-time read outs of inhibition of enzymatic activity (Schenk et al., 2003a,b). Other approach uses the power of combinatorial synthesis to amplify the structural context in which the unique feature of natural product is expressed. Advances in genomics, metabolic engineering (Wiesner et al., 2003) and chemical synthesis offer today exciting new possibilities to exploit the remarkable chemical diversity of nature's small molecules in the quest for new drugs.

However, the introduction of high-throughput synthesis and combinatorial chemistry has precipitated a global decline in the screening of natural products by the pharmaceutical industry (Ortholand and Ganesan, 2004). Indeed, despite the success of ethnopharmacology which permitted to select crude extracts or molecules, pharmaceutical research into natural products has experienced a big decrease during the past two decades (Benoit-Vical, 2005): the underlying reasons for these industry trends are as much commercial as they are scientific, particularly in the case

of research into infection diseases (Projan, 2003). Today's drug discovery environment calls for rapid screening, hit identification and hit-to-lead development. In this environment, traditional resource-intensive natural-product programmes face a distinct competitive disadvantage when compare with approaches that utilize defined synthetic chemical libraries (Koehn and Carter, 2005).

7. Conclusion

As stated in the Roll Back Malaria project, a new drug needs to be discovered every 5 years. This is unlikely to be achieved because the development of a new drug is costly and time-consuming.

The aim of our review was to report the plants traditionally used in malaria treatment in West Africa and for which antimalarial properties have been already demonstrated by the scientific community. This first step was thus to select plants for which pharmacological studies (*in vitro*, *in vivo* and toxicity) have been completed. The ethnopharmacology approach used in search for new antimalarial compounds appears to be predictive (Saxena et al., 2003). Indeed, such plants as *Azadirachta*, *Cryptolepsis*, *Cochlospermum* and *Guiera* appeared to open the way to future research on the prevention and/or treatment of malaria.

However, it should be emphasized that for any compounds or extracts being recommended for the tratment of malaria it is essential that well-controlled clinical trials are undertaken. Such trials would be invaluable for locating new antimalarial drugs.

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Matériel et méthodes

I. Pharmacologie antipaludique africaine

Préalablement à notre travail de recherche, des enquêtes ethnobotaniques sur les pratiques thérapeutiques traditionnelles antipaludiques en Afrique de l'Ouest et Centrale ont été menées.

Ces enquêtes, réalisées par nos collaborateurs ont consisté en des recueils de données auprès des tradipraticiens et des populations locales. Elles visaient entre autres à préciser les schémas thérapeutiques antipaludiques et à identifier les substances utilisées.

I. 1. Sélection et identification des plantes

Plusieurs plantes ont été sélectionnées de par nos collaborations avec les instituts de recherche dans les pays suivants : Sénégal, Niger, Congo-Brazzaville, République Démocratique du Congo et Burkina Faso, et selon les critères ci-dessous :

- efficacité reconnue par l'usage traditionnel,
- usage fréquent par différents tradipraticiens et dans plusieurs zones géographiques,
- identification de la plante effectuée avec certitude par des botanistes,
- facilité d'obtention de la plante tout en préservant la biodiversité végétale.

Chaque espèce étudiée a été identifiée comparativement à des échantillons de référence répertoriés selon l'efficacité reconnue par les populations et tradipraticiens locaux, et déterminée selon la nomenclature internationale par le nom de genre, le nom d'espèce et l'auteur ayant décrit le végétal. Un échantillon témoin a été déposé au laboratoire de référence, avec un numéro d'herbier.

I. 2. Préparations traditionnelles antipaludiques africaines

Les préparations traditionnelles de notre étude sont représentées dans le Tableau 3. Elles sont autant utilisées dans la prophylaxie et que dans le traitement du paludisme. Elles interviennent aussi dans le traitement d'autres pathologies récurrentes de ces zones en l'occurrence les parasitoses intestinales et sanguines.

Les préparations traditionnelles antipaludiques utilisent une ou différentes parties (feuilles, tiges, racines et écorces) d'une ou de plusieurs plantes. Toutes ces préparations traditionnelles sont utilisées par voie orale. D'après les renseignements obtenus au cours des enquêtes ethnobotaniques, quatre modes opératoires sont identifiés.

- La décoction aqueuse qui consiste à déposer le matériel végétal dans de l'eau froide, et porter à ébullition,
- l'infusion aqueuse qui consiste à verser de l'eau bouillante, sur le matériel végétal,
- la macération qui consiste à laisser tremper le matériel végétal dans un liquide,
- l'extraction aqueuse à froid, qui consiste à écraser le matériel végétal dans de l'eau et filtrer la solution obtenue.

Ces modes opératoires réalisés avec les plantes de notre étude, s'inspirent des préparations traditionnelles. Une fois séchée dans les conditions utilisées dans la pharmacopée traditionnelle (dix jours à 33°C en atmosphère sèche), la plante ou partie de la plante est broyée, puis tamisée afin d'obtenir une poudre fine. Les essais sont réalisés après infusion et décoction de ces poudres, méthodes d'extraction traditionnellement utilisées en Afrique.

Pour optimiser le rendement d'extraction et identifier les substances actives, des extractions par des solvants de polarités variables sont effectuées avec toutes les plantes étudiées.

Le potentiel antipaludique des extraits bruts est ainsi évalué par rapport à l'extrait des parties aériennes de la plante *Artemisia annua* (plante dont est issu l'artemisinine).

Tableau 3 : modes opératoires des préparations traditionnelles antipaludiques.

Espèces (Famille); Code herbier	**Préparation**
Sebastiania chamaelae L (Euphorbiaceae) ; Saadou 2430	Décoction aqueuse de 50 g de parties aériennes dans 1L d'eau pendant 10 minutes
Cogniauxia podoloena Baillon (Cucurbitaceae) ; JTB-CP1	Décoction ou infusion aqueuse de 10-50 g de la tige dans 1L d'eau pendant 10 à 30 minutes
Uapaca paludosa Aubrév. et *Léandri* (Euphorbiaceae) ; Coquelin, 1517	Décoction aqueuse de 5 g d'écorces dans 50 mL d'eau distillée pendant 10 minutes (refroidissement à l'air libre)
Phyllanthus niruri L. (Euphorbiaceae) ; Carlier 83	Décoction aqueuse de 5 g de la plante entière dans 50 mL d'eau distillée (refroidissement à l'air libre)
Chrozophora senegalensis (Lam.) A. Juss (Euphorbiaceae) ; 003SN	Décoction aqueuse de 250 g de feuilles dans 2,5L d'eau distillée

II. Culture *in vitro* de *Plasmodium falciparum*

Cinq souches de *Plasmodium falciparum* sont utilisées dans ce travail : FcM29-Cameroun, W2-Indochine, Dd2, FcB1-Colombie et F32-Tanzanie. Elles sont sélectionnées pour leur sensibilité différente à la chloroquine *in vitro*. Le seuil de résistance à la chloroquine est de 100 nM (19).

- **FcB1-Colombie** est chloroquinorésistante, avec une valeur de CI_{50} (concentration qui inhibe de 50 % la croissance parasitaire) de la chloroquine de 120 nM.
- **F32-Tanzanie** est chloroquinosensible, avec une valeur de CI_{50} de la chloroquine de 30 nM.
- **Dd2 (MR4)** est chloroquinorésistante**,** avec une valeur de CI_{50} de la chloroquine de 114 nM.
- **FcM29-Cameroun** est chloroquinorésistante avec une valeur de CI_{50} de la chloroquine de 400 nM.
- **W2-Cambodge** est chloroquinorésistante avec une valeur de CI_{50} de la chloroquine de 300 nM.

La technique de culture continue de *P. falciparum in vitro* utilisée dans nos essais est dérivée de celle mise au point par Trager et Jensen en 1976 (31, 63). Le milieu de culture est le milieu RPMI 1640 contenant 25 mM d'Hepes et 2,05 mM de L-Glutamine (Life Technologies, France). Ce milieu est ensuite enrichi à raison de 5% d'un mélange de sérum humain (Etablissement Français du Sang du CHU de Toulouse). Les globules rouges sains utilisés sont du groupe O (Etablissement Français du Sang du CHU de Toulouse). La culture est réalisée dans des flacons de culture de 25 et 75 mL (TPP, Suisse) et maintenue en atmosphère humide dans une étuve à 37 °C contenant 5% de CO_2. Le milieu de culture est renouvelé quotidiennement. La parasitémie de la souche cultivée est contrôlée sur frottis minces colorés au Diff-quick® (Dade Behring, Suisse) et quotidiennement réajustée à 2% par ajout d'érythrocytes sains.

III. Congélation et décongélation des souches

Les cultures sont régulièrement sauvegardées par congélation dans de l'azote liquide (-180°C) ou au congélateur (– 80 °C). Pour cela, la moitié d'une culture de parasitémie élevée (supérieur à 4%) avec une grande proportion de trophozoïtes jeunes est centrifugée à 1000 g pendant 5 minutes. En effet, les trophozoïtes âgés et les schizontes sont détruits pendant la congélation. Le culot est alors repris dans un volume équivalent d'une solution de congélation contenant 28% de glycérol, 3% de sorbitol (ou mannitol) et 0,65% de NaCl. Le tout stérilisé par filtration sur membrane de 0,22 µm. Cette solution est ajoutée goutte à goutte à température ambiante pour permettre au glycérol de bien pénétrer dans les hématies. Le contenu est ensuite réparti dans des ampoules à congélation (Nunc, 1,8 mL) en aliquots de 0,5 mL par ampoule maximum. Ces ampoules sont plongées dans de l'azote liquide ou placées dans le congélateur à – 80 °C.

Pour utiliser une culture congelée, l'ampoule est retirée de l'azote liquide ou du – 80 °C et décongelée rapidement au bain-marie à 37 °C. Le contenu de l'ampoule, transféré dans un tube 15 mL stérile. Par la suite, une solution de 12% de NaCl stérile est ajoutée goutte à goutte à raison de 0,2 mL pour 1 mL de culture congelée. Après 3 minutes d'incubation à température ambiante, une solution de 1,6% de NaCl stérile est de nouveau ajoutée goutte à goutte à raison de 10 mL pour 1 mL de culture congelée. Après centrifugation à 2000 g pendant 5 minutes, le surnageant est éliminé et le culot repris dans une solution de 0,2% de dextrose et 0,9% de NaCl stérile à raison de 10 mL pour 1 mL de culture congelée. Après une nouvelle centrifugation dans les mêmes conditions, le culot est mis en culture dans les conditions décrites précédemment.

IV. Synchronisation

Afin de pouvoir étudier l'action des molécules testées sur des parasites de même stade, la synchronisation des parasites en culture est effectuée quotidiennement. En effet, la période de synchronisation correspond au temps écoulé entre la concentration des formes âgées par flottation et la lyse des formes âgées résiduelles. Cette période est fixée en fonction de la méthode d'étude choisie.

IV. 1. Concentration des formes âgées

Les stades âgés de *P. falciparum* sont concentrés par sédimentation dans une solution de gélatine (28). La solution utilisée ici est le Plasmion® (Laboratoire Fresenius Kabi, France). Cette technique n'est applicable qu'aux cultures dites K^+ (45) (présence de knobs) (33). En effet, les souches dites K+ forment des knobs (sortes de protubérances “en bouton” visibles en microscopie électronique) à la surface des hématies infectées par les formes parasitaires âgées. Les stades trophozoïtes et schizontes des souches K+ sédimentent plus lentement que les globules rouges non parasités ou contenant des parasites au stade « ring ». La souche FcB1 utilisée dans ce travail étant K+, bénéficie de cette première étape de synchronisation.

La culture est centrifugée (900 g, 10 mn), le culot, mis en suspension dans du milieu RPMI complet à 37 °C à raison de trois fois son volume. Une solution de gélatine à 3% (Plasmion®, Laboratoire Fresenius Kabi, France) est ajoutée à la suspension (10v/v) et la sédimentation s'effectue pendant 30 minutes à 37 °C. La phase supérieure retenant les globules rouges contenant les parasites âgés est prélevée, centrifugée (900 g, 10 mn) et remise en culture avec des globules rouges sains afin de permettre l'invasion par les mérozoïtes issus de l'éclatement des rosaces.

IV. 2. Lyse des formes âgées

Après un laps de temps permettant l'invasion des globules rouges, les formes âgées résiduelles sont éliminées par lyse osmotique (32) au D-Sorbitol (Merck, Allemagne). En effet, la perméabilité membranaire des globules rouges parasités par des formes âgées de *P. falciparum* est fortement modifiée par rapport aux autres hématies.

La culture est centrifugée (900 g, 10 mn) et une solution aqueuse de D-sorbitol à 5% est ajoutée au culot globulaire dans la proportion de 10 v/v. Les tubes, placés pendant 10 minutes à 37 °C, sont centrifugés (900 g, 10 mn) et le culot remis en culture.

V. Evaluation de l'activité antiplasmodiale *in vitro* sur *P. falciparum* par microméthode radioactive

L'activité antiplasmodiale de chaque molécule est estimée selon une méthode dérivée de celle de Desjardins et collaborateurs (16). Ce test permet de mesurer l'inhibition de la croissance des parasites en culture en présence de diverses concentrations de molécule. Cette évaluation repose sur la mesure de l'incorporation, en fin de test, d'un précurseur des bases puriques marqué au tritium : l'hypoxanthine. L'incorporation de l'hypoxanthine se fait au cours de la phase S du cycle, qui commence à la 30ème heure d'un cycle de 48 heures ; elle est fonction de l'état des *Plasmodium* et constitue de ce fait un bon marqueur de croissance parasitaire. Ce test estt effectué en plaques de 96 puits stériles (TPP, Suisse) selon le schéma de plaque suivant (Figure 8) :

- Les puits extérieurs sont remplis d'eau stérile (200 µL par puits afin de limiter les effets de bord,
- Les puits B2 à G2 et D9 à D11 contiennent les témoins positifs,
- Les puits G9 à G11 contiennent les témoins négatifs.

Tous les autres puits contiennent les dilutions des molécules à tester.

Chaque puits reçoit 100 µL d'une solution à 1% de parasitémie et à 2% d'hématocrite constituée de RPMI à 5% de sérum. Les extraits et molécules à tester sont ensuite distribués dans les puits selon des dilutions croissantes (à raison de 100 µL par puits) et laissés au contact des parasites. Chaque dilution est testée sur 3 puits distincts. Toutes les dilutions sont préparées et testées extemporanément puis après deux jours maximum de conservation au réfrigérateur. Les extraits et molécules sont préalablement solubilisés dans le diméthylsufoxyde (DMSO) (Sigma, France), chauffé à 37 °C. A partir d'une solution mère à 1 mg/mL, des dilutions successives sont réalisées dans du DMSO puis toutes diluées au 1/50 dans du RPMI à 37 °C à 5% de sérum humain. Après distribution, la concentration finale du DMSO dans les puits est alors de 1%. Les puits témoins ayant reçu 100 µL de RPMI à 5% de sérum humain ou RPMI à 5% de sérum humain + 1% de DMSO final. Toutes les dilutions faites avec la chloroquine (témoin au cours des tests antipaludiques) sont réalisées directement dans le RPMI à 5% de sérum humain.

Deux gammes de dilutions sont utilisées dans cette étude :

- 0,05 µg/mL ; 0,5 µg/mL ; 5 µg/mL et 50 µg/mL (pour les extraits bruts végétaux)

- 1 ng/mL, 10 ng/mL, 31,6 ng/mL, 100 ng/mL, 316 ng/mL et 1000 ng/mL (pour les molécules extraites et fractions purifiées).

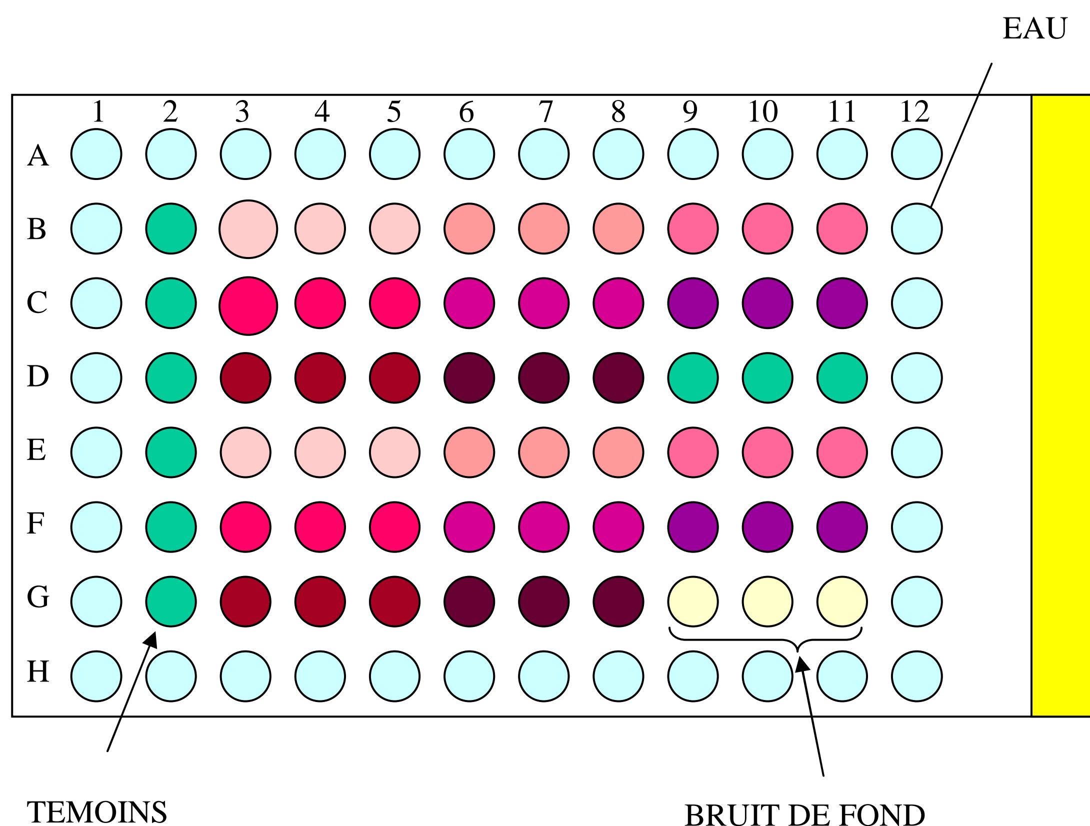

Figure 6 : détail de la répartition des puits pour la microméthode radioactive

La culture parasitée est incubée 48 heures (durée du cycle de *Plasmodium falciparum*) en présence des dilutions dans un incubateur à 37 °C en atmosphère humide à 5% de CO_2. Vingt-quatre heures avant l'arrêt de la réaction, l'hypoxanthine tritiée est ajoutée à raison de 0,25 µCi/puits. Puis la croissance parasitaire est interrompu par la congélation des plaques à – 80 °C pendant au moins 1 heure.

Après décongélation des plaques, le contenu des puits est collecté sur des filtres en fibres de verre (Wallac®, USA) grâce à un collecteur cellulaire (Filter Harvester, USA). Après avoir versé le liquide de scintillation (Perkin Elmer®, USA) à raison de 25 µL/puits, la radioactivité est mesurée par un compteur β automatisé (1450-Microbeta Trilux, USA). Les résultats sont donnés en coups par minute (cpm) par le logiciel Microbeta Windows Workstation®, puis traités sur Microsoft Excel®. Les courbes d'inhibition de la croissance parasitaire en fonction de la concentration des molécules testées sont ensuite tracées.

Les résultats obtenus d'une plaque sont traités de la manière suivante :

- Le bruit de fond, qui correspond à la moyenne des cpm des témoins négatifs est retranché à toutes les valeurs.
- La moyenne en cpm des témoins positifs représente 100% de croissance, soit 0% d'inhibition.
- Pour obtenir l'inhibition de croissance imputable aux molécules testées, la moyenne des triplicatas est ainsi calculée :

$$\%\text{ d'inhibition} = 100 - \frac{\text{moyenne des témoins positifs}}{\text{moyenne du triplicata}} \times 100$$

Cela nous permet de tracer les courbes :

$C_{\%\ \text{inhibition}} = f(\text{logarithme décimal des concentrations testées})$

L'activité antiplasmodiale de chaque molécule est représentée par la concentration à laquelle 50% de la croissance parasitaire est inhibée ; cette concentration est appelée CI_{50} (concentration inhibant 50% de la croissance parasitaire).

VI. Détermination *in vitro* de l'activité stade-spécifique des molécules et extraits testés

Pendant cette étude, le moment d'action de chacun des extraits et molécules d'étude est analysé. Pour ce faire, la souche de *Plasmodium falciparum* FcB1 est utilisée. Quand la parasitémie de la culture est supérieure à 5% avec principalement des formes âgées, la première étape de synchronisation par le Plasmion® s'effectue. Une seconde étape de synchronisation avec le Sorbitol est réalisée 6 à 12 heures plus tard, comme indiqué dans le chapitre IV. Ces deux étapes sont renouvelées jusqu'à avoir une culture très synchronisée avec une parasitémie suffisamment importante pour la réalisation du test. L'expérimentation débute lorsque les parasites sont au stade trophozoïte jeune. Les parasites sont alors mis en plaques 24 puits (TPP®).

La disposition est la suivante.

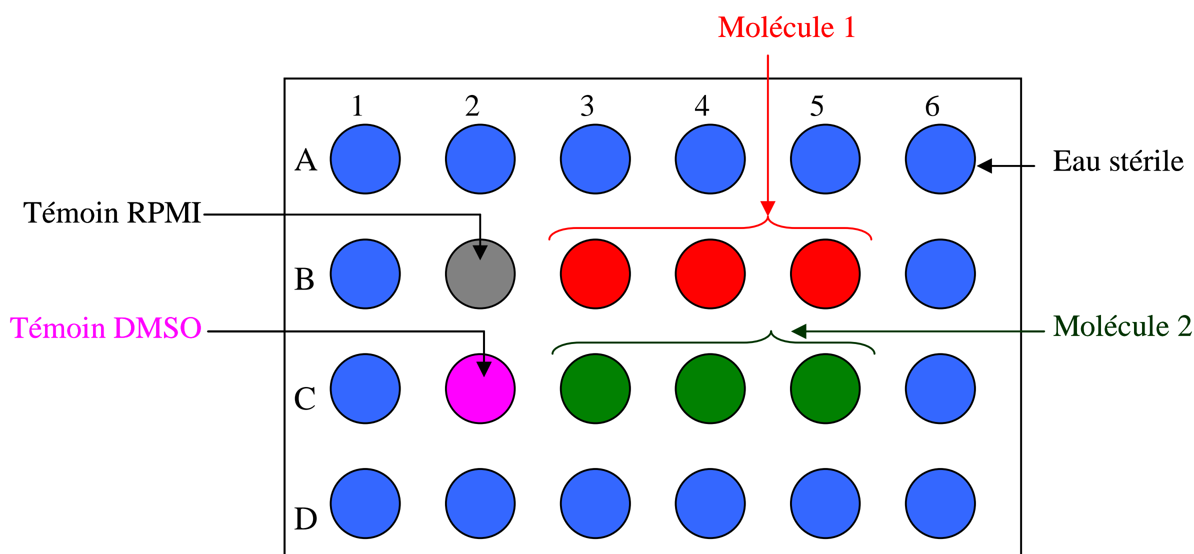

Figure 7 : détail de la répartition des puits pour l'étude du moment d'action

Les puits extérieurs sont tous remplis d'eau stérile (2 mL par puits afin de limiter les effets de bord).

Les puits B2 et C2 contiennent les témoins positifs.

Les puits B3 à B5 et C3 à C5 contiennent des concentrations d'extraits végétaux ou de molécules à tester.

Le cycle du parasite est découpé en périodes de 8 heures et pour chacune de ces périodes une plaque correspondante est préparée. Au temps t = 0 h, chaque plaque reçoit 500 µL d'une solution à 2% d'hématocrite et 0,5 à 1% de parasitémie constituée de RPMI à 5% de sérum. Les puits des plaques sont tous remplis de 500 µL de RPMI 5% sérum humain (SH). Les antipaludiques à tester sont ensuite distribués dans les puits de la première plaque (plaque 1 = 0 à 8 h) selon des dilutions croissantes à raison de 500 µL par puits. Ils sont laissés au contact des parasites 8 h durant. Passé ce délai, trois lavages avec du RPMI s'opèrent sur tous les puits (témoins compris) de la première plaque, et les parasites alors remis en plaque (dans 1 mL de RPMI par puits) pour poursuivre le cycle.

Au même moment, c'est-à-dire à t = 8 h, les différentes dilutions de molécules à tester sont ajoutées à la plaque 2 après avoir retiré 500 µL de milieu RPMI à chacun des puits. L'opération est renouvelée toutes les 8 h pendant les 48 h que dure un cycle parasitaire.

Au bout de ces 48 h, les lavages de la plaque 6 sont faits et 6 heures après (t = 54 h) les frottis de chacun des puits réalisés, et la parasitémie déterminée. Elles permettent d'établir le pourcentage d'inhibition de la croissance parasitaire d'une concentration donnée de molécule à un moment précis au cours du cycle. Il faut noter qu'au cours de l'étude, des témoins antipaludiques (artésunate, chloroquine, artémisinine etc…) sont utilisés aux doses pharmacologiques.

VII. Etude de combinaison de molécules antipaludiques

Les études de combinaisons de molécules utilisent la technique du test de chimiosensibilité. Dans ce test, deux molécules à évaluer en combinaison sont déposées en dilutions sériées et croisées sur une plaque de 96 puits selon le plan de plaque ci-dessous.

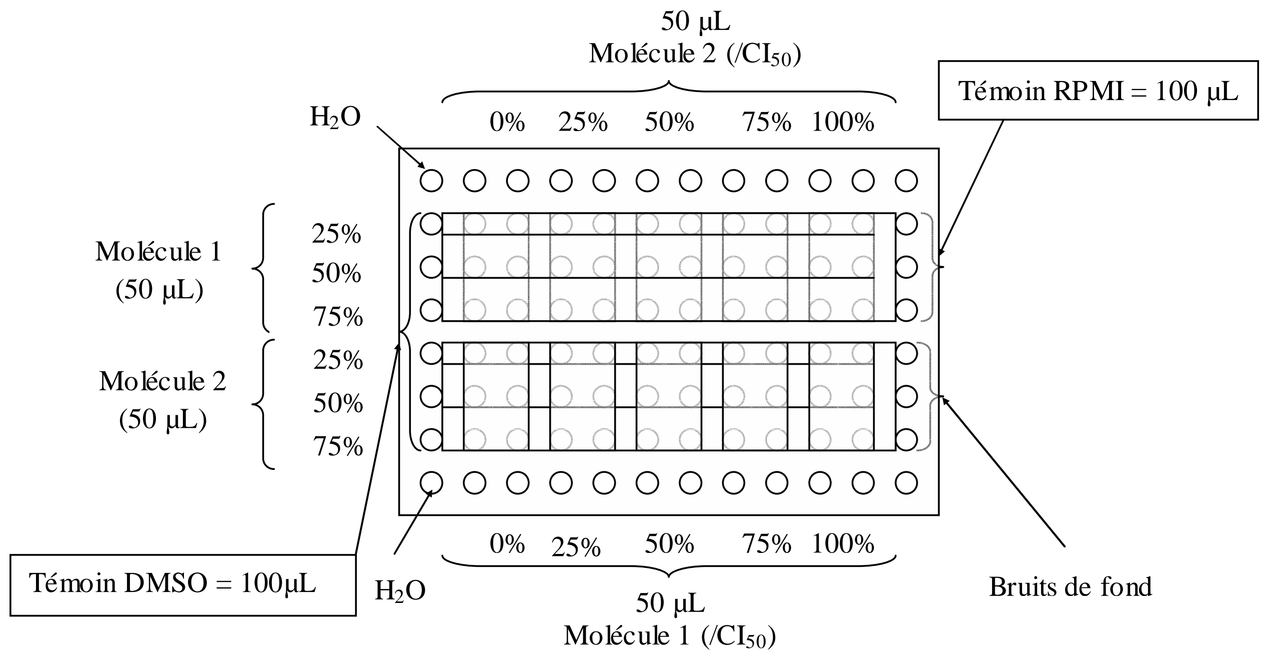

Figure 8 : plan de plaque des tests d'association.

Dans les 72 puits sont déposés 100 µL de sang parasité (2% d'hématocrite et 1% de parasitémie dans du RPMI à 5% SH).

Le témoin DMSO est composé de 50 µL de RPMI à 5% de sérum humain (SH) + 50 µL de RPMI à 5% de SH et à 2% de DMSO.

Le témoin RPMI correspond à 100 µL de RPMI à 5% de SH.

Le bruit de fond n'est constitué que d'hématies non parasitées (200 µL de RPMI à 5% SH et à 2% d'hématocrite).

L'effet potentialisateur des molécules s'évalue sur des courbes dites « isobologrammes ». Pour ce test, différentes concentrations de chaque molécule (correspondant à des fractions des CI_{50} respectives) sont ajoutées à une culture de *Plasmodium falciparum*. Ainsi pour une fraction de la CI_{50} d'une molécule 1, la concentration de la seconde permet d'atteindre la CI_{50} de la combinaison. La valeur CI_{50} de la molécule 2 dans cette combinaison / CI_{50} de la molécule 2 seule est l'ordonnée du point dans l'isobologramme. Puis pour le correspondant de

la molécule 2, la valeur CI_{50} de la molécule 1 dans cette combinaison / CI_{50} de la molécule 1 seule est l'abscisse du point dans l'isobologramme.

