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LOW ANTIPLASMODIAL ACTIVITY OF ALKALOIDS AND AMIDES FROM THE STEM BARK OF *ZANTHOXYLUM RUBESCENS* (RUTACEAE)

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Summary:

The stem bark of *Zanthoxylum rubescens* (syn. *Fagara rubescens*) is used for treating fevers associated with malaria in the Ivory Coast. Three alkaloids: N-nornitidine, 7,9-dimethoxy-2,3-methylenedioxybenzophenanthridine, and bis[6-(5,6-dihydrochelerythrinyl)] ether; and two amides: zanthomamide and lemairamide, were isolated from the stem bark of this plant. These compounds were screened *in vitro* against the chloroquine-sensitive 3D7 strain and the chloroquine-resistant FCM29 strain of *P. falciparum*. N-nornitidine was found to be inactive. 7,9-dimethoxy-2,3-methylenedioxybenzophenanthridine, lemairamide and zanthomamide showed weak activity with average IC_{50} values ranging from 45.6 µM to 149.9 µM. Bis[6-(5,6-dihydrochelerythrinyl)] ether was the most active of the tested compounds with mean IC_{50s} of 14.9 ± 1.4 µM in FCM29 strain and 15.3 ± 3.4 µM in 3D7 strain (~ 58 to ~ 1130 times less active than chloroquine respectively). The anti-*Plasmodium* activities of the tested alkaloids of *Z. rubescens* were low; and do not encourage the use of this plant as antimalarial.

KEY WORDS : *Zanthoxylum rubescens*, Rutaceae, alkaloids, amides, bis[6-(5,6-dihydrochelerythrinyl)] ether, malaria.

Résumé : FAIBLE ACTIVITÉ ANTIPLASMODIALE DES ALCALOÏDES ET AMIDES ISOLÉS DE L'ÉCORCE DE TIGE DE *ZANTHOXYLUM RUBESCENS* (RUTACEAE)

Zanthoxylum rubescens *(syn.* Fagara rubescens*) est utilisée en médecine traditionnelle pour traiter la fièvre associée au paludisme en Côte d'Ivoire. Trois alcaloïdes (N-nornitidine, 7,9-dimethoxy-2,3-methylenedioxybenzophenanthridine et bis[6-(5,6-dihydrochelerythrinyl)] ether), et deux amides (zanthomamide et lemairamide) isolés de l'écore de tige de cette plante ont été testés* in vitro *pour évaluer leurs activités sur deux souches de* Plasmodium falciparum. *Les résultats obtenus ont montré que N-nornitidine est inactive et que l'activité antiplasmodiale de 7,9-dimethoxy-2,3-methylenedioxybenzophenanthridine, lemairamide et zanthomamide, est faible avec des CI50 de 45,6 µM à 149,9 µM. Bis[6-(5,6-dihydrochelerythrinyl)] ether a été la plus active parmi les cinq molécules testées, cependant avec des moyennes de CI50 à 14,9 ± 1,4 µM contre la souche* P. falciparum *FCM29 (résistante à la chloroquine) et de 15,3 ± 3,4 µM contre la souche* P. falciparum *3D7 (sensible à la chloroquine). Bis[6-(5,6-dihydrochelerythrinyl)] ether est ainsi de 58 à 1130 fois moins actif que la chloroquine. Cette étude montre que l'activité anti-*Plasmodium *de ces alcaloïdes et amides de* Z. rubescens *ne justifie pas son utilisation de cette plante comme antipaludique.*

MOTS CLÉS : Zanthoxylum rubescens, *Rutaceae, alcaloïdes, amides, bis[6-(5,6-dihydrochelerythrinyl)] ether, paludisme.*

Malaria, known as *djèkoidjo* or *ahankan* according to Baoulé and Agnis dialects, remains a major public health problem in the Ivory Coast. Our ethnobotanical survey in the southern part of the country (Aboisso region) revealed that the decoction from the stem bark of *Zanthoxylum rubescens* – an Ivorian indigenous plant – is used to treat and to prevent *djèkoidjo* (mainly fever). Also, this plant is used as toothache remedy (Fish *et al.*, 1974), or to prevent abortion in pregnant women (Penali, personal communication). Since bitter compounds such as alkaloids have shown antiplasmodial activity (Addae-Kyereme *et al.*, 2001; Andrade-Neto *et al.*, 2003; Kassim *et al.*, 2005; Randrianarivelojosia *et al.*, 2003; Sener *et al.*, 2003), we isolated and assessed the *in vitro* antiplasmodial properties of alkaloids and amides from, *Z. rubescens*. The purpose of the investigation was to identify the schizonticidal effects of these compounds against *P. falciparum*.

