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Antiplasmodial Activity and Cytotoxicity of Semi Purified Fractions from *Zanthoxylum zanthoxyloïdes* Lam. Bark of Trunk

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Abstract: The aim of the study is to investigate through traditional medicinal plants the possibility for discovery and development of new active and safe antimalarial drugs. For ecological reasons, bark of trunk of *Zanthoxylum zanthoxyloides* instead to roots was used by traditional healers in Burkina Faso to treat malaria or fever and recent study showed that crude alkaloid extract from the bark of trunk displayed good antiplasmodial activity. The bio-guided chromatographic fractionation of this crude alkaloid extract with solvents yielded 11 semi purified fractions which were tested for their antiplasmodial activity and cytotoxicity, respectively against *Plasmodium falciparum* W_2 strains and K562S cells maintained in continuous culture and using flow cytometer. Non polar fractions 2, 3 and 4 displayed good antiplasmodial activity with IC_{50} ranging from 1.91 to 4.32 μg mL^{-1} and little toxicity with selectivity index ranging from 3.03 to 6.15. These data allow further investigations in terms of purification, isolation and development of new antiplasmodial compounds from these semi purified fractions and development of improved phytomedicine.

Key words: *Zanthoxylum zanthoxyloides*, chromatography, fractions, antiplasmodial activity, cytotoxicity

INTRODUCTION

Malaria is a major health problem in many developing countries and is responsible for 300 to 500 million clinical cases worldwide (Rogier and Trape, 1999) with around 1 million deaths each year, mainly in sub-Saharan Africa (Snow *et al*., 1999; Rowe *et al*., 2006). The rising resistance of *Plasmodium falciparum* to affordable antimalarials such as chloroquine (Gansane *et al*., 2009a, b) makes discovery and development of new active and safe antimalarial drugs increasingly important. Therefore research into new antimalarial drugs from natural products is worthy priority in sub-Saharan Africa. *Zanthoxylum zanthoxyloides* Lam. (Rutaceae) previously named *Fagara zantoxyloides* Lam. is very well known species, widely used in the African pharmacopoiea, frequently mentioned and for which crude extracts, semi purified and pure compounds from roots have already been evaluated *in vitro* against Plasmodium strains (Kassim *et al*., 2005). For ecological reason, the bark of trunk, is also used by the local population of Comoe, western part of Burkina Faso for the treatment of fever or malaria (Traore *et al*., 2009) and the antiplasmodial activity of crude alkaloid extract of bark of trunk has been previously evaluated and showed a good IC_{50} = 1.16 μg mL^{-1} against W_2 *Plasmodium falciparum* strains (Gansane *et al*., 2009a, b). The aim of this study was to realize the bio-guided chromatographic fractionation of the crude alkaloid extract derived from *Zanthoxylum zanthoxyloides* bark of trunk and to evaluate antiplasmodial property and toxicity, respectively against W_2 *Plasmodium falciparum* resistant strains and K562S cell of semi purified alkaloids fractions obtained by chromatography methods.

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MATERIALS AND METHODS

Place and duration of study: The fractionation and *in vitro* testing with semi purified fractions were performed from March to October 2009, respectively in the Department of Pharmacology and Toxicology, University of Ouagadougou (Burkina Faso), Burkina Faso and in the laboratory of Parasitology in the Faculty of Pharmacy, University of the Mediterranea (France).