Détermination des CI_{50} :

- De chaque % de CI_{50} de la molécule 1 : CI_{50} exprimée en % de la CI_{50} de la molécule 2
- De chaque % de CI_{50} de la molécule 2 : CI_{50} exprimée en % de la CI_{50} de la molécule 1

Construction des points de l'isobologramme **:**

- Ordonnée : % de la CI_{50} de la molécule 2 pour le % X de molécule 1
- Abscisse : % de la CI_{50} de la molécule 1 pour le % X de la molécule 2

Comparaison avec la diagonale de l'additivité et conclusion de l'effet d'association de deux molécules (cf figure ci-dessous).

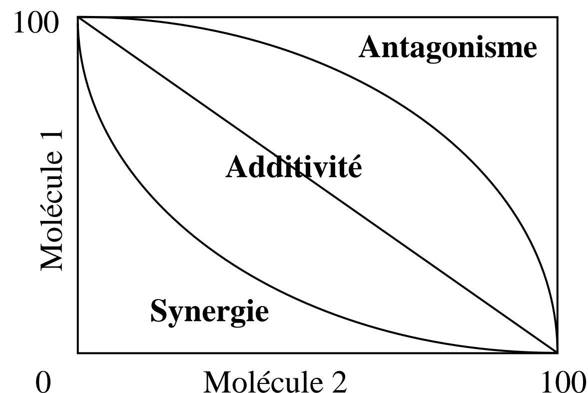

Figure 9 : représentation graphique des isobologrammes.

Les points situés en dessous de la diagonale 100%-100% (diagonale d'additivité) indiquent une synergie entre les deux molécules. Inversement les points situés au dessus de la diagonale indiquent un effet antagoniste.

VIII. Réversion de la chloroquino-résistance

Il a été constaté que certaines molécules aux propriétés antipaludiques pouvaient avoir une activité qui se modifiait en fonction de certains paramètres et notamment dans le cas d'association avec d'autres composés. Une activité est dans certains cas potentialisée, ou dans d'autres diminuée, du fait d'une synergie ou d'un antagonisme spécifique. C'est le cas de la chloroquine associée au vérapamil, qui présente une synergie d'activité sur des souches chloroquinorésistantes. Cette potentialisation est en fait dûe à la réversion de la chloroquinorésistance par le vérapamil, qui agirait comme inhibiteur du canal calcique PfCRT du parasite. Des tests de réversion de la chloroquinorésistance sont effectués, suivant le protocole de Martin (36), avec nos extraits et molécules que nous avons sélectionnés durant les étapes précédentes de , ceci dans le but de récupérer l'efficacité de la chloroquine. La souche chloroquinorésistante W2 mise en présence du vérapamil hydrochloride (Sigma, France) et de la chloroquine sont utilisés comme témoin positif au cours de l'expérimentation. Le témoin négatif étant la souche chloroquinosensible F32, sans effet du vérapamil sur sa chloroquinosensibilité.

- Gamme de dilution pour la souche sensible (F32)

  - **La chloroquine**

0.01 µM – 0.025 µM – 0.05 µM – 0.1 µM

  - **Le vérapamil**

0.1 µM – 0.25 µM – 0.5 µM

  - **La molécule ou extrait de plante à étudier**

Gamme : 10% de la CI_{50} – 20% de la CI_{50} – 40% de la CI_{50} – 50% de la CI_{50}

- Gamme de dilution pour la souche chloroquinorésistante (W2)

  - **La chloroquine**

0.1 µM – 0.25 µM – 0.5 µM – 1 µM

  - **Le vérapamil (même que celle de la souche F32)**

0.1 µM – 0.25 µM – 0.5 µM

- **La molécule ou extrait de plante à étudier**

Gamme : 10% de la CI_{50} – 20% de la CI_{50} – 40% de la CI_{50} – 50% de la CI_{50}

Le pourcentage d'inhibition et la CI_{50} sont calculés et les résultats obtenus permettent de déterminer la CR_{50} (concentration réduisant de 50% la CI_{50} d'une molécule sur une souche chloroquinorésistante).

IX. Etude de la cytotoxicité des molécules et des extraits de végétaux actifs

Ce test est basé sur la mesure de l'activité des déshydrogénases mitochondriales, enzymes fonctionnelles uniquement dans les cellules vivantes. Le principe de ce test est basé sur la réduction d'un sel de tétrazolium XTT (sodium 3,3 (1(1-phényl amino carboxyl)-3-4-tétrazolium bis (4-méthoxy-6 nitro)) benzène sulfonique acide hydrate) par les déshydrogénases mitochondriales en présence d'un agent couplant d'enzymes (le cœnzyme Q). La réduction du sel de tétrazolium XTT libère des cristaux de formazan, un composé hydrosoluble jaune orangé. Une altération de la viabilité cellulaire entraîne alors une modification de la quantité de formazan produite et la densité optique (DO) (490nm) est directement proportionnelle au nombre de cellules vivantes présentes.

Le protocole utilisé est le suivant, des cellules Vero E6 (cellules rénales de singe vert africain), MRC5 (cellules embryoniques pulmonaire humaine) et KB (carcinome épidermoïde humaine), sont réparties en plaques de 96 puits à raison de 5 x 104 cellules par puits (200 µL). Après 48 h de culture (tapis cellulaire à confluence), 50 µL de dilution sériée de solution de molécules ou d'extraits de végétaux à tester sont déposés par puits (en triplicata). Les cellules ensemencées dans les puits témoins de la plaque ne sont pas exposées aux molécules (témoins de viabilité). Après 48 h d'incubation, le milieu de culture est éliminé et le tapis cellulaire lavé deux fois avec du PBS (Sigma, France) pour éliminer le sérum fœtal de veau (supplément du milieu de culture cellulaire) et le rouge de phénol, car ces deux substances interférent dans la réaction de transformation du XTT. 50 µL d'une solution préparée extemporanément à 0,5 mg/mL de XTT (Sigma, France) et 40 µg/mL de coenzyme Q (Sigma) dans du DMEM (milieu de culture modifié de Dulbecco) sans rouge de phénol sont déposés sur le tapis cellulaire et la plaque est incubée pendant 3 heures à 37°C. La réaction est arrêtée avec 100 µL de sodium dodecyl sulfate (SDS) 10% (Sigma, France). Le nombre de cellules viables étant proportionnel à la densité optique mesurée, la concentration cytotoxique pour 50% de la population cellulaire (CI_{50}) est ainsi déterminée.

X. Maintien de *Plasmodium vinckei petteri in vivo*

La souche de *Plasmodium* de rongeurs utilisée dans ce travail est *Plasmodium vinckei petteri* (fournie par le Dr. I. Landau, Musée National d'Histoire Naturelle de Paris, France) (65). L'ampoule contenant le sang d'une souris parasitée (parasitémie proche de 30%), congelée avec une solution de RPMI à 10% de DMSO dans l'azote liquide, est décongelée dans le bain marie à 37°C. Le contenu est alors inoculé par voie intrapéritonéale à deux souris Swiss femelles (Janvier, France), pesant environ 30 g. La parasitémie est alors analysée tous les deux jours pour évaluer l'état d'infestation des souris. Pour cela, une goutte de sang caudal est prélevée pour effectuer un frottis. Lorsque l'une des deux souris a une parasitémie suffisante (environ 30% de parasitémie), un prélèvement de sang est effectué (technique de prélèvement sinus rétro-orbital) avec de l'héparine (Héparine Choay®, 25 000 U.I. /5mL), et 50 µL de ce sang est inoculés à deux nouvelles souris par voie intrapéritonéale.

XI. Détermination des DE_{50} et DL_{50}

L'activité antiplasmodiale *in vivo* est évaluée selon le test suppressif de 4 jours d'après la technique de Peters (13, 46) sur la souche murine *Plasmodium vinckei petteri*. Des souris femelles (Swiss albinos, 8 semaines) sont infectées par voie intrapéritonéale à J_0 par 2×10^7 globules rouges parasités (resuspendus dans 1 mL de NaCl). La molécule à tester est ensuite administrée aux animaux 3 heures après l'infection et tous les jours à cette même heure jusqu'au quatrième jour (J_4). Les molécules solubilisées dans le DMSO, sont diluées au demi dans une solution à 0,9% de NaCl. Le traitement est alors administré par voies orale et intrapéritonéale. Pour chaque dose testée, 5 souris sont infectées et traitées ainsi que 5 témoins (traitées par la solution excipient). A J_4, la parasitémie de chaque souris est évaluée sur frottis colorés au DiffQuick®. Ces parasitémies permettent de tracer les courbes d'inhibition de la croissance parasitaire en fonction du logarithme décimal des doses de molécules administrées. A partir de ces courbes, les doses inhibant 50% de la croissance parasitaire sont déterminées. Ces valeurs, nommées DE_{50}, sont établies par comparaison avec les parasitémies obtenues chez les souris témoins (traitées par un volume identique d'excipient).

La toxicité murine des molécules ou extraits végétaux est aussi évaluée. Les souris (de type Swiss albinos femelles non consanguines) non infectées sont traitées par voies orale et intrapéritonéale avec des concentrations variables des extraits ou des molécules à tester pendant 4 jours. Le nombre de décès et signes cliniques sont enregistrés tous les jours jusqu'au 60ème jour, et la dose provoquant le décès de la moitié de la population murine saine (DL_{50}) est calculée.

Toute expérimentation animale est faite sous le contrôle d'un comité d'éthique et dans une animalerie agréée suivant les normes européennes. Les copies d'accords pour les différentes expérimentations sont en annexes (Annexe 3).

Résultats

Evaluation de l'activité antipaludique des plantes africaines utilisées dans le traitement du paludisme et des molécules extraites

Grâce à la collaboration de notre équipe à Toulouse dirigée par le Docteur Francoise Benoit-Vical (Chargée de recherche Inserm) et des ethnobotanistes situés en Afrique de l'Ouest (Sénégal et Niger) et Centrale (République Démocratique du Congo, Congo-Brazzaville), plusieurs plantes ont été sélectionnées, récoltées, traitées selon les procédés traditionnels et acheminées à Toulouse pour étude pharmacologique et à Gif-sur -yvette pour fractionnement chimique.

Des enquêtes ethnobotaniques ont permis de sélectionner des plantes largement utilisées en Pharmacopée antipaludique en Afrique de l'Ouest et Centrale. Elles ont été menées par des ethnobotanistes :

- à Dakar sous la direction du Dr Rita Nongonierma de la Faculté des sciences et Techniques de l'Université Cheick Anta Diop de Dakar (Sénégal)

- à Niamey par l'équipe du Professeur Ikhirit de la Faculté des sciences de l'Université A Moumouni de Niamey,

- au Congo-Brazzaville, par le Dr Jean Théophile Banzouzi de l'ONG « Médecins d'Afrique » et du groupe CERMA (centre d'étude et de recherche des médecins d'Afrique),

- et en République Démocratique du Congo avec l'équipe du Professeur Lusakibanza du Laboratoire de pharmacologie de l'Université de Kinshasa (RDC).

Ces plantes ont été sélectionnées de part leur distribution élargie en Afrique sub-saharienne dans le traitement du paludisme.

La sélection des extraits actifs des plantes répertoriées, ainsi que le fractionnement chimique bio dirigé pour identifier les molécules, ont été effectués en collaboration des phytochimistes de l'Institut de Chimie des Substances Naturelles du CNRS à Gif-sur-yvette

(selon les plantes par les docteurs Christiane Poupat, Maria Concepcion-Garcia et Jean Théophile Banzouzi).

Des extraits actifs des plantes de polarités variables ont été séparés par des séries de chromatographies sur couche mince ou sur colonne, et les fractions obtenues testées *in vitro* sur les souches de laboratoire. Les extraits les plus actifs ont de nouveau fait l'objet de séparation, par les mêmes méthodes chromatographiques précitées en variant sur les concentrations de solvants d'élution. Les fractions les plus actives sur les souches de *P. falciparum* de laboratoire ont été caractérisées par résonance magnétique nucléaire, ultraviolet, infrarouge et spectrométrie de masse.

Il ressort des résultats de tests d'activités antipaludiques *in vitro* obtenus que le DMSO dilué au $1/50^{ème}$ est le solvant de dilution idéal des extraits d'origine végétale et des molécules extraites pour une bonne reproductibilité des tests antipaludiques *in vitro*.

Le potentiel antipaludique des extraits bruts était contrôlé en utilisant comme témoin l'extrait méthanolique des parties aériennes de la plante *Artemisia annua* (plante à partir de laquelle est extraite l'artémisinine).

I. Publication 3 : *Chrozophora senegalensis*

Au Sénégal, une étude ethnobotanique menée dans la région de Dakar pour inventorier les espèces de plantes utilisées dans le traitement du paludisme a permis de sélectionner 7 plantes remplissant les critères décrits ci-dessus. Il s'agit de *Cissampelos mucronata, Maytenus senegalensis, Terminalia macroptera, Bidens engleri, Ceratothera sesamoides, Chrozophora senegalensis* et *Mitracarpus scaber*. Au cours de cette étude, les tests d'activité antipaludique *in vitro* n'ont concerné que les plantes : *Bidens engleri, Ceratotheca sesamoides, Chrozophora senegalensis* et *Mitracarpus scaber* L'activité antipaludique de ces quatre plantes, n'ayant jamais été rapportée auparavant par d'autres équipes. Les autres plantes ayant fait l'objet d'études antérieures publiées par d'autres équipes sont exclus de notre travail. (*Cissampelos mucronata* (21), *Maytenus senegalensis*, *Terminalia macroptera* (43) ;(55). Les parties des plantes d'étude sont extraites dans trois solvants de polarité variable (éther de pétrole, acétone et éthanol) et testées sur deux souches chloroquinorésistantes de *P. falciparum* (FcM29-Cameroun et FcB1-Colombie). Quelque soit la souche testée, les CI_{50} de *Bidens engleri* varient entre 9 et plus de 50µg/ml, celles de *Ceratotheca sesamoides* de 4 à

plus de 50µg/ml, de *Mitragyna scaber* de 7 à plus de 50µg/ml et de *Chrozophora senegalensis* de 1 à 50µg/ml..

Pour *Bidens engleri*, *Ceratotheca sesamoides* et *Mitracarpus scaber*, les extraits à l'éther de pétrole donnent une meilleure activité qu'à l'éthanol et acétone. Les meilleures CI_{50} sont obtenus avec les extraits bruts aqueux des tiges et feuilles de *Chrozophora senegalensis* (Euphorbiaceae), avec une bonne efficacité *in vitro* (CI_{50} égale à 1.8±1µg/mL) et un bon index de sélectivité (13) comparé aux autres plantes. L'absence de toxicité *in vivo* et la détermination du moment d'action au cours du cycle érythrocytaire du parasite (forte inhibition de la croissance parasitaire entre la $12^{ème}$ et $44^{ème}$ heure), correspondant au métabolisme des protéines et des acides nucléiques parasitaires, de l'extrait aqueux des feuilles de *Chrozophora senegalensis* justifient l'usage traditionnel de cette plante.

Les études *in vivo* n'ont démontré aucune activité des extraits aqueux et éthanolique de *Chrozophora senegalensis*, même à la dose de 500 mg/kg/j. Il faut noter que le test suppressif de Peters est souvent peu approprié à l'étude de l'activité antipaludique *in vivo* des extraits végétaux sur modèle murin.

Ce travail est publié dans « Journal of Ethnopharmacology » 116 (2008) 43-48.

Du fait de ces propriétés antipaludiques, et de son usage élargi en Afrique de l'Ouest, les tests de prophylaxie sur modèles murins de paludisme avec l'extrait aqueux des feuilles de *Chrozophora senegalensis* ont été menés par la suite. Les résultats non publiés dans cette article ont montré qu'après traitement des souris par voie intrapéritonéale pendant quinze jours avant inoculation des parasites *Plasmodium vinckei petteri* (1 million/µL par voie intrapéritonéale), les souris avaient une réduction de la parasitémie à J6 de 76% comparée aux témoins traités avec l'excipient (solution de DMSO diluée au demi dans du sérum physiologique), et se maintenaient en vie jusqu'à J60.

Le fractionnement chimique bio dirigé de l'extrait aqueux des feuilles de *chrozophora senegalensis* a permis par la suite d'identifier un polyphénol : l'acide ellagique comme molécule majoritaire.

L'étude des propriétés pharmacologiques de l'acide ellagique est rapportée dans la publication 6.

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Evaluation of Senegalese plants used in malaria treatment: Focus on *Chrozophora senegalensis*

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Abstract

An ethnobotanical study was conducted in the Dakar area of Senegal to investigate the species used in the treatment of malaria. Seven plants are principally used: *Cissampelos mucronata*, *Maytenus senegalensis*, *Terminalia macroptera*, *Bidens engleri*, *Ceratotheca sesamoides*, *Chrozophora senegalensis* and *Mitracarpus scaber*. From a bibliographic study, it had been shown that the *Cissampelos mucronata*, *Maytenus senegalensis* and *Terminalia macroptera* have already been studied by several authors, and so only *Bidens engleri*, *Ceratotheca sesamoides*, *Chrozophora senegalensis* and *Mitracarpus scaber* were evaluated in the present study. For each plant, extracts were prepared with different solvents and tested *in vitro* on two chloroquine-resistant *Plasmodium falciparum* strains. Crude extracts from the leaves and the stems of *Chrozophora senegalensis* showed the best *in vitro* results. The IC_{50} value of an aqueous extract of *Chrozophora senegalensis* was 1.6 μg/ml without cytotoxicity. The *in vivo* antiplasmodial activity of *Chrozophora* extracts was determined by both the oral and the intraperitoneal ways. The stages of *Plasmodium* cycle targeted by *Chrozophora* were then studied *in vitro*. These results could justify the traditional use of this plant in malaria treatment.
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Keywords: Malaria; *Chrozophora senegalensis*; Senegal; Traditional medicine

1. Introduction

Malaria is one of the most important diseases of the 21st century with an annual death rate of approximately 3 million (WHO and Bloland, 2001). Crude extracts of natural products appear to be promising therapeutic candidates in the hope of finding remedies that can be prepared and administered locally at very low cost. Indeed, natural products have played a key role throughout the history of antimalarial drug discovery as a source of both novel compounds and scaffolds for the development of blockbuster drugs (Benoit-Vical, 2005).

Within this context, four plants traditionally used for the treatment of malaria in the Dakar area (Senegal) were studied for their antiplasmodial activity.

2. Materials and methods

2.1. Plant material

Cissampelos mucronata A. Rich. (Menispermaceae), *Maytenus senegalensis* Lam. (Celastraceae), *Terminalia macroptera* Guill et Perr. (Combretaceae), *Bidens engleri* L. (Euphorbiaceae), *Ceratotheca sesamoides* Endl (Pedaliaceae), *Chrozophora senegalensis* (Lam.) *A. Juss* (Euphorbiaceae) and *Mitracarpus scaber* Zucc. (Rubiaceae) were collected in the Dakar area (Senegal). The vouchers specimens are, respectively, 011SN, 013SN, 017SN, 001SN, 007SN, 003SN and 014SN (Herbarium of Faculté des Sciences et Techniques,

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UCAD, Dakar, Sénégal). Samples were air-dried and powdered.

2.2. Preparation of plant extracts

Only the plants *Bidens engleri*, *Ceratotheca sesamoides*, *Chrozophora senegalensis* and *Mitracarpus scaber* were extracted and tested on *Plasmodium*. For each plant, three crude extracts were obtained, by soxhlet extraction during 48 h, using 250 g of plant powder with 2.5 l of either petroleum ether, or acetone or ethanol. Extracts from *Chrozophora* were divided into extracts from the leaves and from the stems. For the water extract of the leaves of *Chrozophora*, a decoction was carried out in hot distilled water kept at 100 °C for 30 min. After filtration, the extracts were evaporated to dryness under reduced pressure. For the tests, stock solutions of the extracts (1 mg/ml) were prepared in dimethylsulfoxide (Acros Organics, Belgium) and then diluted in RPMI 1640 (Gibco). The dilutions were checked to confirm that the extracts did not re-precipitate under these conditions. All the extracts were tested *in vitro* on *Plasmodium falciparum* immediately after dissolving in DMSO and RPMI. The same extracts were also kept at 4 °C for 3–8 days, and then tested again.

2.3. In vitro culture of Plasmodium falciparum and antiplasmodial activity

The two strains of *Plasmodium falciparum* used were: FcB1-Colombia chloroquine-resistant, with a 50% inhibitory concentration (IC_{50}) for chloroquine of 60 ± 20 ng/ml and FcM29-Cameroon, a highly chloroquine-resistant strain ($IC_{50} = 400 \pm 15$ ng/ml), and were cultivated continuously using standard culture methods (Trager and Jensen, 1976; Van Huyssen and Rieckmann, 1993). The antiplasmodial activity of the plant extracts was evaluated by the radioactive micro-method previously described (Desjardins et al., 1979; Benoit et al., 1996). Each geometric mean IC_{50} corresponded to four independent experiments.

2.4. In vitro cytotoxicity tests

The Vero cells were serially cultured in MEM (minimum essential medium, with Earle's salts), purchased from Seromed and containing 10% foetal calf serum, 2 mM L-glutamine, 60 μg/ml penicillin G, 60 μg/ml streptomycin sulphate and 40 μg/ml gentamycine. For the test, KB cells were grown as monolayers in 24-well plastic plates (25,000 cells seeded per well in 1 ml medium). Serial dilutions of the stock solutions from the ethanol extractions of the compounds were made in the medium and added to the cell cultures in a volume of 10 μl per well, immediately after plating the cells. All the cultures were incubated at 37 °C in a 95% air–5% CO_2 humidified incubator. After 3 days, cell viability was determined by the addition of 100 μl/well of a 0.02% solution of the vital dye neutral red in medium, followed by a further 8–16 h incubation. The cell monolayers were washed with phosphate-buffered saline, lysed with a 1% sodium lauryldodecyl sulphate solution and the extracted dye quantified photometrically at 540 nm, using an ELX800 microplate reader (BIOTEK Instruments, Inc.) as originally described by Borenfreund and Puerner (Borenfreund and Puerner, 1985).

2.5. In vivo toxicity

The toxicity of aqueous extracts of the leaves of *Chrozophora* was evaluated via the oral route. Healthy Swiss 8-week-old female mice (Elevage Janvier, Le Genest Saint Isle, France) were treated once a day for 4 consecutive days with 500 mg/kg/day. The mice were then observed for 60 days to evaluate the eventual effects of *Chrozophora*. Mortality and other signs of toxicity such as prostration were recorded if they occurred.

Animals were kept in plastic cages at room temperature and moisture, under illuminated environment of 12:12 h dark/light cycle. They were fed with a standard diet and had access to tap water *ad libitum*. All procedures involving animals were fully conform to European regulations (EEC directive 86/609 dated 24/11/1986). The experiments involving animals were carried out in the animal room of the Parasitology Department of Rangueil Hospital (Toulouse, France) which is under the control of the National Veterinary Services. All *in vivo* studies were approved by the French Institutional Animal Experimentation Ethic Committee #MP/01/28/07/04/R/02/06.

2.6. In vivo antiplasmodial activity

Plasmodium vinckei petteri was intraperitoneally inoculated to mice with 2×10^7 infected erythrocytes on day 0. The treatment dose was given 3 h after infection on day 0 and was repeated once daily for 3 days, as a "4-day-blood schizonticidal test". Two modes of administration of the treatment (intraperitoneal and oral route) were studied. For each group (one drug, one dose, one route), five female Swiss albino mice (Elevage Janvier, Le Genest Saint Isle, France) were studied; their parasitemia and mortality were followed during 60 days. This *in vivo* experiment included control groups: a sentinel group (the same batch of mice as treated mice but that were not infected and not treated), a drug-free group (mice that were infected with *Plasmodium* but that received no treatment), and an excipient group (mice that were infected with *Plasmodium* and that received no drug treatment but that did treated with the excipient used for the extracts tested). As drug control, five mice were treated by 5 mg/kg/day of artesunate (Gift of Sanofi-Aventis, France) by the both ways. On day 4, thin blood smears were made from mouse-tail blood and stained by Giemsa in order to evaluate parasitemia levels.

2.7. Stage of the erythrocytic life cycle affected by the extract of Chrozophora leaves

Cultures of FcB_1K^+ (which express knobs during the schizonte-stage) strains were synchronised to a 4 h period. The method of synchronisation consisted in the alternative synchronisation of young forms with 5% D-sorbitol and of late forms with Plasmion (Lelievre et al., 2005). An aqueous extract of *Chrozophora* leaves was tested in 24-well plate with culture at

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Table 1
*In vi*tro antiplasmodial activity of extracts from four Senegalese plants against two chloroquine-resistant *Plasmodium* strains

Plant	Extract	Yield (%)	FcM29-Cameroon strain		FcB1-Colombia strain	
			Mean of IC_{50} (μg/ml)			
			Fresh	Stored	Fresh	Stored
Bidens engleri	Petroleum ether	5.88	12 ± 2	18 ± 1	14	9
	Acetone	8.02	36 ± 1	41 ± 1	>50	>50
	Ethanol	8.30	>50	>50	>50	>50
Ceratotheca sesamoides	Petroleum ether	2.15	20 ± 5	23 ± 8	15	15
	Acetone	9.64	28 ± 3	28 ± 3	>50	33
	Ethanol	11.03	>50	27	>50	4
Chrozophora senegalensis (*leaves*)	Petroleum ether	3.56	>50	>50	nd	>50
	Acetone	9.92	17 ± 2	16 ± 0	21	9
	Ethanol	10.20	13 ± 3	18 ± 20	12	nd
	Water	35.06	1.9 ± 0.1	1.8 ± 0	1.6 ± 0	1.8 ± 0.1
Chrozophora senegalensis (*stems*)	Petroleum ether	1.14	13 ± 2	13 ± 2	15	11
	Acetone	6.60	14 ± 2	17 ± 0	15	5
	Ethanol	5.36	10 ± 1	21 ± 9	17	nd
Mitracarpus scaber	Petroleum ether	5.01	15 ± 0	>50	7	18
	Acetone	4.95	38 ± 0	10 ± 0	nd	17
	Ethanol	6.73	/	/	35 ± 0	40
Artemisia annua	Ethanol	18	1.5 ± 1	1.6 ± 1	nd	nd
Chloroquine			400 ng/ml		60 ng/ml	

nd: not done.

0.5–1% parasitemia (hematocrit, 1%). Cultures were subjected to 8-h pulses (corresponding to one-sixth of the erythrocytic cycle time) with the *Chrozophora* extract (0.5, 2 and 10 μg/ml). After being pulsed, the cultures were washed three times with culture medium and then returned to normal conditions until the next cycle. At time zero plus 60 h (the ring stage of the next erythrocytic cycle), parasitemia was calculated by visual examination and counting Giemsa-stained smears (Benoit-Vical et al., 2007). The results are expressed as an inhibition percentage of parasitic growth.

3. Results and discussion

In traditional African medicine, healers often use plants to treat diseases identified as malaria. We report here on an ethnobotanical study that was conducted in the Dakar area in Senegal to investigate the species used in the treatment of malaria. Seven plants are principally used: *Cissampelos mucronata*, *Maytenus senegalensis*, *Terminalia macroptera*, *Bidens engleri*, *Ceratotheca sesamoides*, *Chrozophora senegalensis* and *Mitracarpus scaber*. However a bibliographic study had shown that extracts of *Cissampelos mucronata* (Gessler et al., 1994), *Maytenus senegalensis* (Gessler et al., 1994) and *Terminalia macroptera* (Mustofa et al., 2000; Sanon et al., 2003) had already been studied for their antiplasmodial properties by several authors. Therefore, we only investigated the *in vitro* antiplasmodial activity of *Bidens engleri*, *Ceratotheca sesamoides*, *Chrozophora senegalensis* and *Mitracarpus scaber*.

Each plant was extracted with three different solvents (petroleum ether, acetone and ethanol) and *Chrozophora senegalensis* leaves were extracted with water as well. The IC_{50} values obtained *in vitro* with these extracts on both chloroquine-resistant *Plasmodium falciparum* strains are shown in Table 1.

3.1. Activity of the different plants

Whatever the *Plasmodium falciparum* strain and the solvent, the IC_{50} for *Bidens engleri* ranged from 9 to >50 μg/ml, for *Ceratotheca sesamoides* from 4 to >50 μg/ml, for *Chrozophora senegalensis* leaves from 1.6 to >50 μg/ml, for *Chrozophora senegalensis* stems IC_{50} from 5 to 21 μg/ml and for *Mitracarpus scaber* IC_{50} from 7 to >50 μg/ml. The best results were obtained with leaves of *Chrozophora senegalensis* extracted with water, with an IC_{50} value of 1.6 μg/ml. An ethanol extract of *Artemisia annua* that was tested as an antimalarial control gave an IC_{50} of 1.5 μg/ml, which is in accordance with literature (O'Neill et al., 1985; Phillipson and Wright, 1991).

The antiplasmodial activity of the four plants studied here (*Bidens engleri*, *Ceratotheca sesamoides*, *Mitracarpus scaber* and *Chrozophora senegalensis*) has never been reported by any other authors. *Mitracarpus scaber* was known for its antimicrobial and hepatoprotective properties (Bisignano et al., 1999; Germano et al., 1999; Ali-Emmanuel et al., 2000), and another species (*Bidens pilosa*) from the same genus as *Bidens engleri* has been studied for its antiplasmodial properties (Andrade-Neto et al., 2004).

3.2. Activity according to the solvent

For *Bidens engleri*, *Ceratotheca sesamoides* and *Mitracarpus scaber*, extraction with petroleum ether gave better activity than with acetone or ethanol, showing that the majority of active

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principles in these plants are apolar. This result has previously been reported (Munoz et al., 2000; Mbatchi et al., 2006) even if water and ethanol are the only solvents used in African traditional medicine. By contrast, with *Chrozophora senegalensis* leaves, the active principles are concentrated in the polar fraction with the best results obtained with the water extract, and for the stems, all the activities were similar whatever the solvent used for the extraction.