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MATERIALS AND METHODS

PLANT MATERIAL

The stem bark of *Zanthoxylum rubescens* Planch. Ex. Hook f. (syn. *Fagara rubescens* Engl.) (Rutaceae) was collected from the Lamto forest in the

central part of Ivory Coast at the beginning of the rainy season in May 2004. Plant identification was confirmed by Professor Aké Assi by comparison with authentic specimens at the "Centre National de Floristique" of the University of Cocody, Abidjan, Ivory Coast where a voucher specimen was retained (TK023/04).

Chemical analysis

Air dried, milled stem bark of *Zanthoxylum rubescens* (562.6 g) was extracted successively with hexane, dichloromethane, ethyl acetate and methanol in a Soxhlet apparatus for 24 hours with each solvent. Isolation and purification of compounds from the crude extracts were performed using gravity column chromatography over silica gel (Merck Art. 9385). Thin layer chromatographic analysis was carried out on 0.2 mm silicagel, aluminium-backed plates (Merck Art. 5554) and spots were visualized under UV light and then by spraying with anisaldehyde spray reagent followed by heating. The hexane extract was separated using a hexane/ethyl acetate solvent mixture (85/15); the dichloromethane extract was separated with a dichloromethane/methanol (99/1) solvent mixture while the methanol extract was separated using a dichloromethane/methanol mixture (97/3). Low resolution mass spectra were obtained on Agilent GC MS 5975 instrument and ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 400 MHz NMR spectrometer. Optical rotations were measured at room temperature in chloroform using a Perkin-Elmer 241 polarimeter with a 10 cm flow tube. IR spectra were recorded with a Nicolet Impact 400 D spectrometer on sodium chloride plates and calibrated against an air background. UV spectra were obtained on a Varian DMS 300 UV-visible spectrometer.

In vitro antimalarial testing

Test compounds were dissolved in methanol/water 50:50 in the presence of citric acid to make up a stock solution of 6.4 mg/ml. Serial dilutions were made in distilled water to obtain needed solutions. Test compounds were applied to culture plates which were dried under the hood before testing. The final dilution contained less than 16 µg/ml of citric acid, which had no measurable effect on parasite survival in this system. Samples were tested in 96-well plates in triplicate at final concentrations of 64, 16, 8, 4, 2, and 0.5 µg/mL. Tests were performed three times for each compound. Chloroquine diphosphate (Sigma Chemical, St Louis, MO, USA) was used as a control.

3D7 (chloroquine-sensitive) and FCM29 (chloroquine-resistant) strains of *Plasmodium falciparum* were cultured in a gas mixture containing 5 % CO_2, 5 % O_2, and 90 % N_2 at 37° C according to the Trager and Jensen method (Andrianantenaina *et al.*, 2002; Trager & Jensen 1976). The culture medium RPMI 1640 (Gibco-BRL Laboratories, Grand Island, USA), supplemented with 10 % heat-inactivated human type AB^+ serum (Abcys, Chausson, Paris), 25 mM $NaHCO_3$, 2 mM glutamine and 25 HEPES (Sigma, St. Louis, MO, USA) was used. Parasites cultures were maintained in type O^+ human red blood cell suspensions obtained from healthy local donors (Centre of Blood Transfusion, Military Hospital, Antananarivo, Madagascar).

The *in vitro* antiplasmodial tests were performed using the isotopic method (Desjardins *et al.*, 1979; Randrianarivelojosia *et al.*, 2002). The cultured malaria parasites were synchronized by use of D-alanine (Braun-Breton *et al.*, 1988). Parasitemia was adjusted at 0.5 % by adding non-infected type O^+ non-infected red blood cells. *P. falciparum*-containing red blood cells were suspended in the complete RPMI 1640 medium with 10 % (v/v) AB^+ human serum at 1.5 % hematocrit. *In vitro* testing was performed in 96-well plates (200 µl parasite suspension per well). Parasite growth was assessed by adding tritium labelled hypoxanthine at 1 µCi per well (Amersham Bioscience, Saclay, France) to the culture medium. Plates were incubated at 37° C for 42 hours in a humidified modulator incubator chamber MIC-101 (Billups-Rothenberg, Del Mar, California, USA) flushed with a gas mixture containing 5 % CO_2, 5 % O_2, and 90 % N_2. Afterward, plates were frozen and then defrosted, and each well was harvested onto fiberglass paper (Wallac®, Turkey, Finland). The incorporated tritium labelled hypoxanthine was determined using a beta counter (Wallac 1450, Turkey, Finland). Growth curves were obtained, and the 50 % inhibitory concentration (IC_{50}) values were calculated by use of a log-probit approximation (Randrianarivelojosia *et al.*, 2002). The IC_{50}s were converted from µg/ml into µM. The Students' t test was used for statistical comparison between mean IC_{50}s. The difference was considered significant for *P* values under 0.05. To target promising antiplasmodial compounds, we considered plant extracts with IC50 values > 64 µg/ml as "inactive".