Semi purified alkaloids fractions: *Z. zanthoxyloïdes* (Lam.) *Zepen.* and *Timber* was selected following socio-anthropological and ethnobotanical surveys conducted in 2006 in the province of Comoe, located in western Burkina Faso, 440 km from Ouagadougou, the capital city (Traore *et al*., 2009). Crude total alkaloid extract was obtained from bark of trunk of *Zanthoxylum zanthoxyloides* as described by Gansane in previous step of this research (Gansane *et al*., 2009). The extract was dissolved in chloroform and fractionated using Preparative Thin Layer Chromatography (TLC) 20×20 on G60 F254 silica gel Merck and cyclohexane/toluene/diethylamin (75/15/10) was used as mobile phase. After solvent migration plates were removed from the mobile phase and dried. The visualization of the separated bands on PTLC was done using short (254 nm) and long (366 nm) wavelength UV light to reveal semi-purified alkaloids fractions. Each fraction on silica gel was removed with meticulous care, dissolved in chloroform, filtered on Whatman paper A1 and weighted. TLC analysis was performed for each fraction and based on the results of the thin layer chromatogram, identical fractions were pooled and then submitted to biological evaluations after the evaporation of the solvent under reduce pressure at 40°C giving dried residue of each fraction. Spraying of Dragendorff's reagent on thin layer chromatogram plates were used to reveal the presence of alkaloid in each fraction.

All semi purified alkaloids fractions were solubilized in 100% sterile DMSO (Dimethyl sulfoxide). All solutions were homogenous. The initial concentration of all solutions was 10 μg μL^{-1}. Serial dilutions were made to obtain different concentrations of fractions (25, 12.5 and 6.25 μg mL^{-1}) for *in vitro* parasite testing and 50, 25 and 12.5 μg mL^{-1} concentrations for *in vitro* cytotoxicity assays.

Parasites, media and *in vitro* antiplasmodial tests: The *Plasmodium falciparum* strain used for the *in vitro* tests was W_2, a multi-resistant isolate from Vietnam. The isolate was maintained in continuous culture in the parasitology lab of the pharmacy faculty of the Universite de la Mediterranee, Marseille, France. In 75 cm^2 flasks, the parasite strains were maintained in continuous culture using 20 mL of RPMI 1640 medium supplemented with 10% human serum, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, Gibco-BRL, Paisley, Scotland) and 25 mM NaHCO3 as previously described by Trager and Jensen (1976). Washed human group A+ erythrocytes served as host cells and the cultures were incubated at 37°C in an atmosphere of 6% CO_2, 10% O_2 and 84% N_2 with 90% humidity. Parasitemia was monitored daily and maintained between 1 and 6%. Dilutions, when performed, were done using non-infected A+ erythrocytes stored at hematocrit 50%. Medium renewal and microscopic observation of prepared blood smears fixed with methanol and stained with 10% Giemsa stain were performed daily.

Parasitized blood at a hematocrit of 2% with parasitemia between 1.5 and 2% was suspended in 500 μL culture medium with different concentrations of fractions. A growth control (parasitized blood only), positive controls with chloroquine (the reference drug) at different concentrations (2.5, 1, 0.5 and 0.1 μM) and negative controls with sterile DMSO (0.2, 0.1, 0.05 and 0.025%) were prepared under the same conditions and were used in each series of tests. Nonparasitized blood was used to adjust the flow cytometer (Facsort Becton Dickinson France Paris). Each concentration of extract and control was tested in duplicate in 96 well polystyrene plates (Nunc Brand products, Fisher, Paris, France). Incubation was performed in a CO_2 atmosphere at 37°C during 48 h without medium renewal. After incubation, plates were centrifuged and the supernatants replaced (cells remained in the bottoms of the wells) with 180 μL hydroethidine solution (0.05 mg mL^{-1} in PBS). After 25 min of incubation in the dark at 37°C and three washes with PBS solution, red cells were suspended in 200 μL of PBS. A final dilution of 10 μL of suspended red cells in 1 mL of PBS was used for determination of the number of parasitized cells by flow cytometry (Beckton Dickinson Facsort). The $IC_{50\text{-Plasmodium}}$ was defined as the concentration of an extract that produced 50% inhibition of parasite growth in comparison with a control culture with DMSO. The $IC_{50\text{-Plasmodium}}$ was determined from dose-response.