3.3. Activity according to the part of the Chrozophora senegalensis plant

Whereas the leaves of *Chrozophora senegalensis* are traditionally used in Senegal to malaria treatment, sometimes the leaves and stems of this plant are both used to malaria treatment. For this plant, only the leaf extract in petroleum ether showed no activity ($IC_{50} > 50$ μg/ml), while the leaf extract in water showed a very high antiplasmodial activity ($IC_{50} = 1.6$ μg/ml). All the other extracts from both leaves and stems were similar with IC_{50} ranging from 5 to 21 μg/ml.

One of the aims of our study was to determine, as already described for other plants (Benoit-Vical et al., 1998; Mbatchi et al., 2006), the part of the plant that is the most active against *Plasmodium* and it is clear that an ethanol extract of both stems and leaves appear effective against *Plasmodium*. The water extract of the leaves is particularly effective but a water extract of the stems (very little used in endemic area) was not tested for comparison. It is important to note that the water extract of the leaves that was the best antiplasmodial extract gave the best extraction yield (35.06%). Phytochemical studies could determine the chemical composition from the stems and leaves, and thus establish a correlation between activity and composition.

3.4. Effect of storage on activity

Extracts tested directly after dissolving in the media and extracts stored at 4 °C shown similar results and the antiplasmodial activity was retained. Only the ethanol extract from *Ceratotheca sesamoides* showed an increased activity on both malaria strains after few-days storage at 4 °C, compared with fresh extract. This could be due to an increase in the solubility of this extract during the storage period. It should be noted that short-term storage of the extracts does not reduce the antiplasmodial activity, which is particularly important in areas of endemic malaria, where the extracts are in daily use.

3.5. Activity according to the strain

No significant difference in the activity of the extracts was observed between the two *Plasmodium falciparum* strains used. It is likely that components from the extracts of the four Senegalese plants interfere with *Plasmodium* by a different mechanism than chloroquine.

3.6. Cytotoxicity study

The *in vitro* cytotoxicity on the Vero cell line is shown in Table 2. All the extracts from *Mitracarpus scaber* were cytotoxic, with IC_{50} values around to 10 μg/ml values similar to those obtained against *Plasmodium falciparum*. Thus, *Mitracarpus scaber* does not seem to have a specific action against the parasite.

Except for leaves of *Chrozophora senegalensis*, petroleum ether extracts are the most toxic whatever the plant tested. Acetone and ethanol extracts from *Bidens engleri*, *Ceratotheca*

Table 2
In vitro cytotoxicity of three concentrations of the extracts from the four Senegalese plants studied, against Vero cell line

Plant	Extract	Concentrations tested (μg/ml)		
		10	50	100
Bidens engleri	Petroleum ether	54[a]	96	95
	Acetone	0	0	nd[b]
	Ethanol	0	0	nd
Ceratotheca sesamoides	Petroleum ether	19	59	nd
	Acetone	0	0	nd
	Ethanol	0	20	nd
Chrozophora senegalensis (leaves)	Petroleum ether	0	0	21
	Acetone	7	0	5
	Ethanol	0	0	2
	Water	16	22	23
Chrozophora senegalensis (stems)	Petroleum ether	12	44	81
	Acetone	0	0	21
	Ethanol	11	0	12
Mitracarpus scaber	Petroleum ether	53	88	nd
	Acetone	71	85	nd
	Ethanol	40	51	nd
Etoposide (2×10^{-5} M)			49[a]	

[a] Percentage inhibition of Vero cell line.
[b] nd: not done.

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Table 3
In vivo antiplasmodial activity of crude extracts from *Chrozophora senegalensis* and *Artemisia annua* against *Plasmodium vinckei petteri* determined by the 4-day suppressive test

Route	Extracts tested	10 mg/kg/day	100 mg/kg/day	200 mg/kg/day	500 mg/kg/day
PO[a,b]	Aqueous extract of *Chrozophora senegalensis* leaves	nd[d]	14[e]	16	19
PO[b]	Ethanolic extract of *Chrozophora senegalensis* leaves	8	2	nd	nd
PO[b]	Ethanolic extract of *Artemisia annua* leaves	nd	nd	20	22
IP[c,f]	Ethanolic extract of *Chrozophora senegalensis* leaves	65	Toxic	nd	nd
IP[f]	Ethanolic extract of *Chrozophora senegalensis* stems	22	9	nd	nd

[a] PO: oral way.
[b] Control: artesunate (5 mg/kg/day) by PO route: 99% inhibition.
[c] IP: intraperitoneal way.
[d] nd: not determined.
[e] Percentage of parasitemia inhibition in comparison to the level in the nontreated control animals at day 4.
[f] Control: artesunate (5 mg/kg/day) by IP route: 99% inhibition.

sesamoides and the stems of *Chrozophora senegalensis* showed little toxicity even at high concentrations.

For the extracts of the leaves of *Chrozophora senegalensis*, whatever the dose and whatever the solvent, no extract was toxic. Indeed, even for the higher concentrations tested (100 μg/ml) of these extracts, the percentage of cell growth inhibitions were ranged from 2 to 23%. The IC_{50} obtained for cytotoxicity against the Vero cells of the extracts of the leaves of *Chrozophora senegalensis*, were thus largely superior to 100 μg/ml. Therefore, the extracts of *Chrozophora senegalensis* leaves show a very promising security index (cytotoxicity/activity ratio). Indeed, the leaf aqueous extracts traditionally used in malaria treatment present a security index superior to 53 (>100/1.9).

3.7. *In vivo toxicity*

No mortality, or any signs of toxicity were observed during a period 60 days after mice were treated for 4 consecutive days with 500 mg/kg/day *per os* of the aqueous leaf extract of *Chrozophora*. Thus, the oral use of the *Chrozophora* extract *in vivo* seems not to be toxic.

3.8. *In vivo antiplasmodial activity*

Whatever the vegetal extract tested by oral way, any extract has showed *in vivo* antiplasmodial activity even at the higher concentration of 500 mg/kg/day during 4 consecutively days (Table 3). In parallel, this weak *in vivo* activity of the polar extracts from the very famous plant *Artemisia annua* had already been reported (Wright, 2005). This weak *in vivo* antiplasmodial activity was often reported for many vegetal extracts whereas their promising *in vitro* activity and their wide traditional use for malaria treatment were known. We can venture the hypothesis that the absorption of crude vegetal extracts and especially of polar extracts, on mouse model and by oral way, is very bad. It could be also voiced that natural crude extracts with their complex mixture of several compounds could explain their high *in vitro* activity thanks to potential synergism but fragile chemical structure could be damaged during *in vivo* assays (Soh and Benoit-Vical, 2007). Furthermore, these *in vivo* results are often not dose-dependent (see the both ethanolic extracts of leaves and of stems of *Chrozophora senegalensis*). The lack of dose–response for these extracts since 100 mg/kg/day, both by oral and ip ways, could be explained by a lack of solubility of the *Chrozophora senegalensis* extract for these dosages.

Unlike the oral way, the ethanolic extract of leaves of *Chrozophora senegalensis* has showed by intraperitoneal way, a very interesting result with 65% of parasitemia inhibition with only 10 mg/kg/day (Table 3). The ED_{50} (50% effective dose value is the dose that led to 50% parasite growth inhibition, respectively, in comparison to the level in the nontreated control animals) of this extract was thus largely under 10 mg/kg/day. The difference of *in vivo* antiplasmodial activity obtained by the two routes tested could be due to the lower bioavailability of these extracts administered by oral route. Moreover, the dosage of 100 mg/kg/day by intraperitoneal way was toxic for the treated mice. This information about the animal toxicity by the intraperitoneal route is reduced by the fact that African traditional medicine uses exclusively the oral route.

3.9. *Effect of the aqueous extract of Chrozophora on the erythrocytic life cycle*

During the erythrocytic life cycle (Fig. 1), the highest activity the *Chrozophora* extract was between the 12th and the 44th h for the two highest concentrations. We noticed a peak of activity with the lowest concentration between the 28th and the 44th h. The period between the 12th and the 44th h of the erythrocytic life cycle corresponds to the trophozoïte form of the parasites. Compared with the timing of the onset and cessation of DNA, RNA and protein synthesis (Fig. 2), the activity period of the

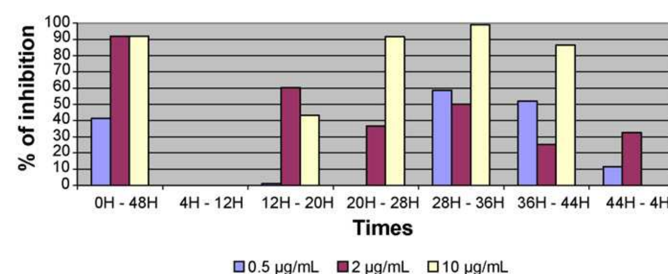

Fig. 1. Stage of *Chrozophora* action with respect to the erythrocytic life cycle.

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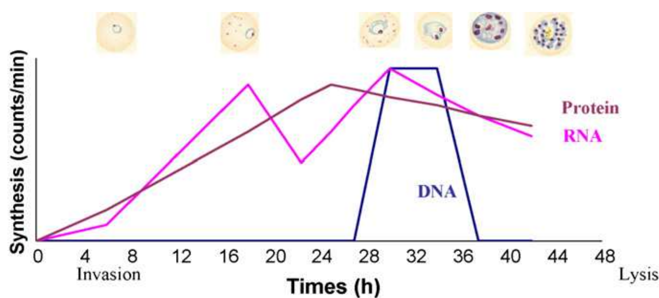

Fig. 2. Approximate timing of the onset and cessation of DNA, RNA and protein synthesis during the development of *Plasmodium falciparum* in synchronised cultures (adapted from Arnot and Gull (1998)).

aqueous extract of *Chrozophora* is marked by the synthesis of DNA, which prepares parasites for future divisions.

Moreover, the *Chrozophora* extract showed a second period of activity (but weaker than the first one) between the end of the first cycle and the beginning of the second cycle (44–48 h period). This observation suggests that *Chrozophora* could act on the liberation of the ring stage (44–48 h period) or the invasion (0–4 h period) of new erythrocytes by young forms (merozoites).

4. Conclusions

This study shows that *Chrozophora senegalensis* has high antiplasmodial activity against two chloroquine-resistant *Plasmodium falciparum* strains *in vitro*, without toxicity *in vitro* and no toxicity *in vivo* by oral way in mice. Moreover, our visual observation of the *Plasmodium falciparum* life cycle identified the stages disrupted by the *Chrozophora* extract to be DNA synthesis and the period of the liberation-reinvasion of merozoites period. It would be very interesting to identify and extract the active principles from the aqueous extract of *Chrozophora* leaves.

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II. Publication 4 : *Phyllanthus niruri*

Une autre enquête ethnobotanique en République Démocratique du Congo (RDC), a montré l'usage élargi d'une plante dans le traitement du paludisme. Il s'agit de *Phyllanthus niruri* (Euphorbiaceae).

La RDC est un pays riche en flore végétale, où persiste une vieille tradition dans l'usage des plantes médicinales. En collaboration avec des ethnobotanistes et tradipraticiens locaux, une enquête ethnobotanique a permis de sélectionner la plante *Phyllanthus niruri* de part son usage élargi dans le traitement du paludisme et des symptômes associés en Afrique Centrale et de l'Ouest. La plante entière de *Phyllanthus niruri* est utilisée dans plusieurs pays africains contre la dysenterie, les douleurs intestinales, le paludisme (38). Des précédentes études avec les extraits de la plante entière, ont rapporté des activités antibactériennes, antifongiques et antivirales (11). Son activité antiamibienne est démontrée (61). Des tests avec les extraits de la plante entière de *Phyllanthus niruri*, originaire d'Inde, démontrent une activité médiocre sur la souche *Plasmodium berghei berghei* (30), contrairement aux activités antipaludiques *in vivo* rapportées par Tona et coll. (62) pour le même extrait. Son activité analgésique est démontrée (56).

L'usage de la plante entière entraîne inévitablement sa destruction massive. Notre étude consiste à explorer l'activité antipaludique des parties de la plante pour tenter de préserver sa biodiversité.

Des extraits par des solvants de polarité variable sont obtenus de trois régions différentes du Congo et des trois parties de la plante (tige, feuilles et racines).

Ces extraits sont testés sur la souche chloroquinorésistante FcM29-Cameroun pour l'activité antipaludique et sur les cellules KB et Vero pour la cytotoxicité *in vitro*. Les tests antipaludiques *in vitro* montrent une activité prometteuse des extraits de la plante entière avec une CI_{50} de 26 µg/mL. Ce résultat a aussi été obtenu au cours d'études antérieures au Congo avec cette plante (61). Des extraits des tiges et racines de la plante montrent une activité antipaludique *in vitro* médiocre quelque soit la région de récolte. Par contre un très bon index de sélectivité des feuilles de la plante a été obtenu. Il semble que l'activité antipaludique des extraits polaires des feuilles de *Phyllanthus niruri* serait due à la présence de l'acide ellagique, du lupeol et des lignans, qui ont déjà démontré une intéressante activité antipaludique *in vitro*

et *in vivo* (72) ;(14). Cette information est particulièrement importante pour valider l'usage traditionnel et la préservation de la plante.

Ce travail est actuellement soumis pour publication au Journal « Fitoterapia »

Le fractionnement chimique bio dirigé des feuilles de la plante est en cours. L'acide ellagique est retrouvé dans certaines fractions actives.

Antiplasmodial activity of various parts of the plant *Phyllanthus niruri* according to its geographical distribution

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Abstract

Phyllanthus niruri L. (Euphorbiaceae) originating from Democratic Republic of Congo is commonly used for the treatment of malaria. Ethanolic and aqueous extracts were obtained from various parts of the _P. niruri_ plant (stems, leaves and roots) collected in three different areas in the Congo (Kisantu, Kimwenza and UNIKIN (University of Kinshasa area)). Extracts were tested on the chloroquine-resistant strain FcM29-Cameroon. The IC_{50} values obtained for extracts ranged from 11 to >50µg/ml. Differences in antiplasmodial activities of these plant extracts harvested in different zones have been demonstrated. Moreover, whereas the whole plant is traditionally used, only leaves extracts presented real _in vitro_ antiplasmodial activity without any cytotoxicity. This information is particularly important for the conservation of this plant because the leaves are affordable and their use is less damaging to plant stocks.

Keywords: ethnopharmacology, *Plasmodium falciparum*, harvest areas, parts of plant

1. Plant

Phyllanthus niruri L. (Euphorbiaceae) was harvested from January to April 2006 in the rainy season respectively by Nzeza, A. Carlier and Mukendi in 3 different areas of the DRC, Democratic Republic of Congo (Kisantu, UNIKIN, Kimwenza). The botanical identification was assured by Mr Nlandu of the INERA (Institut National pour l'Etude et la Recherche Agronomiques) herbarium – University of Kinshasa. The herbarium samples were deposited at the herbarium under the numbers respectively 72 bis (Kisantu), 66 (Kimwenza) and 83 (UNIKIN). The fresh plant material was dried on mats on the floor at ambient temperature, avoiding direct sunlight. Once dried, the plants were crushed and packaged before being sent to France for extraction and biological testing.

2. Uses in traditional medicine

Phyllanthus niruri is used for antimalarial treatment in all areas of the DRC and in other countries of Sub-saharan Africa. In the DRC, local treatment against malaria or its associated symptoms consists of using a decoction of the whole plant with a mixture of roots, stems and leaves of *P. niruri*.

3. Previously isolated classes of constituents

Phyllanthus niruri: alkaloids (29)4], terpenoids [6], four lignans (phyllanthin, hypophyllanthin, phyltetralin and niranthin) [5].

4. Tested material

The various parts of the plant (roots, stems and leaves) were extracted in water and ethanol according to the traditional methods of preparation. Ethanol extracts were obtained by simple maceration of 30g of powder in 300 ml of ethanol over 24 hours. The operation was

repeated twice on the residues. The three successive extracts were mixed together and then concentrated at reduced pressure at 35°C until a syrupy liquid was obtained. This liquid was taken up in 20 ml of distilled water and then freeze-dried to obtain a homogeneous dry extract.

Aqueous extracts were prepared by simple decoction of 5 g of plant powder in 50 ml of boiling distilled water. The mixture was boiled for 10 min before being filtered through filter paper, and then centrifuged at 3000 rpm for 20 min. Each aqueous extract thus obtained was freeze-dried and stored at -20°C before the pharmacological tests. The extraction yields are given in Table 1.

5. Studied activity

The objective of this study was to screen the *in vitro* antiplasmodial activity and the cytotoxicity of aqueous and ethanolic extracts of different parts (roots, stems and leaves) of *P. niruri* coming from 3 areas (Kisantu, Kimwenza and UNIKIN) of the DRC. The antiplasmodial activity of the *P. niruri* was evaluated on the chloroquine-resistant strain FcM29-Cameroon by the radioactive microdilution method described by Desjardins et al [3]. The IC_{50} values were determined graphically using concentration *versus* percent inhibition curves (6)].

Cytotoxicity tests were made on KB and Vero cells. The IC_{50} was calculated as the concentration of compound eliciting a 50% inhibition of cell proliferation [2].

6. Used organisms

In vitro antiplasmodial activity on Chloroquine-resistant (IC_{50} for chloroquine of 290 nM) strain of *Plasmodium falciparum* FcM29-Cameroon. *In vitro* cytotoxicity on KB (human epidermoid carcinoma) and Vero (monkey African green kidney) cells.

7. Results

Reported in Table1.

8. Conclusion

The low cytotoxicity and the antiplasmodial efficacy of *P. niruri*, principally the aqueous extracts, against *Plasmodium falciparum in vitro* validate the wide use of this plant in traditional medicine against malaria in the DRC, where malaria is endemic. The biological activity of the leaves is particularly important for the conservation of this plant because leaves are affordable and their use does not damage the plants and limit the supply as the use of the roots would.

The promising results of the aqueous extracts of leaves of *P. niruri* whatever the geographical region justify our continuing research to determine the active principles responsible of their antiplasmodial activity.

Table I: Antiplasmodial activities and cytotoxicity of the different parts of *Phyllanthus niruri* from three different areas

Plant part tested (area)	Extraction solvents	Extraction yield (%)	IC_{50} (µg/ml) against *Plasmodium*	IC_{50} (µg/ml) on cell lines: KB / Vero
P. niruri stems (Kimwenza)	Ethanol	5.8	22±4 [a]	100 / >100
	Water	10.3	14±4	>100 / >100
P. niruri stems (Kisantu)	Ethanol	3.8	>50±0	100 / >100
	Water	6.5	11±2	>100 / >100
P. niruri stems (UNIKIN)	Ethanol	5.8	>50±0	100 / >100
	Water	8.9	16±0	>100 / >100
P. niruri leaves (Kimwenza)	Ethanol	17.7	25±4	85 / 100
	Water	16.7	19±3	>100 / >100
P. niruri leaves (Kisantu)	Ethanol	6.5	19±0	75 / 100
	Water	11.4	14±1	>100 / >100
P. niruri leaves (UNIKIN)	Ethanol	13.9	22±5	100 / >100
	Water	18.8	16±1	>100 / >100
P. niruri roots (Kimwenza)	Ethanol	5	>50±0	100 / >100
	Water	6.3	>50±0	>100 / >100
P. niruri roots (Kisantu)	Ethanol	3	>50±0	>100 / >100
	Water	6.2	>50±0	>100 / >100
P. niruri roots (UNIKIN)	Ethanol	5.1	>50±0	100 / >100
	Water	4.3	>50±0	>100 / >100
P. niruri whole plant	Ethanol	ND [b]	26±11	ND
Chloroquine (antimalarial drug control)			290.10^{-3} µM	ND
Artemisinin (antimalarial drug control)			7.10^{-3} µM	>300 µM
Taxotere (anticancer drug control)			ND	$2.5.10^{-4}$ µM

a: IC_{50} in µg/ml ± sd (standard deviation) obtained from at least 3 independent experiments ; b: not determined

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III. Publication 5 : *Cognauxia podolaena*

Cognauxia podolaena (Cucurbitaceae) est une liane traditionnellement utilisée dans le traitement du paludisme. L'une des missions du CERMA (Centre d'études et de recherche des médecins d'Afrique) est de promouvoir l'exploration phytochimique des plantes médicinales africaines, ceci en collaboration avec des réseaux de tradipraticiens. Le Congo-Brazzaville est une des régions d'Afrique présentant une biodiversité végétale très riche, où réside une connaissance ancienne de l'usage des plantes médicinales dans le traitement des pathologies, en l'occurrence la lutte contre le paludisme. Des plantes répertoriées au cours d'une étude ethnobotanique précédente, ainsi que des tests antipaludiques des extraits éthanoliques et au dichlorométhane, ont permis de sélectionner 7 plantes actives (CI_{50} entre 10 et 50µg/ml) : il s'agit de l'écorce de *Cassia siamea*, des racines et feuilles de *Landolphia lanceolata*, des racines de *Cognauxia podolaena*, des feuilles de *Millettia versicolor*, des feuilles de *Pseudospondias microcarpa*, des feuilles de *Uapaca paludosa* et des feuilles de *Vernonia brazzavillensis* (38).

L'activité antipaludique de *Cognauxia podolaena* n'a jamais été démontrée. En médecine traditionnelle congolaise, ses racines sont utilisées dans le traitement du paludisme, et d'autres parasitoses tropicales (Informations données par les tradipraticiens congolais).

Un premier screening pharmacologique de la plante entière et des parties (tige, feuilles et racines) de la plante a permis de valider l'activité antipaludique de la tige (CI_{50} : 8.8 µg/mL), supérieure aux autres parties de la plante (racines avec des CI_{50}. respectives de 27 µg/ml) Le fractionnement chimique bio dirigé effectué en collaboration avec les phytochimistes de l'Institut de Chimie des Substances Naturelles (ICSN) a permis d'identifier trois molécules actives issues des extraits au dichlorométhane de la tige de la plante. Il s'agit de la cucurbitacine B, la cucurbitacine D et l'acide epibryonolique, avec des CI_{50} respectives de 1.6, 4 et 2 µg/mL. Ces molécules ont été identifiées par la RMN, l'Infrarouge et par la spectrométrie de masse.

Les études de toxicité ont révélé que seule l'acide epibryonolique avait une activité spécifique contre *Plasmodium*, avec un index de sélectivité supérieur à 10, comparé aux deux autres molécules qui avaient un index de sélectivité inférieur à 1.. C'est la première description de l'acide epibryonolique pour son activité antipaludique.

L'article a été publié comme « Letter » dans « Planta Medica » 2008 74 1-4.

Cogniauxia Podolaena: Bioassay-Guided Fractionation of Defoliated Stems, Isolation of Active Compounds, Antiplasmodial Activity and Cytotoxicity

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Abstract

Cogniauxia podolaena Baill. (Cucurbitaceae) is traditionally used in Congo Brazzaville for the treatment of malaria. We assessed the antiplasmodial activity of the plant and isolated some of the compounds responsible for this activity. It was the first time that a chemical study of this plant has been undertaken. Three triterpenes were isolated: cucurbitacin B (**1**), cucurbitacin D (**2**) and 20-epibryonolic acid (**3**) and their structures were assigned from spectroscopic evidence and comparison with published data. The crystallographic structure of **3** was determined. All fractions and compounds obtained in this study were assayed for antiplasmodial activity (on FcM29, a chloroquine-resistant strain of *P. falciparum*) and cytotoxicity (on KB and Vero cell lines). The IC_{50} values of **1**, **2** and **3** are 1.6, 4 and 2 µg/mL on FcM29. Both **1** and **2** have a high cytotoxicity whereas **3** shows a better selectivity index.

Key words

Cogniauxia podolaena · Cucurbitaceae · Cucurbitacins · 20-epibryonolic acid · antiplasmodial activity · cytotoxicity

Cogniauxia podolaena Baill. is an African liana traditionally used by Congolese tradipractitioners in the treatment of numerous ailments, including malaria. In this paper, we assessed the antiplasmodial properties of the plant and isolated some of the compounds responsible for the antiplasmodial activity of *C. podolaena* stems. A first pharmacological screening of the whole plant and of the roots confirmed the antiplasmodial activity of *C. podolaena* [1] since we found an IC_{50} lower than 27 µg/mL for the root extracts obtained with various solvents. Since many recipes use defoliated stems, we chose them for the current phytochemical investigation. We used solvents covering a wide range of polarity, in order to be sure to extract all the active compounds. This work is part of the bioassay-guided phytochemical research of active compounds initiated by the NGO (Non-Governmental Organisation) Médecins d'Afrique within the CERMA.

Table 1 shows, for each extract, fraction and pure compound, the following data: extracted mass and purification yield, antiplasmodial activity and cytotoxicity. From the four crude extracts obtained with solvents of increasing polarity, CPR2 (DCM) was the only one having an interesting antiplasmodial activity, with an IC_{50} of 8.8 µg/mL. The bioassay-guided fractionation of CPR2 gave 14 fractions (from CPR2A to CPR2N) with IC_{50} values ranging from 1.8 µg/mL to 17.5 µg/mL. Five fractions (CPR2F, H, I, K, L) showed the best activities but only one, CPR2H, gave a real increase in the activity, with an IC_{50} of 1.8 µg/mL. After preparative HPLC, CPR2 H afforded 3 active compounds, **1** (21.6 mg), **2** (3 mg) and **3** (2 mg), obtained as white needles. These purified compounds were moderately active against *P. falciparum,* with IC_{50} values of 1.6, 4 and 2 µg/mL respectively. The cytotoxicity was high for **1** and **2**, more than 89% inhibition with the Vero strain and more than 94% inhibition with the KB strain. Compound **3** was less cytotoxic and thus gave a better selectivity index (cytotoxicity:activity ratio). It could be of therapeutic interest after pharmacomodulation to improve its activity.

The molecular formulae of **1** and **2** were established as $C_{32}H_{46}O_8$ and $C_{30}H_{44}O_7$, respectively, from the ESI data with $m/z = 560.72$ for **1** and 516 for **2**. These compounds have been identified unambiguously as cucurbitacin B for **1** and cucurbitacin D for **2** on the basis of spectroscopic evidence (1 D and 2 D NMR) and direct comparison with the published data [2], [3]. Compound **3** was recrystallized from DCM/MeOH (80/20) as white needles. The molecular formula was established as $C_{30}H_{48}O_8$ from the ESI data with $m/z = 456.38$. The various correlations established by COSY, NOESY, HMQC and HMBC allowed us to finalize the structural determination. These spectroscopic data enabled us to identify **3** as 20-epibryonolic acid, by comparison with the published data [4]. We were able to confirm its structure by X-ray crystallographic analysis (**Fig. 1**). It allowed us to verify the stereochemistry of the asymmetric carbons. Compound **3** has previously been described in the literature, but this is the first determination of its crystal structure.

This work reports the first chemical study of *Cogniauxia podolaena.* Diatewa et al. [5] have studied the hypoglycemic and anti-hyperglycemic activities of leaf extracts, which support the traditional use of the plant against diabetes, but did not isolate the compounds responsible for these activities. Compounds **1**, **2** and **3** have already been isolated from various plants but this is the first report from *C. podolaena*. In the relevant literature, we have not found any studies concerning the antiplasmodial activity of the curcurbitacins, but a patent was filed in 2006 for a preparation containing cucurbitacins B and E, active against the liver stage of *Plasmodium* [6]. Pharmacomodulation of cucurbitacins and their derivatives has already been undertaken, notably for their anticancer potential. Young et al. (2007) reported that addition of different functional groups at both C-3 and C-16 resulted in a several-fold decrease in cytotoxicity [7]. The patents on cucurbitacins already filed indicate the therapeutic interest in these molecules and their potential applications, in particular in malaria treatment.

Test Sample	Weight (g)	Yield (%)	Antiplasmodial activity	Cytotoxicity	
			IC_{50} (μg/mL) on FcM29 strain[a]	% inhibition of KB strain at 10 μg/mL	% inhibition of Vero strain at 10 μg/mL
CPR1	0.480	0.37%	33.2 (13)	94	86
CPR2	**1.930**	**1.48%**	**8.8 (4)**	**97**	**87**
CPR3	3.930	3.02%	> 50	95	86
CPR4	3.450	2.65%	> 50	96	76
CPR2A	0.108	6.35%	> 50	0	0
CPR2B	0.025	1.47%	17.5 (2.1)	29	37
CPR2C	0.007	0.41%	16.0 (2.0)	96	70
CPR2D	0.013	0.76%	13.3 (2.6)	97	96
CPR2E	0.110	6.47%	11.3 (1.7)	98	81
CPR2F	0.049	2.88%	7.1 (2.9)	99	93
CPR2G	0.072	4.24%	11.9 (3.8)	96	89
CPR2H	**0.059**	**3.47%**	**1.8 (1.0)**	**97**	**86**
CPR2I	0.042	2.47%	5.5 (3.0)	96	88
CPR2J	0.014	0.82%	10.0 (2.7)	95	87
CPR2K	0.199	11.71%	6.5 (3.9)	97	89
CPR2L	0.130	7.65%	8.3 (3.4)	98	92
CPR2M	0.228	16.65%	16.5 (2.6)	97	91
CPR2N	0.263	15.47%	10.5 (3.3)	98	92
1	**0.021**	**35.59%**[c]	**1.6 (0.4)**	**94 b**	**89b**
2	**0.003**	**5.08%**[c]	**4.0 (2.0)**	**95 b**	**93b**
3	**0.002**	**3.39%**[c]	2 **(0.8)**	**20 b**	**19.2b**
CPR2H-R	0.027	45.76%[c]	7 **(4)**	98 **b**	90 **b**
Chloroquine	–	–	0.09	ND	ND
Taxotere (2.5×10^{-10} M)	–	–	ND	75	29
Artemisinin	–	–	0.0025	19	39

[a] IC_{50} in μg/mL (standard derivation).
[b] value calculated for 1 μg/mL.
[c] The yield is calculated with regard to the fraction CPR2H.