RESULTS AND DISCUSSION

Five known compounds were isolated from the stem bark of *Zanthoxylum rubescens*. The structures were determined using NMR spectroscopy and MS and confirmed by comparison against literature data as 7,9-dimethoxy-2,3-methylene dioxybenzophenanthridine (Sukari *et al.*, 1999) (3.4 mg), N-nornitidine (Martin *et al.*, 2005) (6.8 mg) isolated from the hexane extract; zanthomamide (88.2 mg), lemairamide (5.0 mg) (Adesina & Reisch 1989; Adesina *et al.*, 1997; Simeray *et al.*, 1985) isolated from the dichloromethane extract; bis[6-(5,6-dihydrochelerythrinyl] ether (Dostal *et al.*, 1995) (6.6 mg) isolated from the methanol extract.

Compounds from *Z. rubensens*	Mean IC_{50} in µM	
	on 3D7	on FCM29
Bis[6-(5,6-dihydrochelerythrinyl)] ether	15.3 ± 3.4	14.9 ± 1.4
7,9-Dimethoxy-2,3-methylenedioxybenzophenanthridine	72.2 ± 13.5	92.4 ± 41.9
Lemairamide	89.7 ± 22.7	101.1 ± 18.7
Zanthomamide	133.8 ± 98.6	149.9 ± 59.5
N-nornitidine	inactive	inactive
Chloroquine diphosphate (control)	0.014 ± 0.008	0.256 ± 0.037

Table I. – *In vitro* response of the chloroquine-sensitive 3D7 and chloroquine-resistant FCM29 strains of *Plasmodium falciparum* to *Z. rubescens* compounds.

The IC_{50} values obtained from testing the various compounds against *P. falciparum* are summarized in Table I. N-nornitidine was found to be inactive; 7,9-dimethoxy-2,3-methylenedioxybenzophenanthridine, and the amides lemairamide and zanthomamide showed mild antiplasmodial activity. Bis[6-(5,6-dihydrochelerythrinyl)] ether (Fig. 1) was found to be the most active of the alkaloids tested against *P. falciparum in vitro*. The IC_{50} value for bis[6-(5,6-dihydrochelerythrinyl)] ether on the chloroquine-resistant strain FCM29 (mean IC_{50} = 14.9 ± 1.4 µM) was not significantly different from the IC_{50} value against the chloroquine-sensitive strain 3D7 (mean IC_{50} = 15.3 ± 3.4 µM). Bis[6-(5,6-dihydrochelerythrinyl)] ether was ~ 58 to ~ 1,130 times less active than chloroquine respectively against FCM29 and against 3D7 strains. In comparison with the chloroquine activity against *P. falciparum* in our study and in comparison with the activity of the benzophenanthridine alkaloid fagaronine from *Fagara zanthoxyloides* against *P. falciparum* with a mean IC_{50} at 18 ng/ml as reported (Kassim *et al.*, 2005), the anti-*Plasmodium* activities of the tested alkaloids of *Z. rubescens* were moderate. Nevertheless, both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* were similarly affected by the *Z. rubescens* alkaloid bis[6-(5,6-dihydrochelerythrinyl)] ether.

Although the mechanism of action of bis[6-(5,6-dihydrochelerythrinyl)] ether is unknown, we suggest that it does not act via the chloroquine pathway. Bis[6-(5,6-dihydrochelerythrinyl)] ether should be investigated further to discover compounds that act against chloroquine-resistant parasites. Traditional medicine practices are based on the treatment of clinical symptoms. Antimalarial plants such as *Zanthoxylum* species are principally used to treat fever although fever may be caused by various infectious pathogens including malaria parasites, viruses or bacteria.

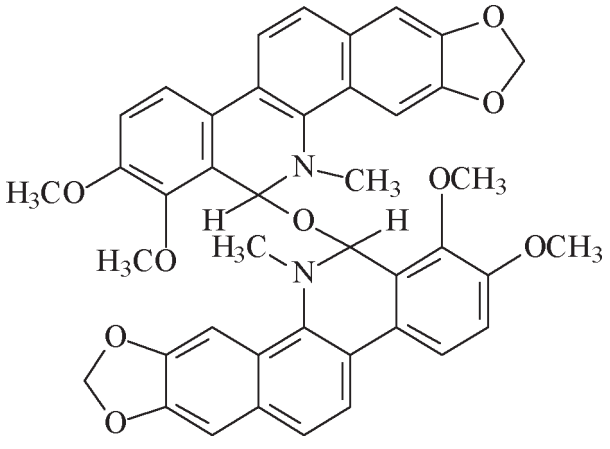

Fig. 1. – Chemical structure of bis[6-(5,6-dihydrochelerythrinyl)] ether.

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