Cell lines, media and cytotoxicity tests: K562S cells, which derived from human chronic myeloid leukemia, were maintained in continuous culture in RPMI 1640 medium (Eurobio, Paris, France) supplemented with 10% fetal bovine serum (Eurobio, Paris, France), 25 mM HEPES, 25 mM $NaHCO_3$ and a 1% mix of 200 mM l-glutamine, 10,000 IU mL^{-1} penicillin and 10 mg mL^{-1} streptomycin (Sigma). The tests were performed in 1 mL of

medium containing 1.5×105 cells mL^{-1} in contact with each fraction at different concentrations (50, 25 and 12.5 µg mL^{-1}). A growth control (cells and medium only), positive controls with doxorubicin at different concentrations (4.300, 0.430, 0.086, 0.040 and 0.215 µM) and a negative control with sterile DMSO at a final concentration of 0.5% were made for each series of tests. Experiments (concentrations of fractions) and controls were tested in duplicate in 24 well polystyrene plates. Incubation was performed in a CO_2 atmosphere at 37°C for 72 h without medium change. Then, incorporation of *Propidium iodide* into the nucleic acids of dead or dying cells, read by flow cytometer, was used to determine the $IC_{50\text{-}K562S}$ (inhibiting concentration of 50% of the growing K562S cells compared to control culture) for each fraction.

A Selectivity Index (SI), corresponding to the ratio between cytotoxic and antiparasitic activities, was evaluated for each tested extract according to the following formula:

$$SI_{Plasmodium} = IC_{50\ K562S}/IC_{50\ Plasmodium\ W2}$$

Data analysis: Data were entered and analyzed using Microsoft Excel 2007. Parasites viability for all concentrations tested was calculated by subtracting the control value (value obtained with negative control). A concentration-response curve (percentage of parasitemia or percentage of cells proliferation versus log concentration) was plotted for each fraction and 50% inhibitory concentration IC_{50} was calculated compared to control by using table curve 5.0 Software.

RESULTS

Crude alkaloid extract and semi purified fractions: The first fraction obtained from *Zanthoxylum zanthoxyloides* bark of trunk was made with 40 g of powdered bark of trunk giving 668 mg of crude alkaloid. The visualization of the separated bands on PPTLC at 254 and 366 nm wavelength UV light revealed the presence of 15 bands representing semi purified alkaloids fractions. The polarity of fractions for this system of solvents decreased with the increases of fractions number. Fraction 1 (117.7 mg) is the more polar and fraction 15 the less polar. Based on the results of the thin layer chromatogram of each fraction, we pooled fractions 11, 12, 13, 14, 15 because of similarities of profile and a total of 11 fractions were submitted to biological evaluation. The weight of these semi purified fractions was summarized in Table 1. The revelation test using Dragendorff's reagent showed the presence of alkaloid in all semi purified fractions with high intensity of coloration in fractions 1, 2, 3, 4, 9 and 11. Fractions 5, 6, 7, 8 and 10 did not react with Dragendorff's reagent.

Table 1: Weight of fractions obtained from crude alkaloid extract of *Z. zanthoxyloïdes*

Alkaloids fractions	Weight (mg)
Fraction 1	192.7
Fraction 2	41.6
Fraction 3	41.1
Fraction 4	6.5
Fraction 5	5.0
Fraction 6	11.3
Fraction 7	13.1
Fraction 8	6.7
Fraction 9	25.1
Fraction 10	13.4
Fraction 11	47.0

Table 2: *In vitro* antiplasmodial activity of semi purified fractions from *Z. zantoxyloïdes* bark of trunk against W2 strains of *Plasmodium falciparum*, antiproliferative activity on K562S cells and selectivity index (SI)