Table 1 Extraction and purification yield, antiplasmodial activity and cytotoxicity of extracts and compounds from *Cogniauxia podolaena*

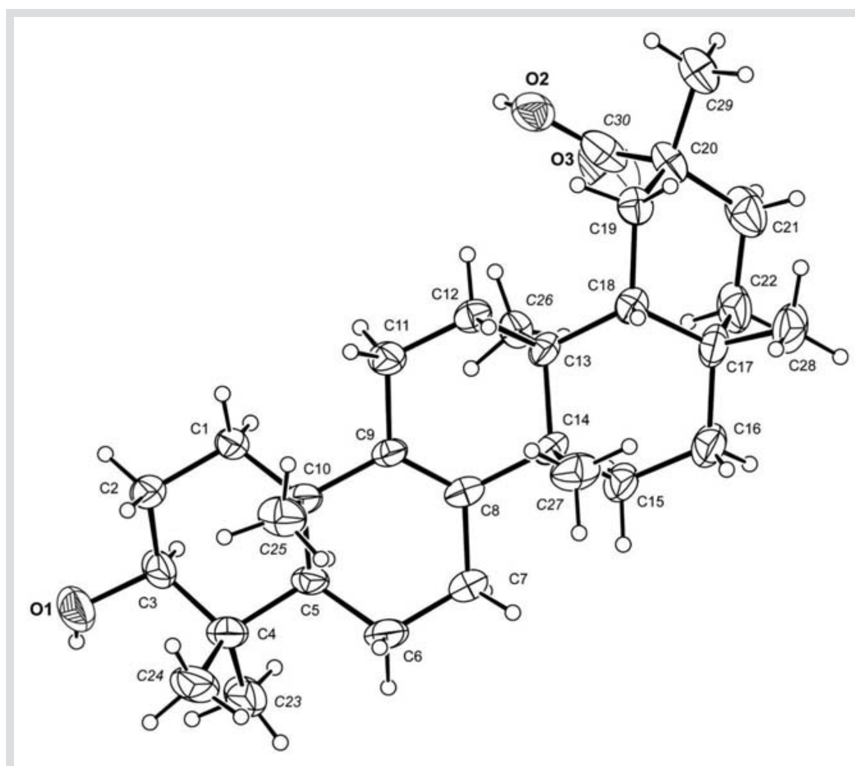

Fig. 1 Crystallographic structure of 20-epibryonolic acid.

Material and Methods

Plant collection and preparation of extracts: The plant was collected in January 2006 in Mansimu, not far from Brazzaville (Congo). The stems were dried for a month on a cement floor shielded from the light at room temperature. The botanical identification was made by staff of Médecins d'Afrique and a voucher specimen was deposited at our herbarium under the number JTB-CP1. The dried stems were crushed with an electric crusher and then packaged in a plastic bag to be sent to ICSN-CNRS (France). 130 g of plant powder were successively extracted with pentane (3 × 1.5 L) to give the extract CPR1, with dichloromethane (3 × 1.5 L) to give CPR2, and with methanol (3 × 1.5 L) to give the extract CPR3. The residue was then extracted with water (500 mL for 10 min at 100 °C, cooling for 4 h then filtration through filter paper). The organic extracts (CPR1, CPR2, CPR3) were concentrated at reduced pressure then dried. The aqueous extract was centrifuged for 20 min at 3000 rpm and the supernatant filtered and freeze-dried to give the dry extract CPR4. The four extracts were kept at 4 °C before the pharmacological tests.
Purification and chromatographic analysis: We chromatographed 1.70 g of the most active extract (CPR2) on a silica column (height = 32 cm, diameter = 5 cm, silica weight = 135 g), using sequential elution with a mobile phase consisting of a mixture of the following solvents: heptane/ethyl acetate/methanol. Their proportions were: heptane/AcOEt (50/50) for the fractions CPR2A to CPR2J; AcOEt for the fractions CPR2K and CPR2 L and finally AcOEt/MeOH (80/20) for the fractions CPR2 M and CPR2N. The chromatographic monitoring was performed on Merck TLC Silicagel 60 F_{254} (migration solvent: heptane/AcOEt (50/50), spray reagent: p-anisaldehyde). The yields for all the fractions and products are given in ▶ **Table 1**.
Fifty mg of the most active fraction (CPR2 H) were subjected to preparative HPLC on a Sunfire C18 column (19 mm × 150 mm, particle size: 5 μm, flow rate: 17 mL/min), using an acetonitrile-water linear elution program (10 – 100 % over 30 min), with UV detection at 190 – 360 nm. This HPLC resulted in the three compounds **1**, **2** and **3** as well as a residual fraction (CPR2H-R) from the concentration of all the residual solvent which contained several minor compounds. The purity of the compounds was monitored by analytical HPLC on a Sunfire C18 column (4.6 × 150 mm, particle size: 5 μm, flow rate: 1 mL/min, Teb: 45 °C, Tev: 50 °C), using an acetonitrile-water linear elution program (10 – 100 % over 30 min), with UV/ELSD detection.
Structural determination and crystal data for **3** *:* IR spectra were obtained from an Perkin-Elmer Spectrum-BX, FT-IR spectrometer. Mass spectra were measured on an AutoMass Multi spectrometer (Thermo-Finnigan) in the electronic impact method and on an LCT spectrometer (Micromass) in the electrospray method. NMR spectra were measured in $CDCl_3$ on a Bruker AC500 with a cryoprobe. The chemical shifts were analyzed with 1 D NMR methods (^{1}H, ^{13}C, ^{13}C DEPT) and 2 D NMR methods (COSY, NOESY, HMQC, HMBC). A crystallographic study enabled us to confirm the structure of product **3**. Data collection: DENZO [8]; COLLECT [9]. Cell refinement and data reduction: SCALEPACK [5]. Programs used to solve and refine the structure: SHELX-97 [10]. Refinement: on *F2* full matrix least-squares. Diffractometer: Enraf–Nonius Kappa-CCD. $C_{30}H_{48}O_3$, CH_4O, MW 488.73, monoclinic, $P2_1$, a = 16.103(2) Å, b = 6.549(1) Å, c = 27.250(3) Å, β = 99.004(5)°, V = 2838.3(6) Å^3, Z = 4, Mo K_α radiation, λ = 0.7107 Å, μ = 0.073 mm^{-1}, 3594 unique reflections (Friedel merged), 647 parameters, H-atoms refined using a riding mode, R = 0.0693, Rw = 0.1404, S = 1.059, min and max electron residuals = –0.233 and 0.233 e.Å^{-3}. Structural data for **3** have been deposited at the Cambridge Crystallographic Data Centre (CIF file) as supplementary publication: number CCDC 673 243. Copies of the data can be obtained free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: + 44(0)1223 – 336 033 or e-mail: deposit@ccdc.cam.ac.uk].
Culture of the parasites and in vitro antiplasmodial activity studies: The parasites were cultivated continuously using standard culture methods [11] with modifications [12]. The parasites were maintained *in vitro* in human erythrocytes provided by the French Blood Bank (EFS, Toulouse, France), diluted to 1 % haematocrit in RPMI 1640 medium (GIBCO BRL) supplemented with 5 % human serum (EFS). The four crude extracts, the various fractions and the isolated compounds were screened for their antiplasmodial activity by the radioactive micro-method (Desjardins, 1979) on FcM29-Cameroon, a chloroquine-resistant strain of *Plasmodium falciparum,* as described by Mbatchi et al. [1]. All tests were performed at least in duplicate.
Assay of cytotoxicity: The cytotoxicity of the *C. podolaena* extracts and compounds was tested against KB (human epidermoid carcinoma) and Vero (African green monkey kidney) cells in culture as previously described by Mbatchi et al. [1]. The positive control used was taxotere (Rhone-Poulenc).

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IV. Publication 6 : L'acide ellagique

Le fractionnement chimique bio dirigé des feuilles de *Chrozophora senegalensis*, *Sebastiania chamaelae* et *Phyllanthus niruri* a permis d'identifier l'acide ellagique dans les fractions actives.

L'acide ellagique est un polyphénol présent dans des nombreux fruits et légumes tels que les fraises, framboises et noix etc… Au cours des années récentes, les propriétés pharmacologiques de la molécule ont été explorées. Concernant l'activité antipaludique, Verotta et coll. (64) ont démontré l'inhibition de la croissance de *P. falciparum*, par l'acide ellagique extrait de la plante *Terminalia calobuxus* originaire de Nouvelle Calédonie, avec une CI_{50} de 103 à 145ng/ml. Des résultats similaires ont été trouvés par Banzouzi et coll. (4), pour l'acide ellagique extraite cette fois d'une plante Ouest Africaine : *Alchornea cordifolia*. Dell'Agli et coll. (15), après expérimentations *in vitro* utilisant la ß-hématine, suggèrent que l'acide ellagique, de part sa capacité à former des liaisons π- π, agirait comme les quinoléines en inhibant la détoxification de l'hème.

Les études sur les propriétés anticancéreuses sur lignées de cellules tumorales et modèles de tumeurs murines, montrent que l'acide ellagique affecterait l'expression de P53 et P21 et provoquerait l'arrêt de la croissance cellulaire en G1 puis l'apoptose des cellules cancéreuses (3). L'acide ellagique est aussi un puissant antioxydant, ceci en réagissant avec les chaînes portant les radicaux peroxydes (52).

L'activité antipaludique *in vitro* de cette molécule ayant été précédemment rapportée, nous avons exploré les propriétés antipaludiques *in vitro* et *in vivo* de l'acide ellagique, et essayer de comprendre son mécanisme d'action antiparasitaire. Les études pharmacologiques de l'acide ellagique commercial (Acros Organics, Belgique) montrent de très bonnes propriétés antipaludiques *in vitro* sur les souches de *Plasmodium falciparum* de laboratoire quelque soit leur résistance à la chloroquine et à la méfloquine, avec des CI_{50} comprises entre 105 et 330nM, et une synergie intéressante avec les antipaludiques courants (artésunate, chloroquine, atovaquone et méfloquine). La toxicité *in vitro* donne un index de sélectivité supérieure à 400. L'étude du moment d'action au cours du cycle érythrocytaire du parasite suggère que la molécule est très active aux stades parasitaires trophozoïtes et schizontes jeunes. Les tests *in vivo* de l'acide ellagique sur le modèle murin de paludisme par

Plasmodium vinckei petteri montrent aussi un bon index thérapeutique (> 4000) sur les souris traitées par voie intrapéritonéale.

L'acide ellagique démontre aussi une efficacité prophylactique chez les souris contre le paludisme murin ainsi que des propriétés antioxydantes. Ensuite, l'effet inhibiteur de son activité antipaludique par l'antioxydant N-acétyl-L-cysteine nous permet de suggérer un possible effet prooxydant de la molécule dans son action antiparasitaire. Ce dernier reste encore à confirmer dans des études ultérieures.

Néanmoins, les traitements curatifs et prophylactiques contre le paludisme murin par voie orale sont médiocres.

Plusieurs hypothèses sont suggérés à cet effet : la demi vie courte de l'acide ellagique dans la circulation sanguine murine (60), ou la modification de la molécule qui entraînerait une perte de son efficacité antipaludique. Il serait intéressant d'explorer le devenir de l'acide ellagique après traitement par voie orale, et améliorer son efficacité sur modèles murins de paludisme.

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1

In vitro and *in vivo* properties of ellagic acid in malaria

3

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Abstract

25 Malaria is one of the most significant causes of infectious disease in the world. The search for
26 new antimalarial chemotherapies has become increasingly urgent due to the parasites'
27 resistance to current drugs.

28 Ellagic acid is a polyphenol, found in various plant products. In this study, antimalarial
29 properties of ellagic acid were explored. The results obtained have shown high activity *in*
30 *vitro* against all *P. falciparum* strains whatever their levels of chloroquine and mefloquine-
31 resistant (50% inhibitory concentrations ranging from 105 to 330 nM). Ellagic acid was also
32 active *in vivo* against *P. vinckei petteri* in suppressive, curative and prophylactic murine tests,
33 without any toxicity (ED_{50} by the intraperitoneal route inferior to 1 mg/kg/d).

34 The study of the point of action of its antimalarial activity in the erythrocytic cycle of
35 *Plasmodium falciparum* demonstrated that it occurred at the mature trophozoite and young
36 schizont stages. Moreover, ellagic acid has been shown to potentiate the activity of current
37 antimalarial drugs such as chloroquine, mefloquine, artesunate and atovaquone. This study
38 also proved the antioxidant activity of ellagic acid and and by contrast the inhibitory effect of
39 the antioxidant compound, N-acetyl-L-cysteine (NAC), on its antimalarial efficacy. The
40 possible mechanisms of action of ellagic acid on *P. falciparum* are discussed in the light of
41 the results. Ellagic acid has *in vivo* activity against plasmodia but modification of the
42 compound could lead to improved pharmacologic properties, and principally for oral route.

43

44

45 # Introduction

46 New drugs against malaria are urgently needed, and traditional methods of malaria treatment
47 could be a promising source of new antimalarial compounds (4). We have recently initiated
48 several collaborative programs with West Africa countries aimed at selecting by
49 ethnopharmacological methods, plants largely used by traditional healers and the local
50 populations for malaria treatment. The *in vitro* antiplasmodial activities of the crude extracts
51 and fractions (from bio-guided fractionation of the promising crude extracts of plants) enabled
52 us to identified ellagic acid as one of the active ingredients. The *in vitro* antimalarial activity
53 of this molecule has previously reported by other researchers (3), (10). In this study we clarify
54 the high *in vitro* and *in vivo* antiplasmodial properties of ellagic acid, its antioxidant activity,
55 its potential pro-oxidant effect and gain a deeper understanding of its mechanisms of action.

56 ## Materials and methods

57 **Compounds.** Ellagic acid hydrate (Figure 1) was obtained from Acros Organics (Belgium),
58 molecular weight: 302.19 g/mol. The other reagents were as obtained as follows: chloroquine
59 diphosphate salt (Sigma, France), mefloquine hydrochloride (Hoffmann-La Roche,
60 Switzerland), artesunate (Sanofi-Synthelabo, France), artemisinin (Sigma, Germany) and
61 atovaquone (Glaxo Smith kline, United Kingdom).

62

63 ***In vitro* antiplasmodial activity.** Five strains (W2-Indochina, FcM29-Cameroon, FcB1-
64 Colombia, F32-Tanzania and Dd2) of *Plasmodium falciparum* were used to evaluate *in vitro*
65 antiplasmodial activity. These strains were continuously cultured using standard methods
66 (27). The *in vitro* antiplasmodial activity was evaluated by the radioactive micro-method as
67 previously described (11), (7). Each IC_{50} was calculated as the concentration inhibiting 50%
68 of parasitic growth.

69

70 ***In vitro* cytotoxicity tests.** The cytotoxicity of ellagic acid was tested against MRC5 (human
71 diploid embryonic lung cells), KB (human epidermoid carcinoma) and Vero cells (African
72 green monkey kidney) in culture. The cells were grown in Dulbecco's modified Eagle's
73 medium supplemented with 25 mM glucose, 10% (v/v) fetal calf serum, 100 IU penicillin,
74 100µg/ml streptomycin and 1.5 µg/ml fungizone, and kept under 5% CO_2 at 37°C. 96-well
75 plates were seeded with 600 cells per well in 200 µl of medium. Twenty-four hours later,
76 ellagic acid, first dissolved in dimethyl sulfoxide (DMSO) and then diluted in RPMI, was
77 added (final concentration of DMSO was 1%) and the cells incubated for a further 72h.
78 Controls received an equal volume of DMSO. The number of viable cells was then measured
79 at 490 nm with the MTS reagent (Promega, USA) and the IC_{50} was calculated as the
80 concentration of compounds eliciting a 50% inhibition of cell proliferation.

81 ***In vitro* potentiation tests.** The synergy between ellagic acid and chloroquine, artemisinin,
82 artesunate, mefloquine and atovaquone was assessed by potentiation experiments as
83 previously described (6). The chloroquine-resistant strain of *Plasmodium falciparum* W2-
84 Indochina (IC_{50} for chloroquine of 300 nM) was used. Several combinations of ellagic acid
85 and the corresponding other molecules were incubated in 96-well plates and the inhibition
86 was evaluated as described above. The ellagic acid fractional inhibition concentrations (FIC_{50})
87 were calculated by dividing the IC_{50} of the combination by the IC_{50} of ellagic acid alone. The
88 corresponding other molecule fractional inhibition concentrations (FIC_{50}) was also calculated.
89 Potentiation results are the fractional inhibition concentration indicating type of the combined
90 effect of drugs. The final value of the FIC_{50} indicated if the interaction was an additive (FIC_{50}
91 equal to 1), antagonistic ($FIC_{50} >1$) or synergistic (FIC_{50} was <1) effect.
92
93 **Chloroquine and mefloquine-resistant reversion assays.** To know if the synergistic
94 association of ellagic acid with chloroquine and mefloquine were not due to a chloroquine and
95 mefloquine-resistant reversion effect, a chloroquine-resistant reversion test was carried out as
96 a positive control using verapamil hydrochloride (Sigma, France) on the chloroquine-resistant
97 strain W2-Indochina (IC_{50} for chloroquine of 300 nM) and on the chloroquine-sensitive F32-
98 Tanzania (IC_{50} for chloroquine of 30±2 nM), according to the experimental procedures
99 described by Martin *et al.* (16). For a mefloquine resistance reversion assay, the positive
100 control consisted of using penfluridol (Sigma, France) (17, 18), a piperidine analogue, and the
101 *Plasmodium falciparum* strains, FcM29-Cameroon, sensitive to mefloquine (IC_{50}: 7±4 nM)
102 and Dd2, that showed a reduced susceptibility to mefloquine *in vitro* (IC_{50}: 21±3 nM).
103 Amplification assays of the *PfMDR1* gene on the Dd2 and FcM29 strains confirmed that Dd2,
104 showing a reduced susceptibility to mefloquine by expressing an average of three copies

105 compared with the reference FcM29 strain, which expressed only one copy (data not shown).
106 This agrees with previous reports showing the same results (20).
107 The CR_{50}, which is the reversant concentration, was determined in 3 independent
108 experiments.
109

110 **Stage of ellagic acid action in the erythrocytic life cycle.** The strain FcB1-Colombia was
111 synchronized to a 4-hour period. The method consisted of alternatively synchronizing young
112 forms with 5% D-sorbitol and late forms with Plasmion (15). Ellagic acid was tested in 24-
113 well plates with cultures at 0.5 to 1% parasitemia (hematocrit 2%). Cultures were subjected to
114 8-hour pulses (corresponding to one-sixth of the erythrocytic cycle time). The ellagic acid
115 concentrations used were 25, 100 (IC_{50} against FcB1) and 250 ng/ml on the strain FcB1. After
116 being pulsed, the cultures were washed three times with RPMI 1640 medium (Gibco
117 Invitrogen, France) and then returned to normal conditions. At the end of the experiment (the
118 ring stage of the next erythrocytic cycle), parasitemia was calculated by microscopic
119 examination and counting Giemsa-stained smears (5). The results were expressed as a
120 percentage inhibition of parasitic growth.
121

122 **Test of antiplasmodial activity *in vivo*.** *In vivo* assays of ellagic acid were performed on a
123 rodent malaria strain of the parasite (*Plasmodium vinckei petteri*), with the classical 4-day
124 suppressive test after inoculating 2.10^7 parasites/mouse (19). Artesunate (5 mg/kg/d) was used
125 as control. Three doses of ellagic acid were tested (1, 50 and 100 mg/kg/d). Groups of five
126 mice (Swiss female, 8 week-old) were used for each dose. Control mice were treated with the
127 vehicle, either orally or intraperitoneally. Another five mice were kept as untreated controls
128 (same batch, no parasite and no treatment) and finally five mice were inoculated but not
129 treated.

130 Female Swiss mice (Janvier, Fance), weighing around 30g were inoculated intraperitoneally
131 or *per os*, daily for 4 consecutive days. The first treatment started 3 hours after parasite
132 inoculation and the others were given at the same time the following days. Parasitemia levels
133 (parasitized erythrocytes/total erythrocytes) were determined on day 4 with thin blood smears.
134 The ED_{50} was assessed as the dose leading to a 50% inhibition of parasite growth compared
135 with growth in the control (treated with an equal volume of vehicle: 100µL of a mixture 50:50
136 of physiological serum and DMSO).
137 The mice were followed after day 4 until day 60 and every week, parasitemia was determined.
138 Surviving mice treated intraperitoneally or *per os* were monitored every day until day 60.
139
140 ***In vivo* prophylaxis-curative test.** Groups of five mice (Swiss female, 8 week-old) were used
141 and one million parasites (*P. vinckei petteri*) were injected intraperitoneally into mice at day
142 0. Mice were treated with ellagic acid in the vehicle, either orally (100 mg/kg/d) or
143 intraperitoneally (10 mg/kg/d) for four days. For the first group, mice were treated for four
144 days before parasite inoculation (day -4 to day 0) and for the second group, mice were treated
145 for four days after parasite inoculation (day 0 to day +4). The third group was treated for 8
146 days, from four days before parasite inoculation until four days after (day -4 to day +4).
147 Parasitemia levels were determined on day 6 and day 8 with thin blood smears and compared
148 with controls (mice treated with equal volume of vehicle, either orally or intraperitoneally).
149 Surviving mice were followed every day until day 60.
150
151 ***In vivo* toxicity test.** Toxicity was evaluated via the oral and intraperitoneal routes. Healthy
152 Swiss 8-week-old female mice (Janvier, France) were treated with concentrations of ellagic
153 acid between 100 mg/kg/day and 1 g/kg/day, once a day for 4 consecutive days and observed
154 for 30 days, and mortality and any signs of toxicity were recorded.

155

156 Any procedures involving animals were fully conform to Europeans regulations (EEC
157 directive 86/609 dated 24/11/1986). The experiments involving animals were carried out in
158 the animal room of the Parasitology Department of University Hospital (Toulouse, France),
159 which is under the control of the National Veterinary Services. All *in vivo* studies were
160 approved by the French Institutional Animal Experimentation Ethics Committee
161 #MP/R/04/31/11/07 (antiplasmodial activity and prophylaxis-curative test) and
162 #MP/05/05/01/08 (toxicity tests).

163

164 **Modulation of antimalarial activity by N-acetyl-L-cysteine (NAC) and antioxidant**
165 **effects of ellagic acid.** The modulating effects of NAC (Sigma, France) on the antimalarial
166 activity of ellagic acid were assessed on the strain FcM29. The experimental procedures were
167 adapted from the protocol described by Arreesrisom (2). The final concentration of NAC used
168 without effect on parasitic growth was 0.87µM. The final concentration in the culture medium
169 of ellagic acid was 0.18µM (IC_{50} of ellagic acid on FcM29 strain). The antiplasmodial activity
170 was evaluated by the radioactive micro-method.
171 The oxygen-dependent respiratory burst in treated human monocytes was measured by
172 chemiluminescence in the presence of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol)
173 using a thermostatically (37°C) controlled luminometer (Wallac 1420 Victor[2], Finland). The
174 generation of chemiluminescence was monitored continuously for one hour after incubation
175 of the cells with luminol (66µM) and treated with PMA (phorbol myristate acetate) (30µM)
176 and ellagic acid (3.3µM). The measurements were performed in duplicate and statistical
177 analyses were done using the area under the curve expressed in counts x seconds.

178 # Results

179

180 **Ellagic acid inhibits *Plasmodium* growth *in vitro* without cytotoxicity.** The antimalarial
181 activity of ellagic acid *in vitro* on 5 *P. falciparum* strains is shown in Figure 2 and Table 1.
182 The IC_{50} values for ellagic acid (range, 105-330nM) were approximately in the same range for
183 all the *P. falciparum* strains tested whatever their chemo-sensitivity. Cytotoxicity tests *in vitro*
184 showed no cytotoxicity on Vero, KB and MRC5 cells for the dose of 100 µM (data not
185 shown). The security index (cytotoxicity/activity ratio) therefore appeared to be up to 495
186 (range, 303-952).

187

188 **Ellagic acid potentiates the *in vitro* antiplasmodial activity of the major antimalarial**
189 **drugs.** Ellagic acid showed synergistic activity with chloroquine, atovaquone, mefloquine and
190 artesunate, but was slightly antagonist with artemisinin (Table 2).
191 To determine if the synergism of ellagic acid with chloroquine was not due to chloroquine-
192 resistant reversion effect, the chloroquine-resistant reversion by ellagic acid was compared
193 with the effect of the known Chloroquine-resistance reversing compound Verapamil on the
194 W2 strain. The results in Figure 3 show that ellagic acid was not a reversal agent of the
195 chloroquine-resistance as was verapamil, which had a CR_{50} (Reversant concentration which
196 reduced to 50% inhibition concentration) of 0.31±0.07 µM. This reversion effect was not
197 found on a chloroquine-sensitive strain F32 used as negative control for our experiments.

198

199 **Ellagic acid shows reversion on the Dd2 strain with reduced susceptibility to mefloquine.**
200 The mefloquine-resistant reversion effect of ellagic acid was carried out in comparison with
201 the effect of penfluridol on the Dd2 strain. The results in Figure 4 show the reversal effect of
202 ellagic acid on the mefloquine reduced susceptibility strain Dd2 with a CR_{50} of 5±4ng/ml.

203 This reversal effect is more important than the control with penfluridol that reverses only at a
204 CR_{50} of 96±7ng/ml. We did not found this reversal effect with the mefloquine susceptible
205 strain FcM29 used as negative control.
206
207 **Ellagic acid acts on the late stages of the erythrocytic *Plasmodium* life cycle.** During the
208 erythrocytic life cycle, the activity of ellagic acid at the pharmacological doses was between
209 the 24th and the 40th hour. This period in the erythrocytic life cycle corresponds to the
210 trophozoïte and early schizont form of the parasites. This stage of the malaria life cycle is the
211 most metabolically active phase with protein, RNA and DNA synthesis taking place (Figure
212 5).
213
214 **Intraperitoneal ellagic acid shows high curative antiplasmodial activity *in vivo* and also**
215 **prophylactic effects without any toxicity.** Mice infected with *Plasmodium vinckei petteri*
216 were treated by the oral and intraperitoneal routes with 1, 50 and 100 mg/kg/d of ellagic acid.
217 The results showed that with the 4 day-suppressive test the ED_{50} of ellagic acid administered
218 by the intraperitoneal route was inferior to 1mg/kg/d (Figure 6) and at doses of 50 and
219 100mg/kg/d, 100% inhibition of parasite growth was obtained. On the other hand, mice
220 treated orally showed very little inhibition of parasite growth.
221 The parasitemia of surviving mice treated orally and intraperitoneally was monitored from D5
222 to D60. No recrudescence of malaria was observed in mice treated intraperitoneally with 50
223 and 100mg/kg/d of ellagic acid and 5mg/kg/d of artesunate until D60
224 To assess the prophylactic effects of ellagic acid, mice had been treated also either by the oral
225 (100 mg/kg/d) or by the intraperitoneal (10 mg/kg/d) routes with ellagic acid four days before
226 and/or after parasite inoculation. The parasitemia levels at day 6 showed that there was a high
227 protective effect on mice treated intraperitoneally with ellagic acid before parasite inoculation

228 (Figure 7). Mice orally treated, the controls and those that received treatment after inoculation
229 died from a high level of parasitemia seven days after parasite inoculation, whereas mice
230 treated with ellagic acid by the intraperitoneal route before parasite inoculation were still alive
231 at day 9.
232 Because of the interesting antimalarial properties *in vivo*, toxicity tests in mice were carried
233 out, using the doses 100, 250, 500, 750 and 1000 mg/kg/d by the oral and intraperitoneal
234 routes during four consecutive days. The mice were monitored until D30. No mortality was
235 observed whatever the doses of ellagic acid and the route used (data not shown). We
236 concluded that there was no toxicity of ellagic acid by the oral or intraperitoneal routes in
237 mice given an LD_{50} up to 1x4 g/kg/d, and thus the therapeutic index by the intraperitoneal
238 route was up to 1000 for the suppressive test (or superior to 4000 with cumulative doses).
239

240 **Ellagic acid has antioxidant properties but its antimalarial activity is decreased by N-**
241 **acetyl-L-cysteine (NAC).** The modulating effects of NAC (an antioxidant compound) on the
242 inhibition of parasite growth by ellagic acid were assessed on the *Plasmodium falciparum*
243 strain FcM29 with a NAC concentration of 0.87µM. This concentration of NAC used in the
244 incubation medium was in accordance with the dosing scheme described in the literature for
245 the use of NAC as adjunctive treatment (2). In the presence of NAC the inhibition of parasite
246 maturation by ellagic acid was reduced while alone NAC was without effect on the parasitic
247 growth of the FcM29 strain (Figure 8). The antioxidant effect of ellagic acid was measured by
248 chemiluminescence in the presence of PMA, which is a powerful inducer of the production of
249 reactive oxygen species (ROS) in human monocytes. As can be seen in Figure 8 the
250 antioxidant properties of ellagic acid reduced the production ROS.

251 ## Discussion

252 The ellagic acid is a polyphenol found in numerous fruits and vegetables, and this molecule
253 seems to be a primary component of several tannin bearing antimalarial plants found in the
254 African flora (28).

255 The first report of the inhibition of the growth of *Plasmodium falciparum* by ellagic acid
256 extracted from plants (*T. Calobuxus* of New Caledonia) was by Verotta et *al.* (29), who
257 obtained an IC_{50} of ellagic acid, whatever the chloroquine-resistance of strains used, between
258 103 and 145ng/ml (331 and 480 nM). Similar results were found by Banzouzi et *al.* (3) with
259 ellagic acid from *Alchornea cordifolia* (West African plant). These results are in accordance
260 with ours, with our IC_{50} values between 105 and 330 nM. By contrast, no antimalarial activity
261 of ellagic acid (extracted from *Punica granatum L.*) was found by Reddy et *al.* (21). In all our
262 experiments, ellagic acid hydrate was bought from Acros Organics in order to obtain pure
263 (97%), cheap and reproductible batches.

264 Synergism was found between ellagic acid and current antimalarial drugs (chloroquine,
265 artesunate, mefloquine and atovaquone), and this is the first report of this potentiation *in vitro*.
266 This synergy with current antimalarial drugs could enable the quantity of each drug to be
267 reduced during the treatment and thus limit the side effects. Such synergy is interesting in the
268 fight against the emergence of resistance, which is one of the major problems in eradicating
269 malaria. Although ellagic acid showed no reversant activity on chloroquine-resistance, it
270 reversed mefloquine reduced susceptibility in the Dd2 strain. In endemic zones, *Pfmdr1* gene
271 amplification has been implicated in the reduced susceptibility of *P. falciparum* isolates to
272 several drugs such as: quinine, halofantrine and mefloquine (9).

273 Reddy et *al.* (21) and Verotta et *al.* (29) suggested that ellagic acid had negligible
274 cytotoxicity. In our case the ratio between cytotoxicity and activity showed an *in vitro*
275 selectivity index of more than 495.

276 In addition, ellagic acid showed high activity and prophylactic effects *in vivo* in a murine
277 model when administered by the intraperitoneal route and without any toxicity. In curative
278 test, the ED_{50} of ellagic acid on *P.vinckei petteri* is around 1mg/kg/d by the intraperitoneal
279 route. By comparison, artesunate, the most potent artemisinin derivative, under the same
280 conditions (similar vehicle and same murine *Plasmodium* strain), showed an ED_{50} inferior to
281 5mg/kg/d. In prophylactic-curative tests, mice treated by the intraperitoneal route with ellagic
282 acid before parasite inoculation had a high reduction (between 79 and 93%) of parasitemia
283 compared with the controls at day 6, suggesting a prophylactic effect of ellagic acid. Poor
284 activity of ellagic acid was found when administered orally, probably due to lower
285 bioavailability. It has previously been shown in rats that ellagic acid is partially absorbed,
286 metabolized by intestinal flora, and excreted in bile and urine as glucuronide and glutathione
287 conjugates (25). A more recent study in rats indicated that, only 9.6 % of ellagic acid was
288 detected in stomach 1h after oral ingestion and after 2h, only traces were detected. Moreover,
289 no ellagic acid was detected in any of the rat organs/tissues or fluids collected over a 24h after
290 consumption (8). In mice, ellagic acid presents highest concentrations in blood only 30 min
291 after oral administration, with an absorption occurred mostly within two hours (26).
292 Weak level of ellagic acid in blood after oral administration could explain the absence of *in*
293 *vivo* efficacy by this way, in our study. Even with high concentration (1g/kg/day), no
294 antimalarial efficacy was reported explaining rather chemical modification(s) of ellagic acid
295 in stomach or in gastrointestinal tract, with loss of its antimalarial activity.
296 This loss of antiplasmodial activity of ellagic acid after oral administration found in our study
297 is correlated with the limited bioavailability of ellagic acid reported after pomegranate juice
298 ingestion (24). These results are thus not limited to the anti-*Plasmodium* activity of ellagic
299 acid but could be transposed to potential chemopreventive, antioxidant and anti-inflammatory

300 bioactivities of ellagitannins as used by health food consumers (especially in the US) who
301 appreciate pomegranate juice because it is one of the richest sources of ellagic acid.
302 Protection of ellagic acid to the stomach acidity or synthesis of a prodrug, able to release
303 efficient amount of ellagic acid in the plasma could be considered. It may be possible to
304 enhance the oral absorption of the ellagic acid by using metabolites. For example, the oral
305 treatment with ellagitannins, which after hydrolyze, release ellagic acid in the jejunum (12)
306 could also be explored. Indeed, the intestinal flora then metabolizes ellagic acid to
307 metabolites, which were absorbed preferentially with their lipophilicity increased (12).
308 Additional absorption, distribution, metabolism and excretion (ADME) studies might be used
309 in lead optimization to profile compound derivatives or metabolites or even other salt forms
310 (13).
311 The point of action of ellagic acid in the parasite life-cycle corresponded with protein and
312 nucleic acid synthesis. The pharmacological targets of ellagic acid and its mechanism of
313 action on *P. falciparum* are not well known. However, Dell'Agli et *al.* (10) investigated the
314 activity of recombinant plasmepsin II, one of the haemoglobin proteases, and the *in vitro*
315 detoxification of haematin into ß-haematin. They showed that ellagic acid inhibited the
316 formation of ß-haematin like some quinoline antimalarial drugs, because of its ability to form
317 a π - π complex, and had an IC_{50} three times higher than that of chloroquine. Currently,
318 plasmepsin II is not considered to be an antimalarial target.
319 Previous reports on ellagic acid showed its effective antioxidant properties and biological
320 effects including antimutagenic and tumor chemoprotective activities (21), (23).
321 In the present study, the antioxidant activity of ellagic acid was confirmed, using PMA
322 (Phorbol Myristate Acetate) on human monocytes (Figure 9). At the same time, ellagic acid,
323 in the presence of the known antioxidant NAC, showed reduced *in vitro* antimalarial activity
324 (range, 15-40%) (Figure 8). The concentration of NAC used was 0.87µM and at this

325 concentration it had no antiplasmodial effect. This last result could suggest prooxidant
326 properties of ellagic acid in its antimalarial action. Ellagic acid could act both as pro-and
327 antioxidant in the same way as several polyphenols such as curcumin (14, 22). It could be
328 useful to carry out experiments to verify the pro-oxidant effects of ellagic acid, given that
329 another antimalarial molecule (artesunate) seems to act by oxidative effects (2). A clinical
330 report has shown that a molecule with the dual activities of antioxidant and antimalarial, was
331 interesting in the case of severe malaria (30).
332
333 In conclusion, ellagic acid is a natural compound with very good activity against malaria
334 parasites and high synergy with current antimalarial drugs *in vitro*. *In vivo* activity test
335 showed the high therapeutic index by intraperitoneal route. Moreover, its dual antioxidant and
336 pro-oxidant properties of ellagic acid is interesting. However, the improvement of oral
337 antimalarial efficay of ellagic acid could lead this molecule as a new future antimalarial drugs.

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349

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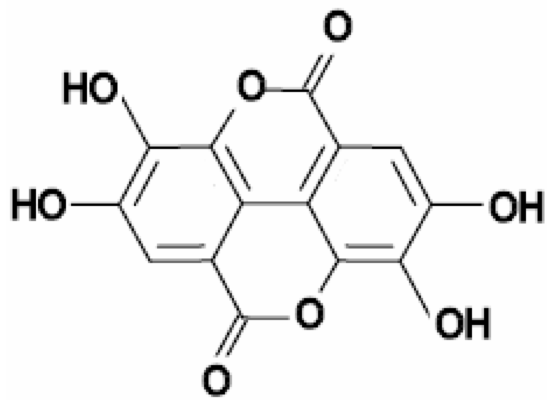

445
446
447
448 Figure 1: ellagic acid
449
450 Chemical formula $C_{14}H_6O_8$; Molecular mass 302.197 g/mol

451

	IC_{50} on F32	IC_{50} on Dd2	IC_{50} on FcB1	IC_{50} on W2	IC_{50} on FcM29
Ellagic acid	330±27	105±27	300±17	330±24	180±20
Chloroquine	29±5	114±29	120±28	299±22	400±12
Artemisinin	12.5±6	13.5±5	12.2±5	13.30±4	12.2±5

452

453 **Table 1**: IC_{50} (in nM) ± standard deviation (SD) of *in vitro* antiplasmodial activity of ellagic

454 acid on 5 *Plasmodium falciparum* strains. Each IC_{50} corresponds to the mean of at least 3

455 independent experiments.

456

Molecule tested with ellagic acid	Mean of FIC_{50}	Kind of combination
Chloroquine	0.63±0.05	Synergistic
Mefloquine	0.64±0.09	Synergistic
Artemisinin	1.26±0.13	Antagonism
Artesunate	0.57±0.13	Synergistic
Atovaquone	0.53±0.21	Synergistic

457

458 **Table 2**: Results of antimalarial agents in combination with ellagic acid on W2 strain.

459 Values are the means of FIC_{50} (Fractional inhibition concentration, which is an interaction
460 coefficient indicating whether the combined effect of drugs is synergistic, additive or
461 antagonistic) and standard deviations for assays run in triplicate on different days. Synergy, if
462 FIC_{50} was <1; additive of FIC_{50} equal to 1 and antagonism if FIC_{50} >1.

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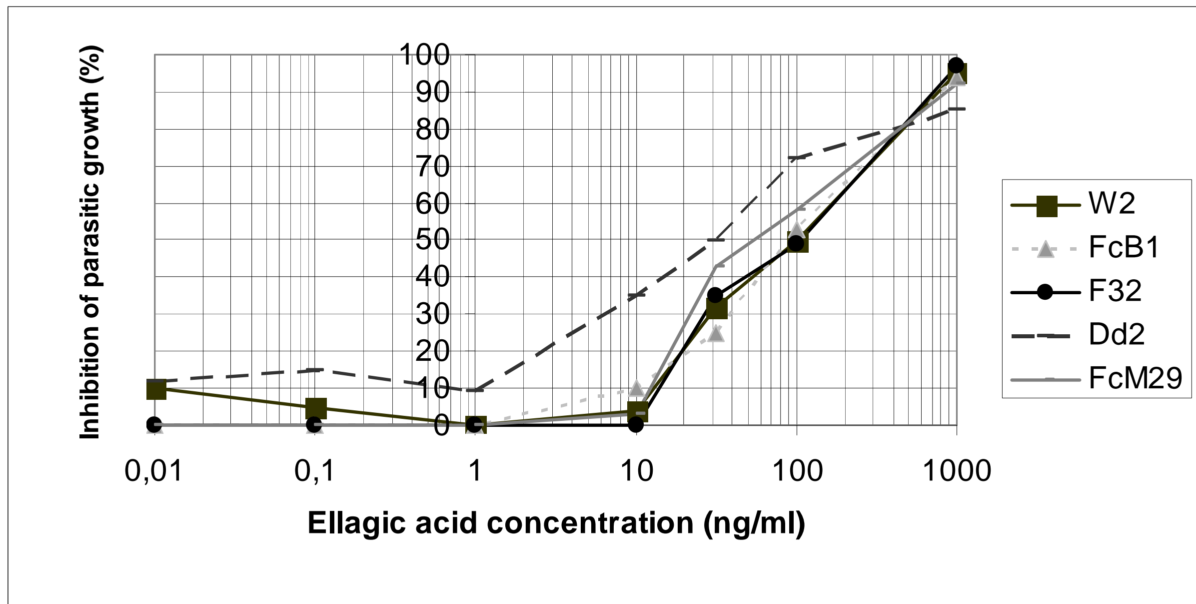

464

465 **Figure 2**: Means of ellagic acid antiplasmodial activity *in vitro* against five *Plasmodium*

466 *falciparum* strains according to at least 3 independent experiments for each strain.

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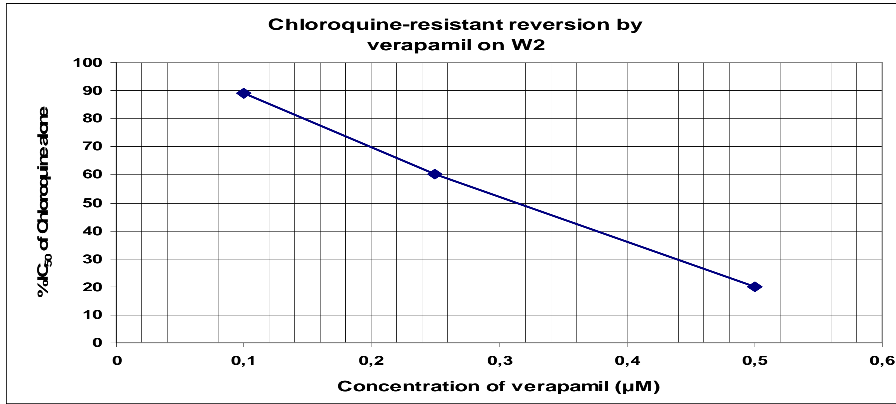

468 Figure 2a

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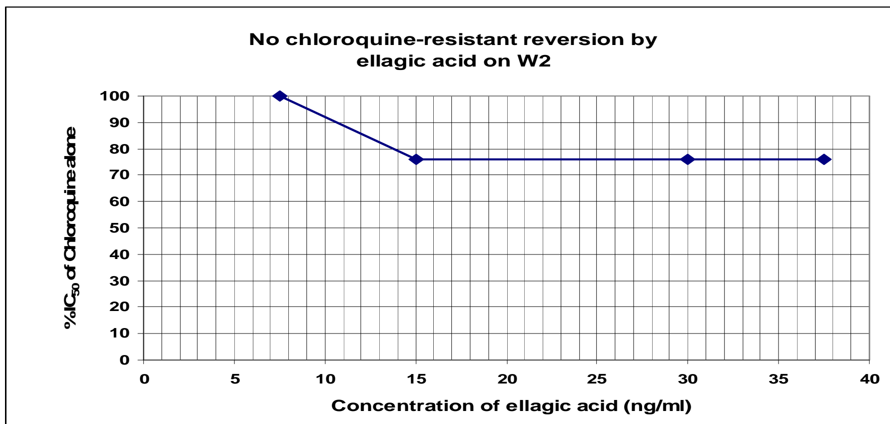

470 Figure 2b

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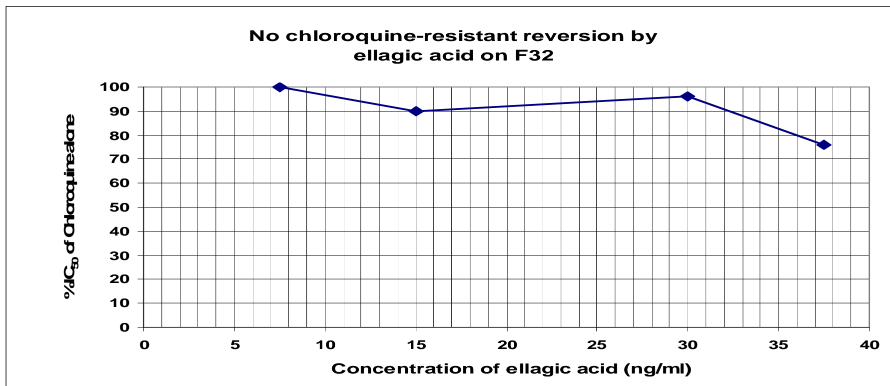


472 Figure 2c

473

474

475 **Figure 3:** Chloroquine resistance reversal by ellagic acid. Values correspond to the mean of
476 three independent experiments. **a** Mean of reversal concentration of verapamil (positive
477 control) on chloroquine-resistant strain W2 (CR_{50} equal to 0.31±0.07 µM); **b** Absence of
478 reversion by ellagic acid on chloroquine-resistant strain W2; **c** Absence of reversion by
479 ellagic acid on the chloroquine-sensitive strain F32 (negative control).

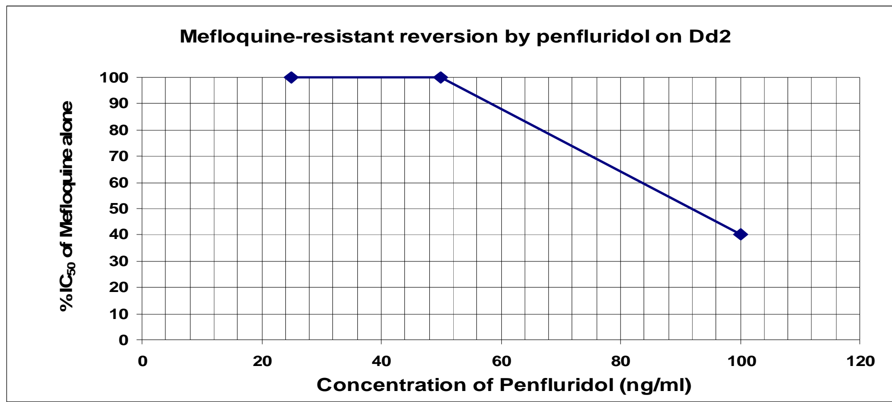
480 

Figure 3a

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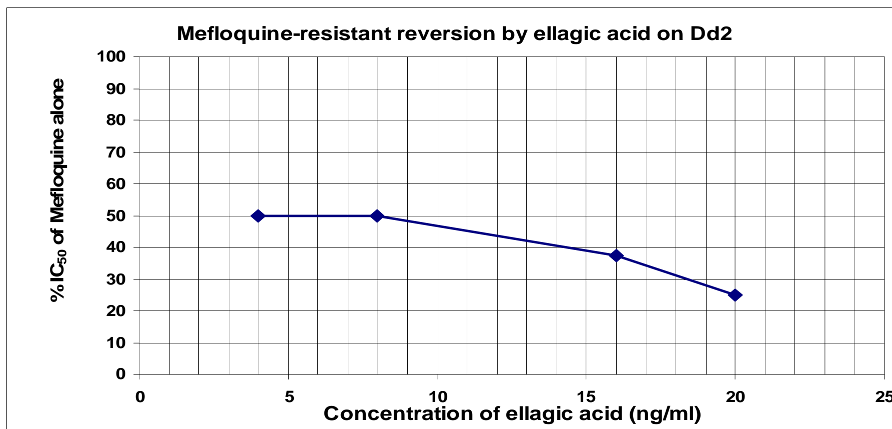
482 

Figure 3b

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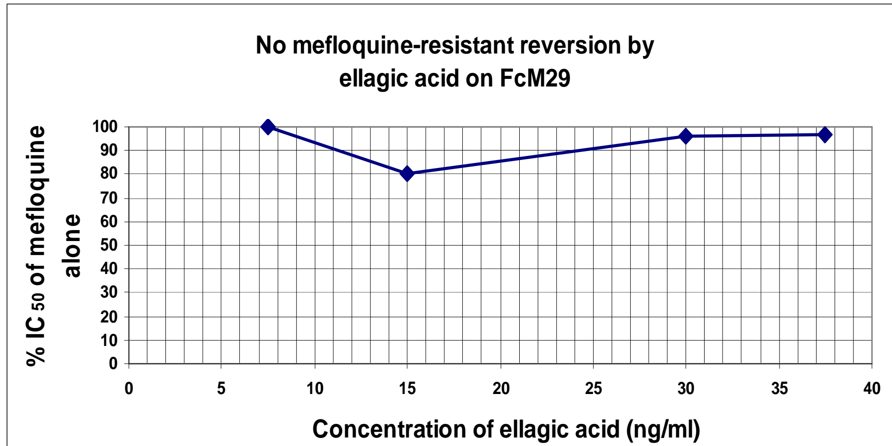
484 

Figure 3c

485

486 **Figure 4**: Mefloquine resistance reversal by ellagic acid. Values are the means of three

487 independent experiments. **a**: Means of reversal concentrations of penfluridol (positive control)

488 on mefloquine-resistant strain Dd2 (CR_{50} for Penfluridol: 96±7 ng/ml); **b**: Means of reversal

489 concentrations of ellagic acid on mefloquine-resistant strain Dd2 (CR_{50} for ellagic acid: 5±4

490 ng/ml); **c**: Absence of reversion by ellagic acid on mefloquine-sensitive strain FcM29

491 (negative control).

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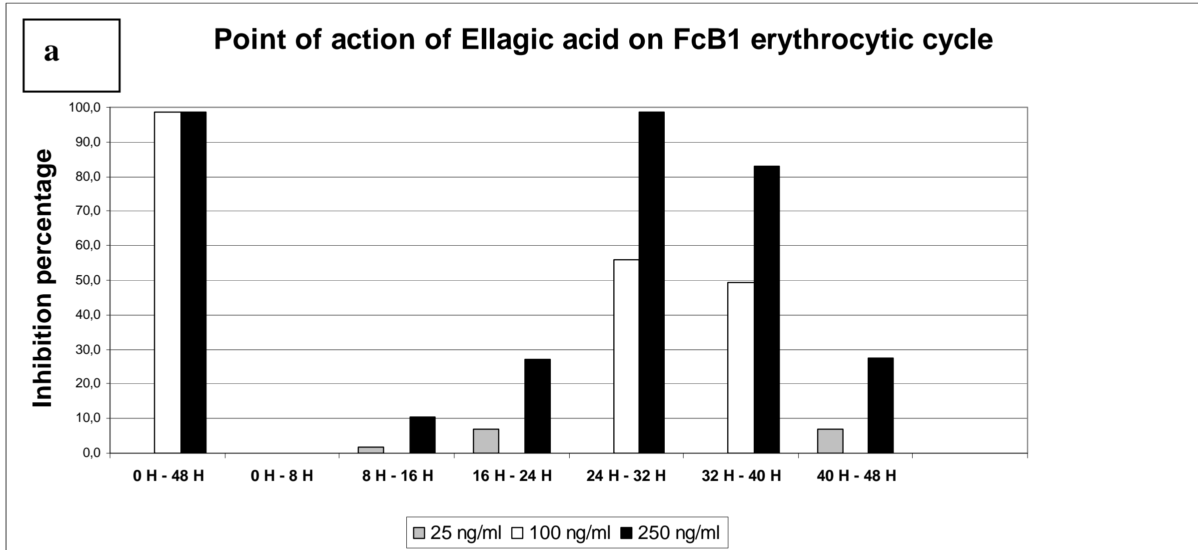

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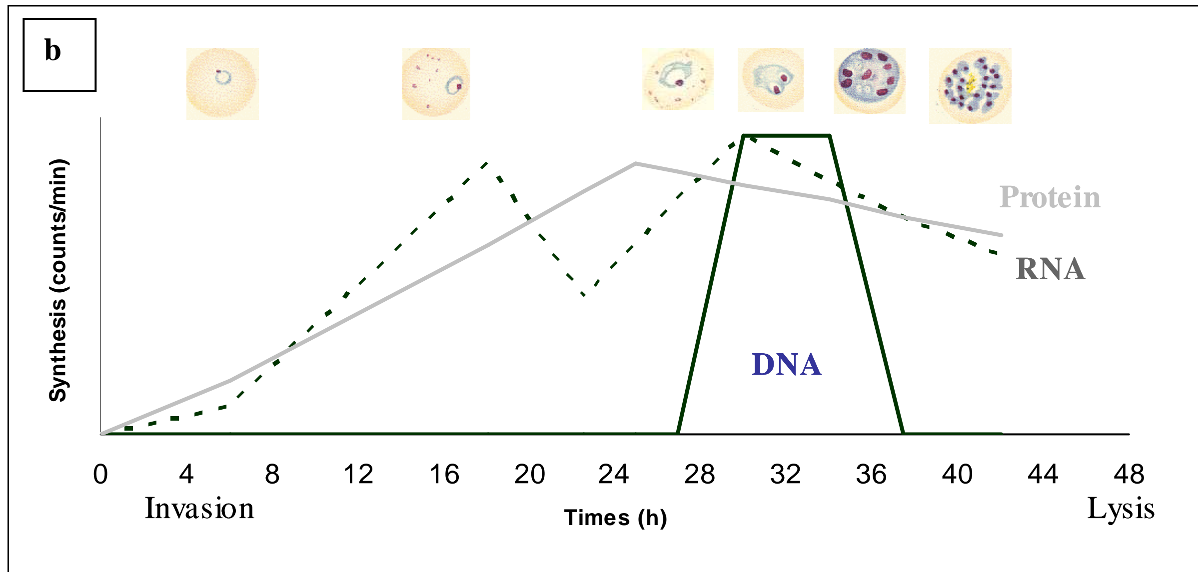

495

496 **Figure 5**: **a** Point of action of ellagic acid on the erythrocytic life-cycle; **b** Approximate

497 timing of the onset of inhibition and DNA, RNA and protein synthesis during the

498 development of *Plasmodium falciparum* in synchronised cultures adapted from (1).

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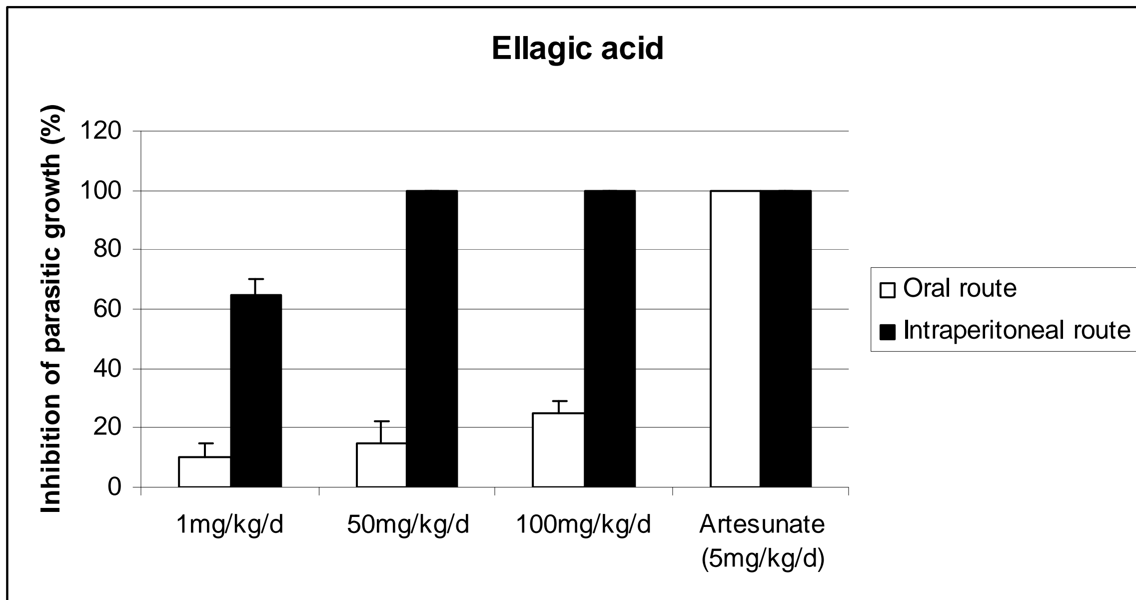

502
503 **Figure 6**: Percentage inhibition of parasite growth *in vivo* compared with untreated control
504 mice, *versus* dose of ellagic acid (or artesunate) at D4 in treatment by the oral and
505 intraperitoneal routes assessed by the 4-day suppressive test.

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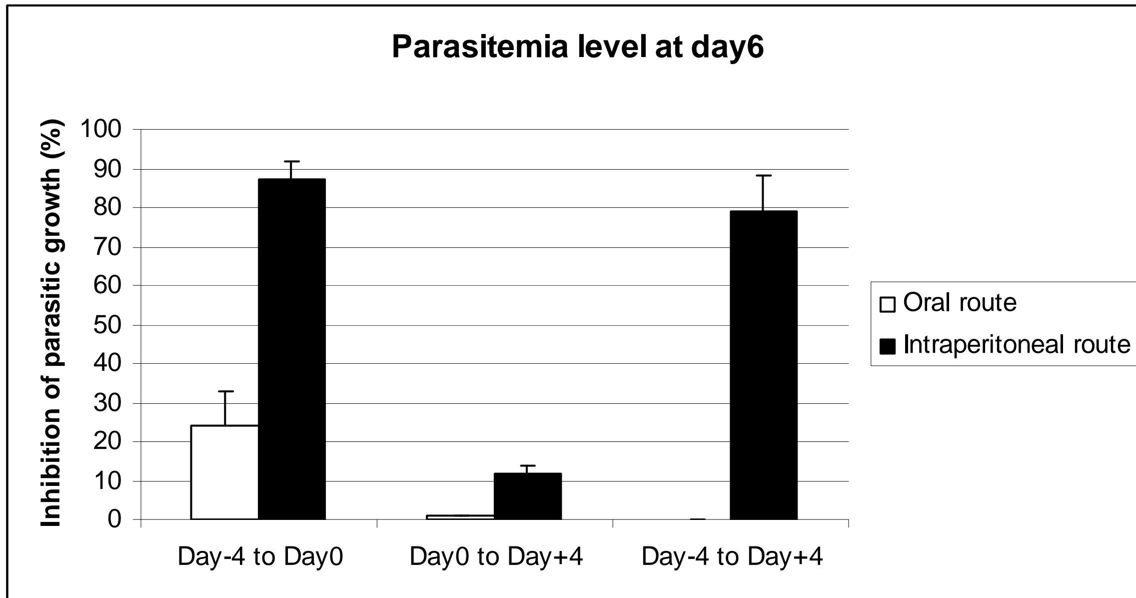

512

513 **Figure 7**: Percentage inhibition of parasite growth *in vivo* and standard deviation at day 6 in
514 all groups of mice infected by *P. vinckei petteri* and either orally (100mg/kg/d) or
515 intraperitoneally (10mg/kg/d) treated by doses of ellagic acid compared with untreated
516 controls. D0 corresponds to the day of *Plasmodium* infection.

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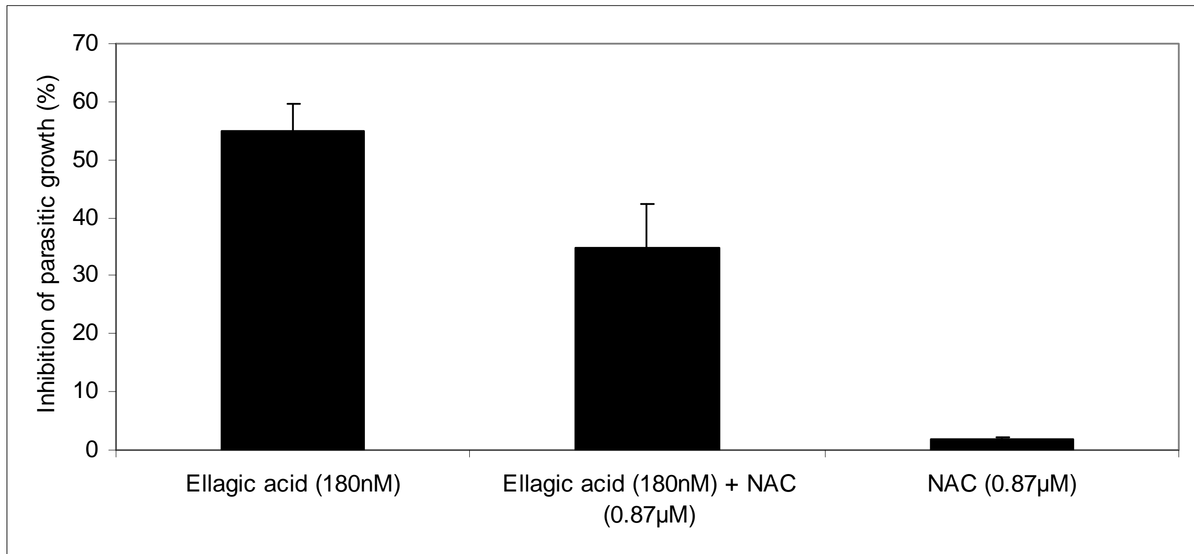

523
524 **Figure 8**: Inhibition effect of ellagic acid antiplasmodial activity by the antioxidant

525 compound N-acetylcysteine (NAC) on the FcM29 strain of *P. falciparum.*

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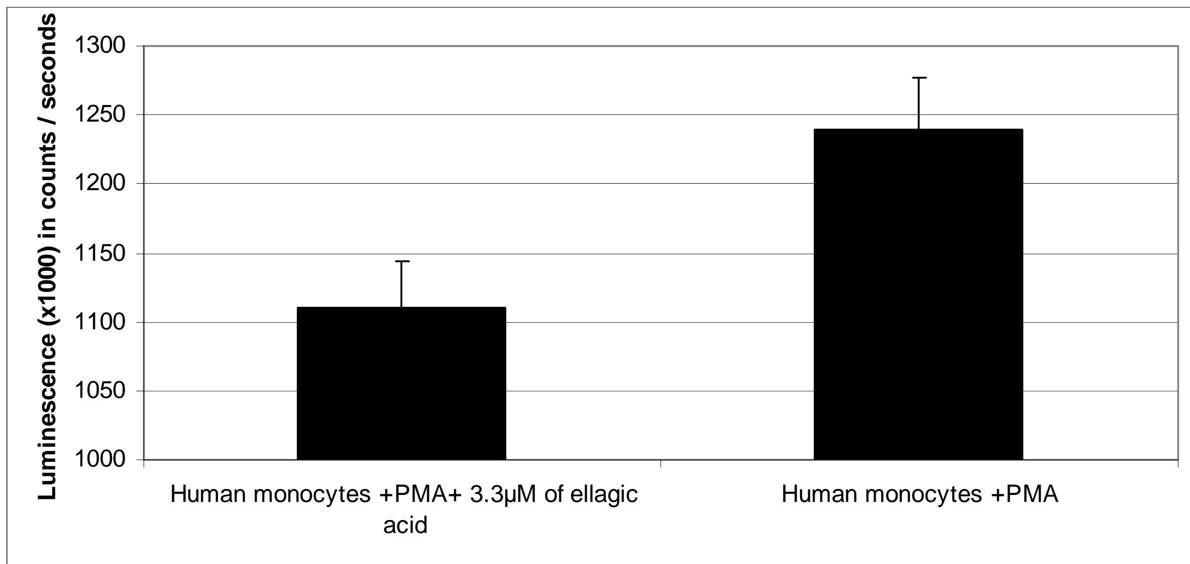

529
530 **Figure 9**: Antioxidant effect of ellagic acid on human monocytes using PMA, which induced
531 strong generation of reactive oxygen species (ROS).

532

V. Publication 7 : La girolline, molécule antipaludique issue de la recherche anticancéreuse

Diverses approches sont explorées pour rechercher de nouvelles cibles antipaludiques. Surtout le recours aux produits naturels. Exemple du taxol (49), molécule antitumorale extraite de *Taxus sp*, qui a montré une très bonne activité antipaludique *in vitro* (CI_{50} sur souches de *P. falciparum* égale à 71 nM). Par analogie, les extraits marins sont une autre source inestimable de molécules d'intérêt éventuelles (58). En 1950, l'Ara-C, agent anti-tumoral du traitement de leucémies et lymphomes, fut l'un des premiers composés dérivés de roduits marins (51). Par la suite, plus de 15 000 composés marins ont été décrits pour leurs propriétés dans différentes applications :

- anti-inflammatoire, comme le manoalide (éponge marine, *Luffariela variabilis*), également bien étudié pour son action sur le psoriasis,
- anti-tumorale, en inhibant de façon non spécifique la croissance cellulaire, comme l'halichondrine B (*Halichondria okadai*), qui interfère avec les microtubules et le latrunculine A (*Latrunculia magnifica*), qui inhibent la polymérisation de l'actine,
- antivirale, comme les papuamides C et D (*Theonella mirabilis*, *Theonella swinhoei*) ou l'avarol (*Dysidea avara)* présentant également une activité sur le psoriasis.

Ces molécules, agissant sur des voies métaboliques parasitaires connues, sont des cibles antipaludiques intéressantes à explorer (23). Mais elles ont l'inconvénient majeur d'être régulièrement toxiques. Notre collaboration avec les phytochimistes de l'ICSN-CNRS, Alain Ahond et Christiane Poupat, qui ont identifié des molécules anticancéreuses d'éponges marines, nous a amenés à étudier les propriétés antipaludiques de ces molécules.

Parmi ces molécules, la girolline a été sélectionnée. Elle est extraite d'une éponge sous marine (*Cymbastela cantharella*) de Nouvelle Calédonie, connue pour ses propriétés anticancéreuses. Elle a en effet montré une intéressante activité antitumorale *in vitro* sur des lignées de cellules tumorales et *in vivo* sur des tumeurs murines.

Les résultats de l'efficacité antitumorale de la girolline ont permis d'envisager des essais cliniques. En dépit d'études sur le chien et la souris montrant peu d'effets toxiques, les essais cliniques de phase I ont rapidement dû être arrêtés à cause d'effets secondaires sévères, essentiellement sur le système cardio-vasculaire. En particulier, la girolline induit une forte hypotension sans effet antitumoral apparent.

Afin de détourner ces effets indésirables, des analogues de synthèse ont ensuite été envisagés, mais n'ont pas donné les activités escomptées à ce jour.

Le docteur Ahond propose l'hypothèse d'une altération de la synthèse protéique en agissant sur le processus de terminaison de la traduction comme mécanisme d'action antitumorale (1). Par analogie, nous cherchons à savoir si les motifs chimiques associés à l'activité antitumorale de la girolline sont associés à l'activité antipaludique.

Les propriétés antipaludiques *in vitro* et *in vivo* de la girolline et de ses dérivés ont été ici explorées. Les résultats des activités antipaludiques *in vitro* sur 4 souches de *Plasmodium falciparum* ont donné des CI_{50} entre 77 et 215 nM quelque soit la chloroquinorésistance des souches, ainsi qu'une synergie avec la chloroquine. De plus l'activité antipaludique *in vivo* sur des modèles de paludisme murin a donné une DE_{50} à 1 mg/kg/j quelque soit la voie d'administration. Néanmoins, l'étude de la toxicité cellulaire (sur la lignée cellulaire KB) et murine (sur la souris et le rat) de cette molécule a mis en évidence un index de sélectivité et un index thérapeutique tous les deux médiocres. L'activité antipaludique des dérivés de la molécule montre que sans le noyau aminoimidazole, aucune efficacité *in vitro* n'est possible. Actuellement, la girolline fait l'objet d'une pharmaco-modulation pour réduire la toxicité en préservant l'activité antipaludique liée au motif chimique aminoimidazole.

Enfin il pourrait être envisagé la production de nouveaux dérivés conservant le cycle aminoimidazole afin d'étudier leur activité et leur toxicité.

L'article sur les propriétés antipaludiques de la girolline a été publiée dans le journal « Planta Medica » 2008 74 438-444.

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Girolline: A Potential Lead Structure For Antiplasmodial Drug Research

Author

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Key words
- Malaria
- drug research
- marine compounds
- *Cymbastela cantharella*
- Axinellidae

Abstract

Girolline is a 2-aminoimidazole derivative extracted from *Cymbastela cantharella* (a New-Caledonian sponge) that has shown antitumor activity. In this study, we investigated its antimalarial activity and the point of action within the erythrocytic cycle of *Plasmodium falciparum*. Initially, we tested girolline and some synthetic analogues *in vitro* against four *P. falciparum* strains. The IC_{50} values of girolline ranged from 77 to 215 nM, and as with artemisinin or chloroquine, girolline inhibited parasitic growth by 100%. Girolline was found to be active at a dose of 1 mg/kg/d (orally and intraperitoneally) *in vivo*. Moreover, there was a significant synergistic effect between girolline and chloroquine *in vitro*. The investigation of the mechanism of action of girolline during the erythrocytic life cycle of the parasite showed that its action targets the synthesis of proteins by the parasite. With such a biological profile, girolline could be considered as a model chemical structure for new candidates in the arsenal of new drugs and in particular of drugs able to fight malaria.

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Dedication
Dedicated to Pierre Potier who initiated marine chemistry in the I.C.S.N. and was fully involved in the girolline project

Bibliography
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Introduction

Malaria is the most prevalent parasitic disease in the world today. About 500 million people present with signs and symptoms of malaria every year and in Africa over more than one million succumb to it annually [1]. The seriousness of the endemic has recently been aggravated by the progressive spread of *Plasmodium falciparum*, now resistant to the most commonly used and affordable antimalarial drugs such as chloroquine.
There will always be a clear demand to search for new antimalarial agents [2]. In this context, research into new antimalarial drug candidates originating from natural sources has been actively pursued. For example, taxol, a natural product from *Taxus sp.* (western yew tree), and one of the best antitumor drugs, also shows very good *in vitro* antiplasmodial activity (IC_{50} = 71 nM against *P. falciparum*) [3], [4].
By analogy, marine extracts that possess antitumor activity represent a new approach in the search for antimalarial drugs. Among these marine extracts, girolline (**1**) (or girodazole), a 2-aminoimidazole derivative, is an original metabolite extracted in 1988 [5] from the New-Caledonian marine sponge, *Cymbastela cantharella* (formerly *Pseudaxinyssa cantharella*). Girolline is known for its antitumor properties because it shows significant activity *in vitro* against several tumor cell lines, and *in vivo* against grafted tumors in mice [6], [7], [8]. Recently, this compound has been isolated from another species of the marine sponge *Axinella brevistyla* collected in western Japan [9].
In the present study, we evaluated the effects of girolline and some of its analogues *in vitro* and *in vivo* against *P. falciparum* and *P. vinckei petteri*, respectively. To complete the study on girolline, we evaluated its cytotoxicity *in vitro* and its acute toxicity *in vivo*. We have also determined the point of action of girolline in the erythrocytic life cycle of the malarial parasite and its synergistic action with chloroquine.
In addition, we have also tested some analogues of girolline and other compounds extracted from the same sponge (*Cymbastela cantharella*).

Materials and Methods

Compounds

Girolline (dichlorhydrate form) **1** [5], dibromocantharelline **10** [10] and hymenialdisine **11** [10] (**Fig. 1**) were extracted from *Cymbastela*

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cantharella, a New Caledonian sponge. The molecular weight of girolline is 262 g/mol and that of hymenialdisine is 324 g/mol. All other compounds are synthetic analogues of girolline: **2** 5-deazathiogirolline (*erythro*) [11], **3** 5-deazathiogirolline (*threo*) [11], **4** (+)-(3*S*,4*R*)-3-benzyloxy-4,5-*O*-isopropylidene-4,5-dihydroxy-1-pentene [12], **5** (2*R*/*S*,3*S*,4*R*)-3-benzyloxy-1,2-epoxy-4,5-*O*-isopropylidene-4,5-dihydroxypentane [12], **6** 2-amino-4-[(1′*S*,2*R*′)-1′-benzyloxy-(2′,3′)-dihydroxypropyl]-thiazole-*N2-tert*-butylcarbamate [12], **7** 2-amino-4-[(1′*S*,2′*R*)-3′-azido-1′-benzyloxy-2′-hydroxypropyl]-thiazole-*N2-tert*-butycarbamate [12], **8** 2-amino-4-[(1′*S*,2′*S*)-3′-azido-1′-benzyloxy-2′-chloropropyl]-thiazole-*N2-tert*-butycarbamate [11], **9** 4-deazathiogirolline [12], **10** dibromocantharelline, and **11** hymenialdisine.

Standard molecules

Artemisinin (Sigma Aldrich), molecular weight: 283.34 g/mol, is a compound extracted from *Artemisia annua* and chloroquine (Bufa), molecular weight: 515.87 g/mol.

Parasites *in vitro*

P. falciparum was cultured continuously according to the modified Trager and Jensen's method [13], [14]. The FcB1-Columbia (gift from the Museum d'Histoire Naturelle, Paris, France), W2-Indochina (gift from the Institut de Médecine Tropicale du Service de Santé des Armées, Marseilles, France) and FcM29-Cameroon (gift from the Museum d' Histoire Naturelle, Paris, France) were considered to be chloroquine-resistant strains (chloroquine 50% inhibitory doses: mean IC_{50} = 120, 299 and 400 nM, respectively). The F32-Tanzania (gift from the Museum d'Histoire Naturelle, Paris, France) was considered to be a chloroquine-sensitive strain with an IC_{50} for chloroquine of 29 nM (mean of more than 10 independent experiments).

Evaluation of IC_{50}

The antimalarial activity was evaluated by the radioactive micro-method described by Desjardins et al. [15] with modifications as reported by Benoit et al. [14]. Drug testing was performed at 3 or 4 different times in triplicate in 96-well culture plates (TPP) on asynchronous cultures. Compounds were dissolved in DMSO (stock solution: 5 mg/mL) and further diluted in culture medium so that the final DMSO (Sigma) concentration never exceeded 2%. This 2% concentration of DMSO did not affect parasite growth. Parasite growth was estimated by [^3H]hypoxanthine incorporation (Perkin Elmer).

The control parasite culture (RPMI with 5% of human serum alone or with 2% dimethyl sulfoxide) was referred to as 100% growth. The IC_{50} values were determined graphically by plotting the log of the drug concentration versus the percentage inhibition of parasite growth.

Evaluation of ED_{50}

In vivo antimalarial activity was determined against *P. vinckei petteri* according to the Peters' 4-days suppressive test [16]. Female Swiss mice (Elevage Janvier), weighing around 30 g, were inoculated intraperitoneally on day 0 (D0) with 2×10^7 parasitized erythrocytes in 100 μL of physiological serum. Drugs were then administered to the animals, intraperitoneally or per os, daily for 4 consecutive days. The first treatment started 3 hours after parasite inoculation and the others were given at the same time the following days. Parasitemia levels (parasitized erythrocytes/total erythrocytes) were determined on D4 with thin blood smears (light microscopy × 1,000).

The ED_{50} is the dose leading to 50% inhibition of parasite growth compared with growth in the control (treated with an equal volume of vehicle, 100 μL of mixture physiological serum and

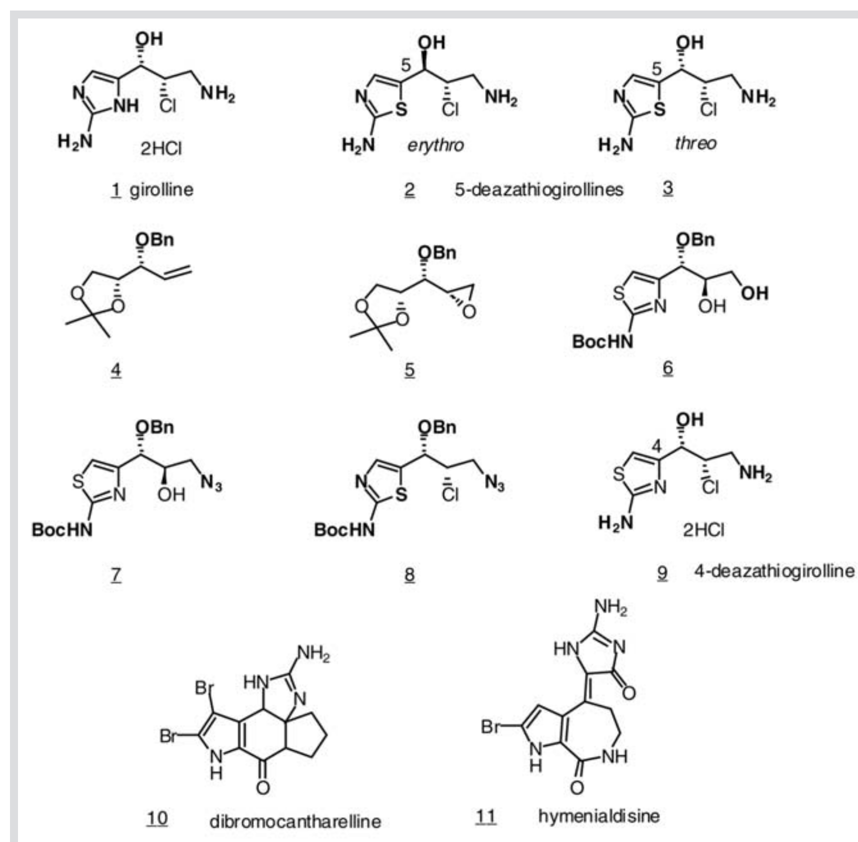

Fig. 1 Structures of the compounds **1 – 11**.

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DMSO). It was evaluated from a plot of activity (expressed as a percentage of activity) versus the dose.
Groups of five mice were used for each dose (except for 0.1 mg/kg/d intraperitoneally: 3 mice). Finally, five doses were tested (0.1 mg/kg/d, 1 mg/kg/d, 10 mg/kg/d, 25 mg/kg/d and 50 mg/kg/d). Each experiment was performed twice. Two control groups were also included: they were both treated with the vehicle, either orally or intraperitoneally. Five mice were kept as untreated controls (same batch, no parasite, no treatment) and five mice were inoculated but received no treatment.
All procedures involving animals conformed fully to European regulations (EEC directive 86/609 dated 24/11/1986). The experiments involving animals were carried out in the animal room of the Parasitology Department of Rangueil Hospital (Toulouse, France) which is under the control of the National Veterinary Services. All *in vivo* studies were approved by the French Institutional Animal Experimentation Ethic Committee # ETH/MP-TLS-1510 – 01/R/01/06.

Acute toxicity evaluation

Acute toxicity was determined after a single injection of drug to non-infected female Wistar rats, weighing around 100 g, and non-infected female Swiss mice, weighing around 30 g (Elevage Janvier). It was expressed as the 50% lethal dose (LD_{50}), which corresponds to the dose leading to 50% death, 10 days after drug injection. Each dose was tested with groups of two to four animals.
The drugs were dissolved in a mixture of DMSO and physiological serum, in equal amounts. Drugs were administered intraperitoneally or orally by gavage (100 μL per mouse and 200 μL per rat). This dose of DMSO did not affect parasite growth or cause death to the mice. Animals were then kept under observation for 60 days. Two control groups were also included: they were both treated with the same vehicle, either orally or intraperitoneally (two rats or three mice per control group). Three mice and rats were kept as controls.

In vitro potentiation tests

The synergy between girolline and chloroquine was assessed by potentiation experiments as already described [17], [18] and tested on the F32 strain of *P. falciparum*. Several combinations of both drugs were incubated in 96-well plates and the inhibition was evaluated as described.

The isobologram was constructed by plotting a pair of fractional IC_{50} values for each combination of girolline and chloroquine. Girolline fractional IC_{50} values were calculated by dividing the IC_{50} of the combination by the IC_{50} of girolline alone, and the corresponding chloroquine fractional IC_{50} values were also calculated. An isobologram as a straight diagonal indicates an additive effect. Points above or below the diagonal indicate antagonistic or synergistic effects, respectively.

Stage of girolline action with respect to the erythrocytic life cycle

Cultures of the strains W2 and FcB1 (which express knobs during the schizont stage) were synchronized on a 4-hour period. The method consisted of alternatively synchronizing young forms with 5% d-sorbitol and late forms with plasmion [19]. Girolline was tested in 24-well plates with cultures at 0.5 to 1% parasitemia (hematocrit 1%). Cultures were subjected to 8-hour pulses (corresponding to one-sixth of the erythrocytic cycle time) of girolline (5, 50 and 250 ng/mL). After being pulsed, the cultures were washed three times with culture medium and then returned to normal conditions until the next cycle. At time zero plus 56 h (the ring stage of the next erythrocytic cycle), parasitemia was calculated by the visual counting of Giemsa-stained smears [20].

Results

In these tests, artemisinin and chloroquine were used as standards. The IC_{50} value of girolline on FcM29 was 35 ng/mL (130 nM) whereas the value for chloroquine was 280 ng/mL (540 nM) and that for artemisinin 2.6 ng/mL (9 nM). As with artemisinin or chloroquine, girolline was able to inhibit parasitic growth by 100% (▶ **Fig. 2**).
We noted that the values for girolline (from 77 to 215 nM) were in the same range for all the *P. falciparum* strains tested, whatever their sensitivity to chloroquine (▶ **Table 1**).
The security index (cytotoxicity/activity ratio) was 2.3 for girolline (▶ **Table 2**). This security index appears to be 50-fold higher for chloroquine and more than 14,000-fold higher for artemisinin than for girolline. The other compounds, analogues of girolline, were also tested (▶ **Table 2**). They are all synthetic analogues except dibromocantharelline (**10**) and hymenialdisine (**11**) that are natural products extracted from *Cymbastela*

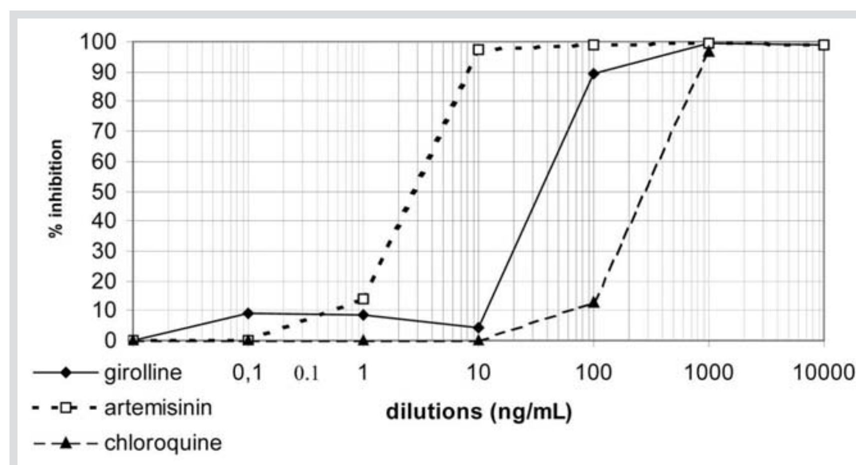

Fig. 2 Percentage inhibition of parasite growth *in vitro* by girolline, chloroquine and artemisinin on FcM29 (a chloroquine-resistant strain).

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Table 1 IC_{50} values for girolline and chloroquine against F32, W2, FcB1 and FcM29 strains

Strains	IC_{50} (nM) for girolline	IC_{50} (nM) for chloroquine
F32-Tanzania	77 ± 37[a]	29 ± 5
W2-Indochina	114 ± 34	299 ± 22
FcB1-Colombia	215 ± 62	120 ± 28
FcM29-Cameroon	130 ± 19	400 ± 12

[a] SEM (standard error of the mean).

Table 2 IC_{50} values for girolline and its derivatives against FcM29-Cameroon

Compounds	IC_{50} (μM) on FcM29	IC_{50} (μM) on cells
girolline	0.13	0.3
2	> 200	> 5
3	> 200	> 5
4	> 50	nd
5	> 50	nd
6	> 50	> 10
7	> 50	> 10
8	> 50	8
9	> 50	>10
10	> 50	nd
11	2.47	nd
artemisinin	9×10^{-3}	> 300
chloroquine	0.4	50

cantharella. For all derivatives, the IC_{50} values were higher than 50 μM, except for hymenialdisine, which had an IC_{50} of 2.47 μM.
When mice were treated intraperitoneally with girolline, 87% inhibition of the parasite growth was obtained with a dose of 1 mg/kg/d. This shows an ED_{50} (intraperitoneally) of girolline significantly lower than 1 mg/kg/d (**Fig. 3**).
When mice were treated orally, 44% inhibition of parasite growth was obtained with a 1 mg/kg/d dose (**Fig. 3**). Orally, the ED_{50} of girolline was around 1 mg/kg/d. For both routes, we have not been able to reach a conclusion concerning the doses of 10, 25 and 50 mg/kg/d because the mice died before the end of the fourth treatment (**Fig. 3**) whereas mouse controls (only treated with vehicle) were kept under observation for 60 days.
The parasitemia of surviving mice treated orally or intraperitoneally with 1 mg/kg/d was monitored from D10 to D40. After D32, no parasites were observed on the stained blood smears and the mice were totally cured at D60 (no recrudescence) (data not shown).
Because in the *in vivo* antiplasmodial test (determination of ED_{50}) toxicity was observed after multiple injections of girolline, we assessed its acute toxicity. Toxicity tests in mice gave an LD_{50} of 8.5 mg/kg/d for the oral route and 17.5 mg/kg/d intraperitoneally (data not shown). Toxicity in rats showed an LD_{50} of 17.5 mg/kg/d and 6.5 mg/kg/d, respectively for the oral and the intraperitoneal routes (data not shown).
The synergy of chloroquine by girolline is shown in **Fig. 4**. All points were below the diagonal, indicating synergistic effects. This was evidence of a significant potentiation effect between girolline and chloroquine.

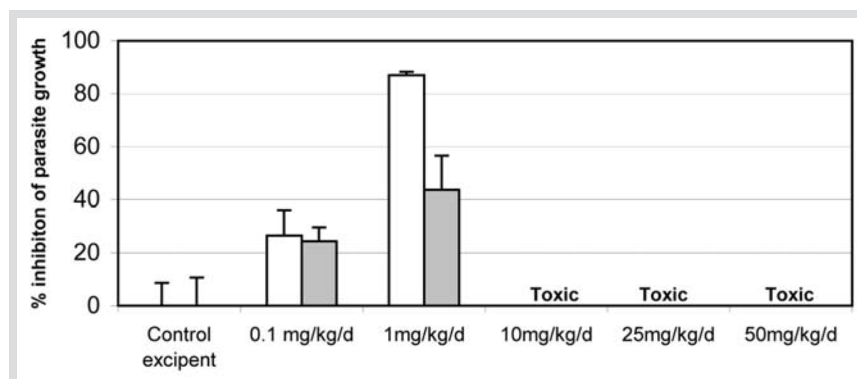

Fig. 3 Percentage inhibition of *in vivo* parasite growth versus doses of girolline at D4 with treatment by the intraperitoneal route (white bars) or by the oral route (grey bars).

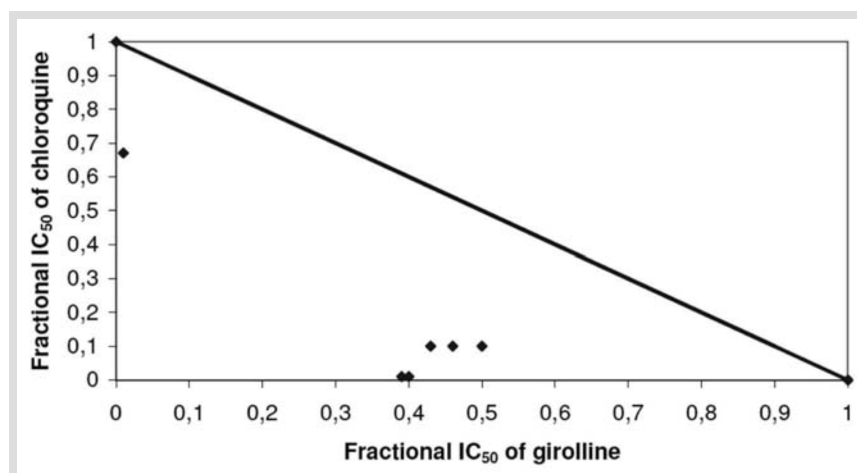

Fig. 4 Potentiation of chloroquine by girolline in the F32 strain. * The straight diagonal indicates an additivie effect.

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During the erythrocytic life cycle, the action of girolline began at the 16th hour for the W2 strain and at the 8th hour for the FcB1 strain. Then the activity increased for both strains and the maximum was reached between the 24th and the 40th hour, when the parasites were at the trophozoite stage (**Figs. 5** and **6**). The trophozoite stage of the malaria life ycle is the most metabolically active phase with protein and DNA synthesis. After the 40th hour, the activity of girolline on both strains was lower. This profile of activity has been compared with the synthesis of proteins, DNA and RNA by *Plasmodium* (**Fig. 7**). The point of action of girolline was confirmed to be within the same period of activity found with both the *P. falciparum* strains.

Discussion

Girolline is an antitumor candidate drug studied for its antimalarial activity. The antiviral activity has also been explored by determining the effects of the compounds on the replication of HIV-1, but the IC_{50} of 4-deazathiogirolline (a synthetic derivative of girolline) on virus type HIV-1 Bal was high with 100 nM, compared with the IC_{50} of AZT (azidothymine), as reference, of 6 nM [12]. However, to ther best of our knowledge, this is the first time that girolline has been studied against *Plasmodium*.

First, we tested the *in vitro* activity of girolline against *P. falciparum*. The antiplasmodial activity of girolline approached that of artemisinin or chloroquine, in totally inhibiting *P. falciparum* growth *in vitro,* whatever the chloroquine sensitivity

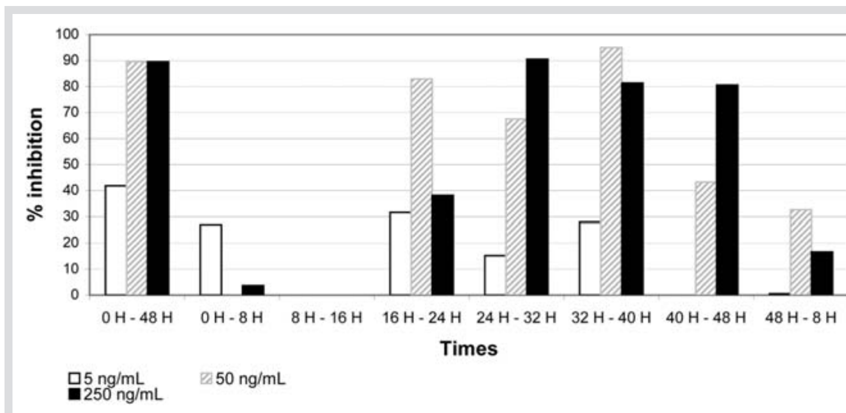

Fig. 5 Percentage inhibition during the erythrocytic life cycle for W2.

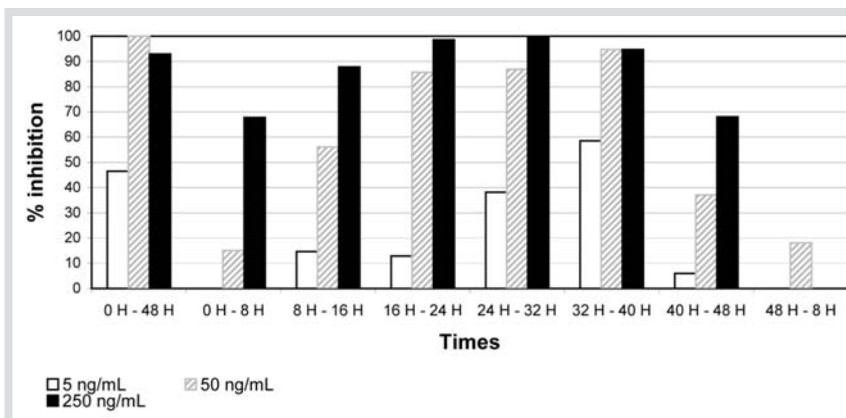

Fig. 6 Percentage inhibition during the erythrocytic life cycle for FcB1.

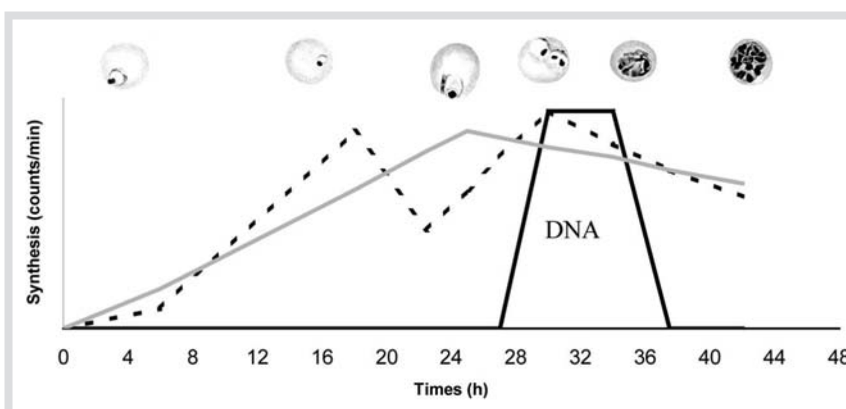

Fig. 7 Approximate timing of the onset and cessation of DNA, RNA and protein synthesis during the development of *Plasmodium falciparum* in synchronised cultures (adapted from Arnot and Gull, Ann Trop Med Parasitol 1998).

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of the strain. Then, the synthesis of two series of analogues (the series of 5-deazathiogirolline and the series of 4-deazathiogirolline), that do not contain the aminoimidazole heterocycle, was undertaken but as for their antitumor activity [11], [12], they were devoid of any action. The aminoimidazole heterocyclic ring thus seems to be necessary both for the antitumor activity [6] and probably for the antiplasmodial properties.

Among the other compounds extracted from the same *Cymbastela*, only hymenialdisine [10] was found to be active against *P. falciparum*. Hymenialdisine is known for its cytotoxicity against 60178y cell lines (ED_{50} = 3.9 μg/mL) by inhibition of cyclin-dependent kinase (IC_{50} = 28 nM) [21].

In addition, girolline showed very high activity *in vivo* in a murine model. The ED_{50} of girolline on *P. vinckei petteri* is mostly inferior to 1 mg/kg/d by the intraperitoneal route and 1 mg/kg/d when administered orally. In comparison, the standard artemisinin showed, in similar conditions with the same murine *Plasmodium* strain, poorer results with an ED_{50} of 4 mg/kg/d and 17 mg/kg/d by the intraperitoneal and oral routes, respectively [20].

However, girolline seems to be quite toxic even if preliminary toxicological studies using dogs and mice have led girolline up to phase I trials suggesting few toxic effects. The phase I anticancer clinical study was stopped because of effects of girolline on the cardiovascular system including severe hypotension in patients [7].

The toxicity of marine compounds has often been reported [22], [23] and studies of compounds from sponges where the activity is close to the toxicity are frequent in the literature. While, *in vitro*, the security index of girolline (2.3) is better than that of pycnidione (1), this compound is seen as a potential lead structure for antimalarial drugs [24]. In addition, the security index of girolline is only 50-fold poorer than that of chloroquine, whereas a recent investigation of different marine invertebrate phyla producing compounds with interesting antiplasmodial activities showed that they had worse *in vitro* selectivity indices, ranging from 125- to 5000-fold poorer than the selectivity index of chloroquine [25].

Additionally, the *in vivo* toxicity of numerous marine extracts has often been reported [25]. For girolline, the LD_{50} (17.5 or 8.5 mg/kg/d for mice, for intraperitoneally and oral routes respectively) is not very far from the ED_{50} (1 mg/kg/d). The therapeutic ratio [interval between toxicity (LD_{50}) and activity (ED_{50})] of girolline is thus narrow (TR = 17.5 for mice for the intraperitoneal route and 8.5 for the oral route). The low LD_{50} in mice was confirmed in rats (oral and intraperitoneal routes). The differences in the pharmacological responses between mice and rats could explain the variations we have found between the intraperitoneal and oral routes with these laboratory animal species.

Furthermore, manzamine A (an alkaloid extracted from several sponge species, with IC_{50} values below 0.96 μM) [26] and xestoquinone (an alkaloid from *Xestospongia* from Vanuatu with an IC_{50} of 3 μM) [27], have both been studied for their antimalarial properties, but they also had very narrow therapeutic ratios. The TR for manzamine A is 10 and for xestoquinone is around 4 and thus lower than girolline (8.5 – 17.5).

We could explain the toxicity of girolline by the fact that in the sea, marine sponges produce metabolites to combat predation, but also to be protected against bacteria, fungi or parasites [28]. Probably to overcome dilution in this aqueous milieu, molecules or compounds are produced at high concentrations by marine organisms. This could explain why these compounds are usually toxic to terrestrial organisms [23] and that only a few marine animals are dependent on sponges for their diet [28].

A first approach to solve the toxicity problems associated with girolline could be the association of girolline with an antiplasmodial drug. Currently chloroquine remains one of the most commonly used antimalarials, but the spread of resistance against this 4-aminoquinoline has forced the WHO to recommend the use of drug combinations. The very high synergy between girolline and chloroquine could enable the quantity of each drug to be reduced during the treatment and thus limit the side effects of girolline. Such synergistic effects between both these drugs are also an advantage particularly sought after in the fight against the emergence of resistance.

The period of girolline's activity corresponded with protein synthesis and suggested that girolline acted by inhibiting the synthesis of the *Plasmodium* proteins. The pharmacological targets of girolline, and its mechanism of action on *P. falciparum*, are not well known. On the one hand, Tsukamoto et al. [29] have suggested that girolline affects the step of recruitment of polyubiquinated p53 to the proteasome and that this phenomenon could be responsible for the antitumor and cytotoxic effects of girolline. On the other hand, the inhibition of protein synthesis by girolline has been reported by Lavelle et al. to be the likely mechanism of action [6]. They and Colson et al. [8] suggest that girolline inhibits the release of peptides from the ribosomes. Recently Schroeder et al. have demonstrated that girolline specifically interacts with the ribosomes of eukaryotes and not with those of prokaryotes [30]. Our experiments during the erythrocytic life cycle, on both W2-Indochina and FcB1-Colombia strains, confirmed that girolline inhibits parasite growth between the 24th and the 40th hour that is during the protein synthesis stage, which could explain its antiplasmodial activity. Finally, several teams [31], [32], [33] have now achieved the total synthesis of girolline.

In conclusion, girolline is a natural compound with very promising activity against malaria both *in vitro* and *in vivo*. Moreover, the specific mode of action of girolline, by inhibiting *Plasmodium* protein synthesis and the high synergistic effect between girolline and chloroquine show that this molecule is of a real interest as research basis for a new class of antimalarials. However its narrow therapeutic ratio could preclude the development of this molecule as such. An important pharmaco-modulation strategy is thus envisaged. Other derivatives, with the preservation of the aminoimidazole heterocyclic ring to maintain activity, are currently being studied with the aim of limiting the toxicity.

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Conclusion et perspectives

L'avenir est dominé par la crainte de l'évolution des chimiorésistances, plusieurs travaux mentionnent les baisses de sensibilités des isolats de *Plasmodium falciparum* vis-à-vis des antipaludiques dans les zones d'endémies. Une étude menée par notre équipe sur les isolats de voyageurs revenus d'Afrique sub-saharienne démontre cette résistance. En effet, de nouveaux principes actifs sont nécessaires pour diversifier l'arsenal thérapeutique antipaludique et traiter les formes sévères de paludisme ainsi que pour lutter contre les parasites poly-chimiorésistants. Il est indéniable que des molécules à mode d'action original peuvent contourner les résistances aux molécules actuelles.

La recherche de nouveaux antipaludiques restent d'actualité car l'une des raisons de l'expansion du paludisme est le manque de traitements efficaces à faible coût. Des nombreuses équipes à travers le monde s'investissent dans cette quête par des stratégies diverses et variées.

Au cours de notre projet, l'ethnopharmacologie des plantes africaines, le fractionnement chimique bio dirigé et le screening des molécules naturelles ayant une activité antitumorale étaient les stratégies d'investigations.

L'utilisation des plantes à visée médicamenteuse est une alternative thérapeutique des populations africaines dans le traitement ou la prophylaxie antipaludique, ceci à cause du coût élevé des médicaments, et parce que l'expérience a montré une efficacité thérapeutique. Par notre collaboration avec l'Institut de Chimie des Substances Naturelles du CNRS, l'activité antipaludique des extraits bruts des plantes sélectionnées a été prouvée, par la suite des extraits actifs des plantes de polarités variables ont été séparés. Le fractionnement chimique des plantes : *Chrozophora senegalensis*, *Phyllanthus niruri*, *Sebastiania chamaela* et *Cognauxia podolaena*, a permis d'identifier des molécules actives (curcubitacine B et D, l'acide ellagique, l'acide gallique etc…). Certaines sont décrites pour la première fois (acide epibryonolique).

Ce travail a permis, d'une part de valider scientifiquement les plantes médicinales sélectionnées au cours des enquêtes menées par des ethnobotanistes en collaboration avec des tradipraticiens en Afrique au Sud du Sahara, et d'autre part, d'identifier les molécules à partir des extraits actifs des plantes retenues en collaboration avec les phytochimistes.

L'acide ellagique a été identifié au cours du fractionnement chimique bio dirigé de plusieurs plantes africaines et peu de travaux ont explorés les propriétés antipaludiques de cette molécule. Les tests d'activités antipaludiques montrent un bon index de sélectivité *in vitro* et un bon index thérapeutique *in vivo* ainsi qu'un effet protecteur chez des modèles murins de paludisme au cours des traitements par voie intra péritonéale. Son activité antioxydante a été aussi démontrée et son activité prooxydante est en cours d'investigation. Néanmoins, l'acide ellagique a démontré une efficacité médiocre au cours des traitements par voie orale des modèles murins de paludisme.

L'acide ellagique semble être une molécule antipaludique prometteuse, par son efficacité et sa faible toxicité cellulaire et murine. D'autres investigations sont en cours, concernant l'amélioration de la biodisponibilité murine au cours des traitements par voie orale. En effet, la facilité d'utilisation et de conservation des médicaments est un élément important pour permettre la dispensation d'un traitement antipaludique en zone d'endémie, c'est à dire dans des pays avec des structures sanitaires généralement précaires.

Notre démarche qui consiste à partir de la plante médicinale pour extraire un principe actif, point de départ dans l'identification de nouvelles molécules a montré la lourdeur et les difficultés inhérentes à cette méthode d'investigation. D'une part, certaines plantes médicinales, largement utilisées dans les localités endémiques du paludisme, validant tous les critères de sélection ethnobotanique, n'ont jamais démontré une efficacité antipaludique sur des modèles expérimentaux de laboratoire. D'autre part, des extraits actifs *in vitro* sur des souches de *Plasmodium* de laboratoire, étaient souvent inactifs sur les modèles murins de paludisme.

D'un point de vue technique, l'évaluation de l'activité antipaludique des extraits de plante s'est faite par micro méthode radioactive, utilisant un radioélément. Cette méthode d'évaluation est très coûteuse et n'est pas pratique pour des équipes de recherche dans les pays du Sud qui ne disposent pas toujours d'une gestion des déchets radioactifs. D'autres méthodologies sont utilisées, pour évaluer l'efficacité antipaludique des molécules, entre autres, l'usage du bromure d'éthidium (intercalant de l'ADN), ou des cibles du parasite tels que l'histidine rich protein 2 (HRP2) et le lactate déshydrogénase du parasite (17).

La détermination de l'activité antipaludique sur des souches de référence de *P. falciparum* pose aussi le problème de la validité des tests *in vitro*. En effet, ces différentes souches utilisées sont adaptées à la culture en laboratoire et pourraient ne plus correspondre à la réalité du terrain. Il serait aussi intéressant d'évaluer l'activité des extraits et molécules actives sur des isolats de patients impaludés en zone d'endémie.

Des études cliniques, pour évaluer l'efficacité des plantes antipaludiques ont été menées par d'autres équipes. C'est le cas en Afrique de l'Ouest de la plante *Cochlospermum planchonii* au Burkina Faso (8) et *Cryptolepis sanguinolenta* au Ghana (70) qui ont montré une efficacité similaire à celle de la chloroquine dans le traitement du paludisme non compliqué. Des molécules ont été identifiées à partir des extraits actifs de *Cryptolepis sanguinolenta* (cryptolepine et autre alcaloïdes) et semblent montré un index thérapeutique médiocre (71). Des questions se posent à cet effet. L'efficacité clinique et parasitologique d'un extrait actif de plante n'est il pas dû à l'interaction de plusieurs molécules ?

Des stratégies alternatives pour optimiser la recherche de nouveaux antipaludiques sont évoquées dans la littérature, comme le choix d'extraits actifs de produits naturels après validation de son efficacité clinique, ou l'exploration de l'effet prophylactique ou immunomodulateur.

Une autre voie d'exploration a concerné les molécules naturelles antitumorales potentiellement antipaludiques. Le screening de ses molécules a permis d'identifier le motif aminoimidazole de la girolline, responsable de l'activité antipaludique. Ce motif ouvre un nouveau champ d'investigation cherchant à améliorer l'index thérapeutique, soit par pharmacomodulation, soit par association de molécules.

Ces deux molécules d'étude (acide ellagique et girolline) agiraient sur la synthèse des protéines parasitaires. Une compréhension plus fine du mécanisme d'action antipaludique des molécules devrait être explorée, mais peu d'outils techniques et biologiques le permettent. La compréhension de la biologie et biochimie du parasite a par ailleurs considérablement évolué ses dix dernières années, avec la découverte de nouvelles cibles potentielles. Le décryptage du génome de plusieurs espèces plasmodiales ouvre ainsi la voie vers de nouveaux outils post génomiques pour enrichir le réservoir de cibles thérapeutiques, et standardiser

l'expérimentation pharmacologique antipaludique, en vue d'uniformiser la recherche de nouvelles molécules.

En conclusion, toute notre démarche expérimentale a permis de valider l'usage de la pharmacopée traditionnelle africaine dans le traitement du paludisme, et d'identifier de nouvelles molécules. La suite consistera à poursuivre l'identification des molécules antipaludiques à partir de produits naturels, et d'explorer de nouvelles cibles sur le parasite, améliorant le screening de nouvelles molécules et la compréhension de leur mécanisme d'action antiparasitaire.

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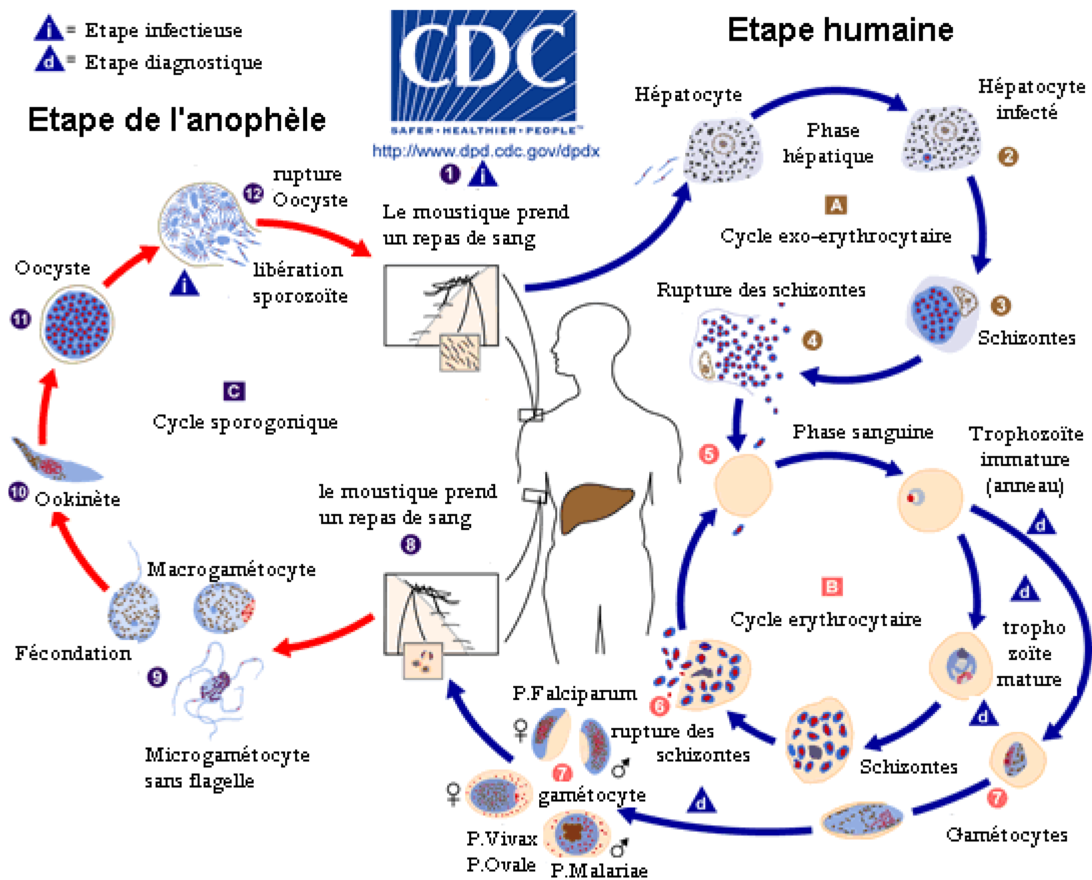
Annexes

Annexe 1 : cycle de *Plasmodium sp.*

D'après le site internet http://www.dpd.cdc.gov



Annexe 2 : Evaluation des niveaux de résistance aux divers antipaludiques en Afrique, entre 2003 et 2007, au laboratoire de Parasitologie du CHU de Toulouse : publication 1

L'article ci-dessous est soumis pour publication dans « Malaria Journal ».

Phenotypic and genotypic evaluation of antimalarial drug sensitivity in *Plasmodium falciparum* isolates from travellers between 2003-2007 in Toulouse University Hospital, France

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Introduction

Malaria is a public health problem that causes thousands of deaths around the world every year. The World Malaria Report for 2008 found an estimated 247 million cases of malaria and 881 000 deaths from the disease, with Sub-Saharan Africa being the worst affected area [1].

Although travellers represent only a small proportion of the global burden of the disease, imported malaria from endemic areas has been rising in Europe [2]. Over the past 20 years, international travel to tropical countries has increased considerably and Sub-Saharan Africa is one of the malaria regions more and more visited [3]. From epidemiological, clinical, and parasitological data collected by a network of 120 laboratories between 1996 and 2003, 21,888 *falciparum* malaria cases were reported among travellers returning to France from areas endemic for the disease. Ninety six deaths have occured, giving a case-fatality rate of 4.4 per 1,000 cases of *falciparum* malaria [4].

Antimalarial drug prophylaxis and treatment have been used in Sub-Saharan Africa for decades. The emergence and the expansion of strains of *P. falciparum* resistant to numerous drugs and the number of antimalarials available have made the choice of chemoprophylaxis and treatment more difficult [5]. Today, for chemoprophylaxis, atovaquone/proguanil, chloroquine/proguanil, doxycycline and mefloquine are used. For uncomplicated and complicated cases of malaria treatment, artemisinin-based combination therapies (ACT) and quinine are most frequently used in Sub-Saharan Africa. Although not recommended in many African countries, chloroquine and sulphadoxine-pyrimethamine are always used in endemic zones.

The recently introduced ACT are highly effective against parasite lines resistant to the other antimalarials. Nevertheless a slight reduction of *in vitro* sensitivity to artemisinins is being observed in areas of mefloquine-resistance [6]. Due to the change in the drug sensitivity of malaria parasites over time, the efficacy of the chemoprophylaxis and treatment regimens

must be closely monitored [7]. The aim of this work was to study the chemosensitivity of *Plasmodium falciparum* isolates from patients with imported malaria after having returned from Sub-Saharan Africa between 2003 and 2007 and hospitalized in the Toulouse University Hospital. The isolates collected were tested with chloroquine, quinine, mefloquine, artemisinin, atovaquone and artesunate in order to compare the degree of variability in parasite sensitivity *in vitro*, and examined for the relationship between *in vitro* sensitivity and molecular markers for resistance (*Pfcrt* T76 and *Pfmdr1* Y86 mutations), and *Pfmdr1* gene copy number.

Material and Methods

1. Isolate choice and clinical follow-up

Clinical isolates

A total of 70 clinical isolates were selected between September 2003 and December 2007 from patients hospitalized in the Toulouse University Hospital (France).

The criteria were:

- parasitemia greater than 0.3%
- returning from Sub-saharan Africa
- known information about visited, origin of the travellers and observance of chemoprophylaxis

A diagnosis of malaria was established in the Parasitology-Mycology Unit of Toulouse on EDTA-anticoagulated venous blood samples of patients by microscopic examination of MGG stained thin blood smears and QBC® (Quantitative Buffy Coat) (Becton-Dickinson, France). The diagnosis was confirmed by real-time PCR [8, 9].

Treatment and follow-up

The decision to hospitalise a patient was taken by physicians according to clinical and biological findings. Uncomplicated malaria was treated with quinine (8 mg quinine base/kg three times a day) administred orally or by intravenous injection in the case of vomiting, for a total of seven days. Patients with complicated or severe malaria (according to 1990 World Health Organisation criteria) were only treated with intravenous quinine (8 mg quinine base/kg three times a day after a loading dose of 16 mg quinine base/kg infused for four hours) for a total of seven days.

2. Phenotypic studies

Determination of *in vitro* sensitivity

Six drugs were tested: chloroquine diphosphate salt (Sigma, France), mefloquine hydrochloride (Hoffman-Roche, France), quinine hydrochloride (Sigma, France), artesunate (Sanofi-Synthelabo, France), artemisinin (Sigma, France) and atovaquone (GlaxoSmithkline, France). A stock solution of chloroquine was prepared in RPMI 1640 medium (Gibco Invitrogen, France), whereas stock solutions for the other five drugs were prepared in DMSO (Dimethyl sulfoxide) (Acros Organics, Belgium).

The suitability of the drugs for *in vitro* testing was monitored every two months, using reference chloroquine-resistant (W2-Indochina, and FcM29-Cameroon) and chloroquine-sensitive (F32-Tanzania) strains maintained in continuous culture and having known responses to the various drugs tested.

The *in vitro* sensitivity of the isolates to chloroquine, artemisinin, artesunate, mefloquine, quinine and atovaquone was determined using the radioactive micro-method [10]. The 50% inhibitory concentration (IC_{50}) using tritiated hypoxanthine uptake was calculated and defined as the concentration at which 50% of the incorporation of tritiated hypoxanthine was inhibited, as compared with the drug-free control wells. Parasitic growth was measured by

using a log probit approximation to determine the IC_{50} values. The threshold IC_{50} for *in vitro* resistance to chloroquine, quinine, and mefloquine were estimated to be 100 nmol/L [11], 800 nmol/L [12], 30 nmol/L [13] respectively, according to the correlation between clinical failures and the *in vitro* resistance. The resistance cut-off for artemisinin was not currently available and. Artesunate cut-off was estimated at 10.5 nmol/L. This *in vitro* threshold value was defined statistically to be greater than 2 standard deviations above the geometric means of IC_{50} obtained with artemether on field isolates from Senegal by Pradines (1998) [14]. This threshold has frequently been used by authors to determine the proportion of reduced sensitivity against artemisinin derivatives. The global median of 96 pooled *P. falciparum* strains and the 90th percentile [15] gave the *in vitro* resistance threshold concentration of atovaquone at 6 nmol/L.

3. Genotype studies

DNA extraction from blood samples

The genomic DNA of parasites was extracted from 200 µl of blood from each isolate with High pure template PCR kit® (Roche, France). Samples were then stored at -80 °C until analysis.

Detection of the *Pfcrt* K76T and *Pfmdr1* N86Y mutations

Mutations were detected in the *Pfcrt* and *Pfmdr1* genes at codon 76 and 86 respectively by real time PCR with hybridation probes.

The principle of the detection of the *Pfcrt* T76 mutation by LightCycler® and details of the assay have already been published [8, 9]. The oligonucleotides used: PlasR iLCS, (5'-AGTATTATTTATTTAAGTGTATGTGT*AAT-3') internally (*) labeled with LCRed 640, antisense primer PlasR (5'-CCTTTTTATTTCCAAATAAGGAAT-3'), and the 3'fluorescent-labeled sensor C (5'-AAGTTCTTTTAGCAAAAATTGTT-3').

For *Pfmdr1*, the detection of mutations has been published [16]. The probes and primer sequences were: forward primer; F86: 5'-TGTATTATCAGGAGGAACATTACC-3', Reverse primer R86: 5'-ACCACCAAACATAAATTAACGGA-3', Fluorescein probe name Sensor86: 5'-ATTAATATCATCATAAATACATG-3', probe name Anchor86: 5'-TCTTTAATATTACACCAAACACAGATAT-3'.

Evaluation of *Pfmdr1* copy number by real-time PCR

The *Pfmdr1* gene copy number was assessed by q-PCR as previously described [6]. The primers that were used were: *Pfmdr1* forward primer:
5'TGCATCTATAAAACGATCAGACAAA-3'; *Pfmdr1* reverse primer:
5'TCGTGTGTTCCATGTGCATGT-3'; *Pfcrt* forward primer:
5'GGAGGTTCTTGTCTTGGTAAATGT-3'; *Pfcrt* reverse primer:
5'TTGAATTTCCCTTTTTATTTCCA-3'. The probes and primers were synthetized by Tib Mobiol (Germany) and Eurogentec (Belgium) respectively
Amplification reactions were carried out in a LightCycler® (Roche, France).
Each reaction was performed in 10 µl with primers, as described below, at a concentration of 0.5 µM for *Pfmdr1* and 0.25 µM for *Pfcrt*. Fast start DNA Sybr green (Roche, France) was used at 1 µl per reaction in 1 µM $MgCl_2$. Each DNA sample was distributed into the reaction mix in a volume of 2.5 µl. The *Pfcrt* gene, present in a single copy per parasite genome, was used as reference gene to analyse the amplification of the target gene *Pfmdr1*. Every run contained triplicates of the same control DNA from FcM29 and Dd2 clones of *P. falciparum* having *Pfmdr1* copy numbers of 1 and 3 respectively [17]. The copy number was then calculated by the $2^{-\Delta\Delta Ct}$ method: $n=2e^{-([Ct\mathit{mdr}-Ct\mathit{crt}]\ \mathrm{isolate})-([Ct\mathit{mdr}-Ct\mathit{crt}]\ \mathrm{FcM29})}$ where, n was the copy number of *Pfmdr1* in the isolate, Ct*mdr*, the detection value in the amplification reaction of *Pfmdr1* and Ct*crt* the detection value in the amplification reaction of *Pfcrt*.

Data analysis

Categorical variables (complicated malaria, *Pfcrt* mutation, *Pfmdr1* mutation) were rated 0 ("no") or 1 ("yes"), and the time period was rated from 1 (2003-2004) to 3 (2007). A bivariate analysis was performed using the $\chi 2$ test for Trend, the Pearson $\chi 2$ test or the Fisher's exact test to compare qualitative data. For continuous parameters (parasitemia, IC_{50}, number of copies), the data distribution was checked by the Skewness and Kurtosis tests and was found to be non-Gaussian. The distributions of these parameters are displayed as medians along with interquartile ranges (med [IQR]). These data were then analysed using the nonparametric Mann–Whitney test between qualitative data and continuous parameters, and the Spearman's rank correlation for the association between the different continuous parameters. Tests were two-sided, and $p<0.05$ was considered significant.

Statistical analyses were performed using Stata™ 9.1 (Intercooled Stata™ 9.1 for Windows, StataCorp, College Station, TX, USA).

Results and discussion

1. Characteristics of cases and clinical follow

The Parasitology and Mycology Department in the Toulouse University Hospital had received 2584 blood samples to diagnosis for malaria between September 2003 and December 2007, from patients coming from endemic areas.

Four hundred and seventy-seven blood samples (18%) gave a positive diagnosis with 83% being *P. falciparum*, 7% *P. ovale*, 5% *P. vivax*, 3% *P. malariae* and 2% polyparasitism (diagnosis data from Parasitology Unit of the Toulouse University Hospital).

In this study, we were interested in 70 of the 477 samples according to our selection. criteria Sixty percent of the samples were for travellers from West African countries, 34% from Central Africa and 6% from East Africa and the Madagascar Islands. 47% of the travellers were of Sub-Saharan African origin and 53% were Caucasian. The patients were between 1 and 68 years old.

Sixty-two (89%) patients had uncomplicated malaria, with 46% of the Caucasian population. Eight (11%) patients had severe malaria with complications such as: acute renal failure (2), seizure (3) and respiratory distress (3). Three patients died. All patients had been living for a long time in France and they were infected during a short stay in an endemic area. Complicated malaria occurred only in the Caucasian population. As shown in Table 1, parasitemia was significantly higher ($p = 0.01$) in the complicated malaria group compared with the uncomplicated group. Parasitemia thus seems really linked with the severity of the malaria [18] The high level of exposure in non-immune patients could explain these results. Acquired immunity may protect African immigrants from severe malaria caused by *P. falciparum* even after several years following non-exposure as reported by Bouchaud [19].

The high median level of parasitemia (3% [1.2-6]) found in the uncomplicated malaria group is explained by our selection criteria (parasitemia > 0.3%).

2. *In vitro* sensitivity

Previous studies in our laboratory showed that *in vitro* cultures of isolates were positive if parasitemia was at least 0.3% and cultures carried out within 48 hours after taking the blood sample (data not shown). The results of the microscopic examinations showed that the parasite loads of the samples included in our study were between 0.3 and 33%. The number of interpretable *in vitro* assays for each drug is presented in Table 2. Identifiable causes of culture failure *in vitro* principally included a recent self-medication with antimalarial drugs or antibiotics, the storage of blood samples and the time period between sampling and the *in vitro* assays.

The *in vitro* sensitivity of isolates against chloroquine, mefloquine, quinine, atovaquone, artemisinin and artesunate was stable (data not shown) between 2003-2004, 2005-2006 and 2007.

3. *Pfcrt* K76T and *Pfmdr1* N86Y mutations

From the 64 samples studied, mutant alleles of the *Pfcrt* gene at position 76 were found in 62.5% and for the *Pfmdr1* gene, mutant-type Y86 alleles were found in 42%. These results are in agreement with those of Durand [20]. The combination of intergenic *P. falciparum* alleles in isolates is shown in Table 3. *P. falciparum* isolates harbouring the wild-type K76 allele and mutant-type Y86 allele were a significant (p= 0.01) but small proportion of the combinations (6%) compared with the other three possible combinations: K76/N86, T76/N86 and T76/Y86 with proportions of 31%, 27% and 36% respectively. All the K76/N86 isolates were significantly more sensitive to chloroquine than T76/N86 and T76/Y86 (Figure 1). This

higher chloroquine sensitivity was also found by Babiker [21]. These parasites could have a poor fitness. None of the Y86 allele samples was from the complicated cases (Table 1).

No significant difference was observed in the evolution of the distribution over the years for both mutant alleles of *Pfcrt* and *Pfmdr1* genes (data not shown). As in the case of the phenotypic tests (IC_{50}), there was no evolution in the genotype assays.

4. Correlation between *in vitro* sensitivity to each molecule and genotypic profiles

During 2003-2007, from 70 assays with chloroquine, 50 were successful. Based on the threshold value of 100 nM, 21 (42%) isolates were chloroquine-resistant, with a median IC_{50} of 241 nM [120-570]. This confirmed the high level of chloroquine-resistance (CQR) in Africa [22].

The IC_{50} values were compared for both haplotypes of the *Pfcrt* gene. The IC_{50} for chloroquine were significantly lower in the K76 population (median IC_{50}=27.02 nM [7.68-35.5] versus 134 [76-480] in the T76 population, $p<0.001$).

In the case of polymorphism for both the *Pfcrt* and *Pfmdr1* genes, as shown in Figure 1, the IC_{50} were significantly lower in the K76/N86 population compared with the T76/N86 and T76/Y86 populations ($p<0.005$ for both populations).

This result confirmed the high implication of *Pfcrt* T76 mutation in the resistance against chloroquine. Poor sampling in our study could explain the lack of effect of the *Pfmdr1* Y86 mutation and thus the absence of a significant difference between IC_{50} values for T76/N86 and T76/Y86.

All these results obtained with chloroquine were consistent with many previous studies in this area, where the majority of countries have been classified in zone 3 of chloroquine resistance [23], [24]. The presence of the wild-type K76 allele in one *in vitro* resistant isolate (IC_{50}= 113

nM) was observed but a similar result was reported by a previous study [25] and would need further confirmation.

In Africa and France, quinine remains the treatment of choice in the management of severe malaria, whereas mefloquine is only used for chemoprophylaxis.

During 2004-2007, from 54 *in vitro* assays with quinine, 30 were successful. Based on the threshold value of 800 nM, 5 (16%) isolates had reduced *in vitro* quinine-sensitivity, with a median IC_{50} of 2015 nM [1529-2519]. Reduced *in vitro* quinine-sensitivity in isolates in our study came from different endemic zones in Sub-Saharan Africa (data not shown). This 800 nM threshold originated from clinical outcomes and the *in vitro* sensitivity of Cambodian isolates to quinine [26]. A high proportion of *P. falciparum* isolates with reduced *in vitro* sensitivity to quinine has been reported in Libreville (Gabon) (up to 32%) with the same threshold as us [27]. Whereas Henry [28] found reduced *in vitro* quinine-sensitivity to *P. falciparum* in 6% of isolates from Senegal with this same threshold.

However, in our study, quinine treatment was 100% effective in the parasitological monitoring of patients carrying uncomplicated or complicated *falciparum* malaria. This justifies its use for the treatment of malaria in Africa and in most European countries. This result showed an absence of a correlation between *in vitro* resistance threshold (800 nM) of quinine and the clinical outcome in patients (data not shown). It seems interesting to reconsider the *in vitro* threshold concerning African isolates of *P. falciparum*.

During 2004-2007, from 51 assays with mefloquine, 32 were successful. Based on the threshold value of 30 nM, 18 (56%) isolates had reduced *in vitro* sensitivity to mefloquine, with a median IC_{50} of 58 nM [43-84]. Whereas a small proportion (17%) of isolates showed reduced *in vitro* sensitivity against mefloquine in Africa as shown by Henry [28], Ndong *et al.*

reported 47.5% reduced *in vitro* mefloquine-sensitivity in Gabon [29]. In West Africa, the failure of antimalarial chemoprophylaxis with mefloquine in travellers, associated with *in vitro* resistance, has been widely observed [30].

From 32 isolates studied with mefloquine *in vitro* assays, 27 were genotyped. For polymorphism of the *Pfmdr1* gene, as shown in Figure 2, the IC_{50} values were significantly lower in the Y86 population than in the N86 population ($p = 0.016$). The same difference was demonstrated in the Madagascar Islands [31] where the *Pfmdr1* N86Y mutation was associated with high sensitivity against mefloquine, and not with other aryl-amino-alcohols. Moreover, the double wild-type K76/N86 showed significantly higher IC_{50} values than double mutated-type T76Y86 (Figure 3).

The activity of atovaquone against *P. falciparum* was demonstrated in the 1990s [32]. The combination of atovaquone and proguanil hydrochloride (Malarone®) is used either chemoprophylactly or as a curative treatment for travellers. The atovaquone-proguanil combination has shown failures in treatment and prophylaxis against *P. falciparum* infections [33]. During 2004-2007, 30 assays with atovaquone were performed. Based on the threshold value of 6 nM [14], 18 (60%) isolates had reduced *in vitro* atovaquone-sensitivity, with a median IC_{50} of 49 nM [16-1000] (Table 2). Henry found in Senegal that 32% of isolates showed atovaquone resistance [28]. This threshold is much debated. Indeed, a recent study by Musset [34] reported a very high atovaquone IC_{50} (8230 nM) associated with the Y268S mutation in atovaquone/proguanil-resistant parasites and recommended the use of two new atovaquone thresholds to discriminate isolates from travellers: 0-30 nM, sensitive; >1900 nM, resistant. With this new threshold, only 17% of isolates would have reduced sensitivity *in vitro* in our study.

The emergence and spread of malaria resistance to chloroquine in Africa and resistance to other antimalarial drugs caused the WHO to introduce artemisinin-based combination therapy (ACT) [35]. During 2003-2007, 49 *in vitro* assays were successfully performed. Currently no cut-off value is established to evaluate artemisinin resistance. The global median of artemisinin IC_{50} obtained here is close to most of the values obtained with laboratory strains or field isolates [7, 36]. The *in vitro* decrease of susceptibility to artemisinin is globally absent in Africa. Nevertheless, some rare "out of range" samples (IC_{50} above 15-30 nM) found in our work lead us to estimate that clones less sensitive to artemisinin already exist. Moreover, Jambou [37] showed that some *P. falciparum* isolates from Senegal have a reduced *in vitro* sensitivity to artemether associated with the S769N *PfATP6* mutation but no studies have shown clinical failure with artemisinin and its derivatives in Africa.

Additionally, it has been shown that inoculum size might cause variations in the *in vitro* assay. This error may be responsible for some of the higher IC_{50} values found here for high parasitemia samples (above 10%).

Some authors have previously published an inverse correlation between CQR and increase in theartemisinin IC_{50}. Here the same artemisinin IC_{50} median was found in the CQR and chloroquine-sensitive (CQS) groups (13.3 versus 13.35 nM). After removing artemisinin IC_{50} values obtained with high parasitemia (>10%), a significative difference can be observed with 13.3 nM for the CQS group and 7.1 nM for the CQR. These results show that isolates with high parasitemia need to be carefully considered.

In parallel, the *in vitro* activity of artesunate was assessed in 43 isolates between 2005 and 2007. Interpretable results were obtained for 21 (48%) isolates. Only 1 isolate showed reduced *in vitro* artesunate-sensitivity, with a very high IC_{50} value of 781 nM. This out of norm value would need to be confirmed by supplementary investigations. Unfortunately

chemosensitivity assays on *P. falciparum* isolates are practicable only once. The question always remains about the choice of the *in vitro* threshold concentration.

No significant differences were found between the polymorphisms of *Pfcrt* and *Pfmdr1* and the *in vitro* sensitivity to atovaquone, quinine, artemisinin and artesunate (data not shown). However, a high CQR (and thus high level of the 76T PfCRT mutation) is correlated with increase of sensitivity to others drugs except quinine [7]

The correlation between reduced sensitivity to mefloquine and amplification of the *Pfmdr1* gene has already been reported in field isolates from Thailand and other Asian countries [38]. In our study the *Pfmdr1* copy number (range: 0.36 to 4.9) was obtained with all isolates with positive *in vitro* assays. The increased *Pfmdr1* copy number was significatively associated with reduced susceptibility *in vitro* against mefloquine (Spearman's rho = 0.67, p = 0.02). Nevertheless, this is not always the case [39]: reduced mefloquine sensitivity *in vitro* (IC_{50} > 30 nM) was obtained with six isolates, without any increase in *Pfmdr1* copy number. By contrast the *Pfmdr1* copy number for 3/18 isolates increased with high *in vitro* sensitivity against mefloquine. No significant correlation was obtained between the *Pfmdr1* copy number and *in vitro* sensitivity against the other anti-malarial molecules. Other known resistance mutations in codons 184, 1034, 1042 and 1246 in the *Pfmdr1* gene and other genes should be investigated in African isolates, to better understand the resistance mechanisms [40].

In our study, even if high levels of reduced sensitivity were found in our isolates (42% for chloroquine, 16% for quinine, 56% for mefloquine and 60% for atovaquone), there was no evolution of the IC_{50} for the 6 molecules tested and no changes in the distribution of *pfcrt* and *pfmdr1* polymorphism between 2003 and 2007.

Moreover, reduced *in vitro* sensitivity is not synonymous with clinical failures, but it mitigates increased vigilance and also enables any important trends in the sensitivity of *P.*

falciparum isolates against current antimalarial drugs to be followed. *In vitro* assays, *Pfcrt* and *Pfmdr1* polymorphisms and *Pfmdr1* amplification could be used as markers to the follow-up of the level of resistance of *P. falciparum* isolates. Although we observed reduced sensitivity to quinine, mefloquine, chloroquine and atovaquone, the choice of resistance threshold seems to be crucially important depending on the endemic zones and the immune status of the patients. This study pleads in favour of supervision in endemic zones of Africa (*in vivo* assays and other known molecular markers), that is essential for making meaningful decisions in formulating any changes in national drug policies concerning therapeutic and chemoprophylactic treatments.

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Table 1: Parasitemia and genotypic profile of isolates from uncomplicated and complicated *falciparum* malaria

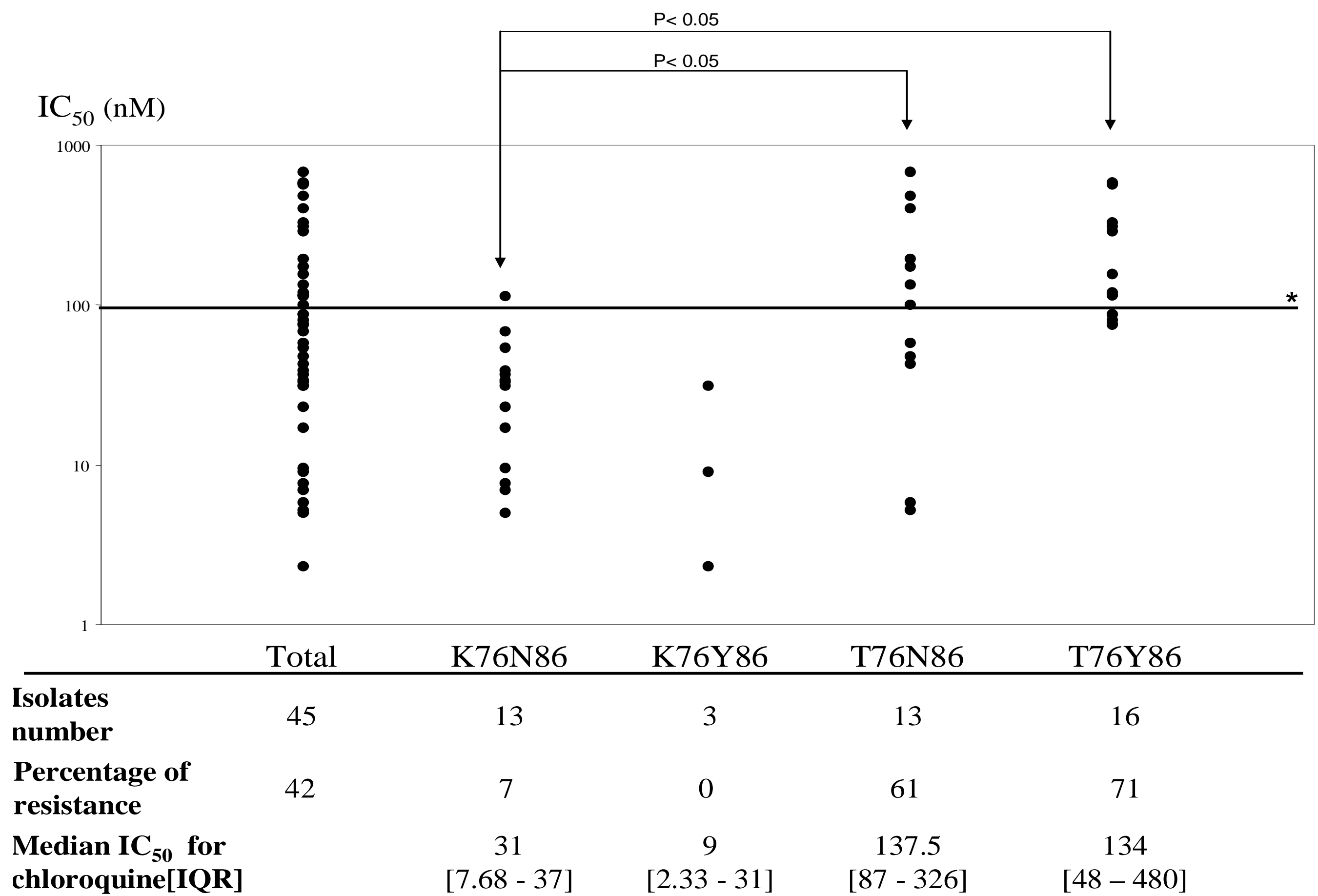
	Uncomplicated malaria	**Complicated malaria**
Number of clinical cases	62	8
Parasitemia (%): median [IQR]	3 [1.2-6]	17.5 [11.5-26]
% of *Pfcrt* T76 genotype	65.5	37.5
% of *Pfmdr1* Y86 genotype	50.9	0

Table 2: Trends of *in vitro* response of *P. falciparum* isolates between 2003 and 2007 and reference strains (3D7, FcM29-Cameroon and W2-Indochina)

	Chloroquine	Quinine	Mefloquine	Atovaquone	Artemisinin	Artesunate
Number of positive cultures by isolate (%)	50 (71)	30 (55)	32 (62)	30 (57)	49 (83)	21 (48)
In vitro resistance threshold (nM)	100	800	30	6	nd	10.5
Global IC_{50} (nM): median [IQR]	76 [31-174]	164 [60-105]	31 [5-647]	11 [2.3-162]	6 [2.3-11]	2 [0.6-6.3]
Min IC_{50}- Max IC_{50}	2.33-680	22-2600	0.02-1085	0.16-2732	0.30-177	0.02-781
IC_{50} of sensitive isolates : median [IQR]	33 [9-54]	150 [52-226]	10.5 [5-13.2]	2 [1-3]	-	2 [0.5-6]
Number of reduced sensitivity isolates (%)	21 (42)	5 (16)	18 (56)	18 (60)	-	1 (4)
IC_{50} of reduced sensitivity isolates : median [IQR]	241 [120-570]	2015 [1529-2519]	58 [43-84]	49 [16-1000]	-	781 [781-781]
median IC_{50} (nM) of 3D7	8	305	39	1.4	10.5	1.2
median IC_{50} (nM) of FcM29	688	429	15.6	0.4	12.5	3.1
median IC_{50} (nM) of W2	528	480	18	0.7	13.3	3.0

Table 3: Association between alleles of *Pfcrt* and *Pfmdr1* among 70 *P. falciparum* isolates

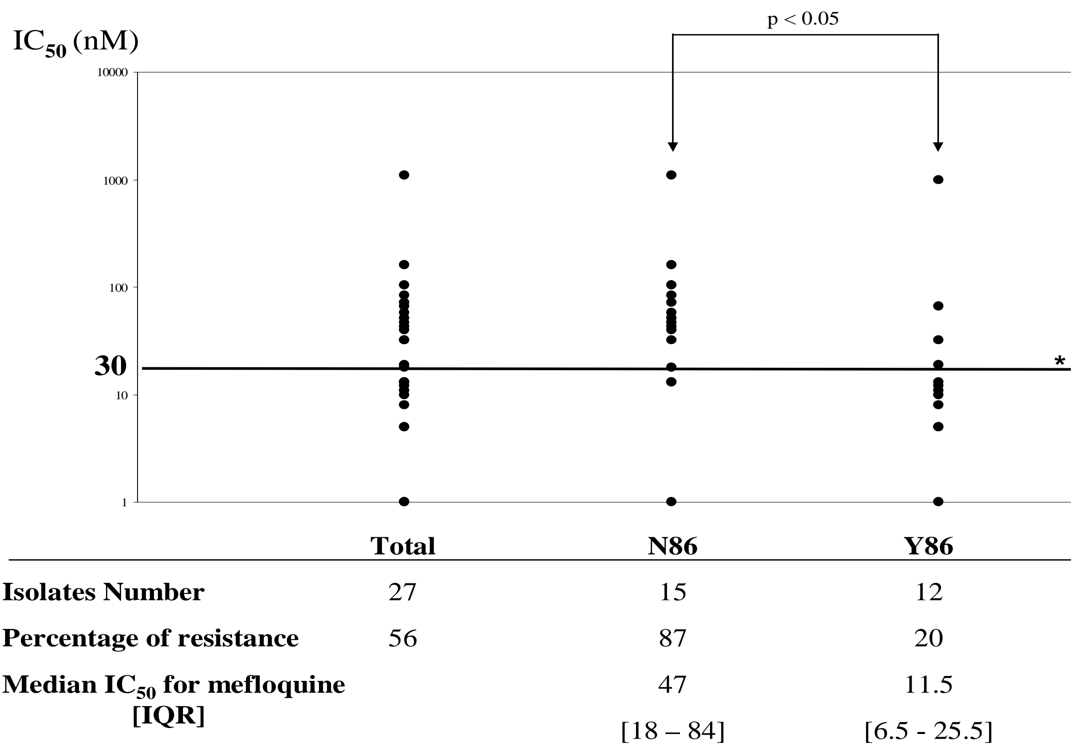
Parasite genotype, *Pfcrt* **and** *Pfmdr1*	**Percentage of isolates**
K76/N86	31% (double wild-type haplotype)
T76/N86	27%
K76/Y86	6%
T76/Y86	36% (double mutant haplotype)



	Total	K76N86	K76Y86	T76N86	T76Y86
Isolates number	45	13	3	13	16
Percentage of resistance	42	7	0	61	71
Median IC_{50} for chloroquine[IQR]		31 [7.68 - 37]	9 [2.33 - 31]	137.5 [87 - 326]	134 [48 – 480]

Figure 1: Relation between *Pfcrt* and *Pfmdr1* polymorphism and *in vitro* chloroquine susceptibility.

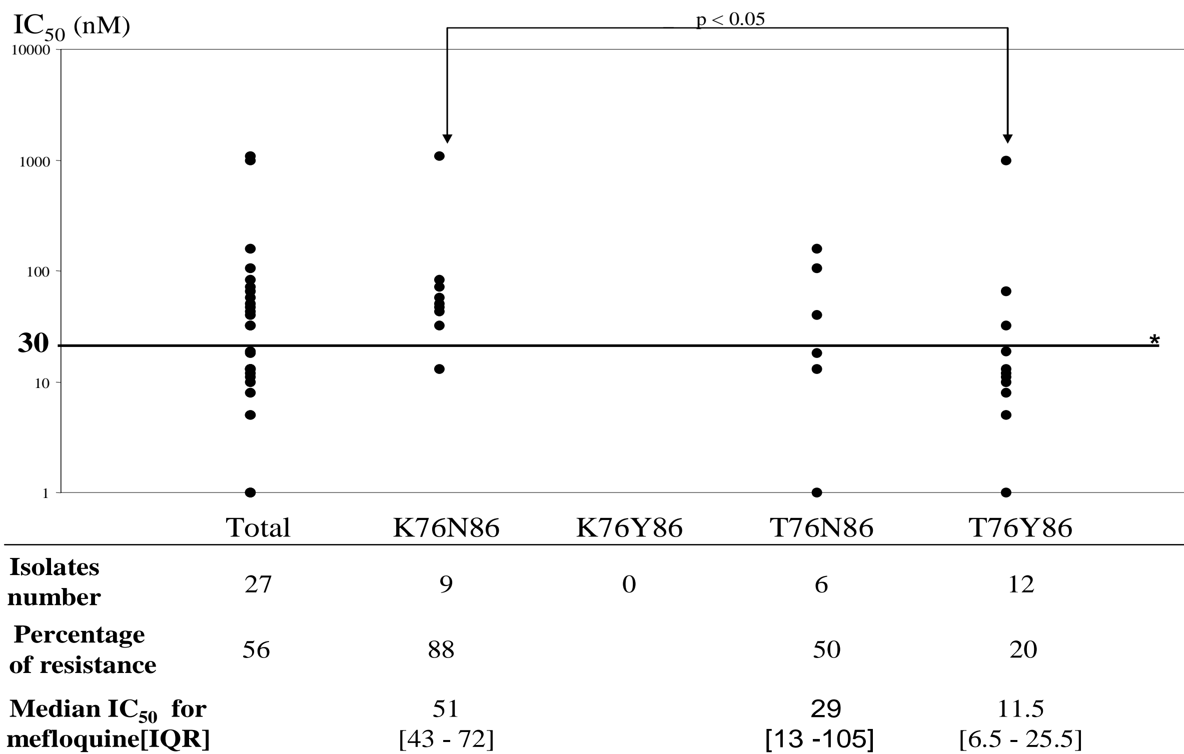
*** = resistance threshold**



	Total	N86	Y86
Isolates Number	27	15	12
Percentage of resistance	56	87	20
Median IC_{50} for mefloquine [IQR]		47 [18 – 84]	11.5 [6.5 - 25.5]

Figure 2: Relation between *Pfmdr1* polymorphism and *in vitro* mefloquine sensitivity.

*** = resistance threshold**



	Total	K76N86	K76Y86	T76N86	T76Y86
Isolates number	27	9	0	6	12
Percentage of resistance	56	88		50	20
Median IC_{50} for mefloquine[IQR]		51 [43 - 72]		29 [13 -105]	11.5 [6.5 - 25.5]

Figure 3: Relation between *Pfcrt* and *Pfmdr1* polymorphism and *in vitro* mefloquine sensitivity.

*** = resistance threshold.**

Annexe 3 : agréments pour l'expérimentation animale

Abstract

Resistance against wide range of antimalarial is a public health problem in endemic zones of malaria.

The search of new antimalarial molecules is crucial. Many strategies are explored today and one interesting approach is to study natural products, particular those that have been used in traditional medecine.

Ethnopharmacology in Sub-Saharan Africa, bioguided fractionation and screening of marine molecules were our strategie in the research of new antimalarial molecules.

Our project consisted of exploring the antimalarial properties of plant extracts and molecules, beginning by *in vivo* and *in vitro* antimalarial activity and toxicity, the parasitic stage of molecules or extract activity and the possible potentiation of molecules with current antimalarial drugs.

The results showed interesting antimalarial activities of extracts and molecules from *Chrozophora senegalensis* (Senegal), *Phyllanthus niruri* (Democratic republic of Congo), *Cognauxia podolaena* (Congo-Brazzaville) and *Sebastiania chamaelea* (Niger).

The molecules indentified by bioguided fractionation were explored.

Girolline showed interesting antimalarial activity *in vitro* and *in vivo* with poor selectivity and therapeutic indexes. Moreover, ellagic acid showed interesting antimalarial activity without toxicity. More experiments are needs to understand his antimalarial mechanisms.

For girolline, an important pharmacomodulation is envisaged with the preservation of the aminoimidazole ring, to maintained antimalarial activity and reduce toxicity.

Résumé

Les résistances de plus en plus nombreuses aux antipaludiques constituent un problème majeur de santé publique. Les besoins en nouvelles alternatives thérapeutiques sont cruciaux. Bien que diverses stratégies soient explorées de nos jours, le screening de nouvelles molécules isolées de produits naturels ou issues de la médecine traditionnelle est de mise.

Notre travail a consisté à la recherche de nouveaux antipaludiques issus de la pharmacopée africaine par la méthode ethnopharmacologique, le fractionnement chimique bio-dirigé et le screening des molécules d'origines naturelles ayant une activité antitumorale.

Les tests de laboratoire ont consisté à l'étude des activités *in vitro* sur souches de *Plasmodium falciparum* de laboratoire et *in vivo* sur modèles murins de paludisme. En parallèle, pour les molécules et extraits actifs présentant un potentiel antipaludique prometteur, leur toxicité a été évaluée *in vitro* et *in vivo*.

Les résultats obtenus nous ont permis de valider l'usage de certaines plantes dans le traitement du paludisme. *Chrozophora senegalensis* (Sénégal), *Phyllanthus niruri* (République Démocratique du Congo), *Cognauxia podoloena* (Congo-Brazzaville) et *Sebastiania chamaelae* (Niger) se sont avérés présenter une réelle activité antipaludique.

Le fractionnement chimique bio-dirigé a permis d'identifier des molécules actives. Deux molécules ont fait l'objet d'explorations plus poussées. Il s'agit de l'acide ellagique et de la girolline. La girolline a montré une activité élevée sur *Plasmodium* mais des index de sélectivité et thérapeutique médiocres. Par ailleurs, l'acide ellagique a montré une importante activité antipaludique *in vitro*, une synergie avec la chloroquine, la méfloquine, l'atovaquone et l'artésunate, et une efficacité *in vivo* sur modèles murins de paludisme après traitement par voie intraperitonéale. Son activité antioxydante a été prouvée.

Des expérimentations sont en cours pour réduire la toxicité de la girolline par pharmaco modulation et comprendre le mécanisme d'action antiparasitaire de l'acide ellagique.