	IC_{50} (µg mL^{-1})		
Fractions	W2	K562S	SI
Fraction 1	9.10	30.72	3.38
Fraction 2	2.44	12.44	5.10
Fraction 3	1.91	11.74	6.15
Fraction 4	4.32	13.11	3.03
Fraction 5	21.36	18.83	0.90
Fraction 6	24.88	15.7	0.63
Fraction 7	10.14	21.11	2.08
Fraction 8	11.26	9.94	0.90
Fraction 9	5.00	5.44	1.09
Fraction 10	24.10	22.28	0.93
Fraction 11	8.62	13.75	1.60

***In vitro* antiplasmodial and antiproliferative effects:** The IC_{50} of chloroquine, the reference drug, was 700 nM for multiresistant W_2 strains of *Plasmodium falciparum*. The *in vitro* antiplasmodial activity of these semi purified fractions is summarized in Table 2. A total of six semi purified fractions collected from the crude alkaloid extract of bark of trunk of *Z. zanthoxyloïdes* (fraction 1, 2, 3, 4, 9 and 11) displayed good activity with IC_{50} less than 10 µg mL^{-1} and high activity for fractions 2, 3 and 4 which displayed IC_{50} less than 5 µg mL^{-1}.

Antiproliferative activity against K562S cells was evaluated for all semi purified fractions. The IC_{50} of doxorubicine, the reference drug for the test, was 0.02 µM. Results of antiplasmodial and toxicity assays of these semi purified fractions are summarized in Table 2. The toxicity of each semi purified fraction was established by analysis of the SI.

DISCUSSION

Zanthoxylum zanthoxyloides is very well known specie, widely used in the African pharmacopoiea, frequently mentioned and that crude extracts from roots have already been evaluated *in vitro* against Plasmodium strains (Kassim *et al*., 2005). In this study we focussed our research on bark of trunk of this plant instead to roots

for ecological reason and sustainability of plants and the antiplasmodial and cytotoxicity activities, respectively against W2 strains and K562 cells of semi-purified fractions obtained by Preparative chromatography were investigated because of good activity and selectivity index of crude alkaloid extract (Gansane *et al*., 2009a, b). To our knowledge his is the first time that semi purified fractions from extract from bark of trunk for *Z. zanthoxyloïdes* is tested for antiplasmodial activity against resistant strains of *P. falciparum.* According to Deharo *et al.* (2001), from a total of 11 semi purified fractions collected, six (fraction 1, 2, 3, 4, 9 and 11) displayed good activity with IC_{50} less than 10 μg mL^{-1} and high activity for fractions 2, 3 and 4 which displayed IC_{50} less than 5 μg mL^{-1} suggesting that the antiplasmodial compounds present an semi polar nature with the system of solvent used for fractionation. These 6 fractions intensely reacted with Draggendorf's reagent confirming the presence of alkaloids like quinine, which is an antimalaria compound isolated from genus Cinchona and formulated to antimalaria drug currently used to treat severe malaria. However no fraction displayed better antiplasmodial activity than the crude alkaloid extract fraction with IC_{50} = 1.16 μg mL^{-1} obtained in previous study (Gansane *et al*., 2009a, b) although this IC_{50} obtained with the crude alkaloid extract was close to that obtained with fraction 3 (IC_{50} = 1.91 μg mL^{-1}). It was so evident that the overall antiplasmodial activity of the crude extract was due to the synergistic effects of these fractions with maybe a great contribution of fraction 3. Purification of these fractions 2, 3, 4 and 9 upon fractionation could allow isolation of pure alkaloid compound with very good activity against malaria parasites. Many pure compounds such as 3,4-O-divanilloylquinic acid, 3,5-O-divanilloylquinic acid, 4,5-O-divanilloylquinic acid and the pure alkaloid fagaronine were isolated from roots of *Z. zanthoxyloides* by previous studies (Ouattara *et al*., 2004; Messmer *et al*., 1972). Fagaronine which is a benzophenanthridine alkaloid was tested against sensitive strain of *Plasmodium falciparum* and displayed IC_{50} = 0.018 μg mL^{-1} (Kassim *et al*., 2005) and the three new isomeric divanilloylquinic acids were tested for their antisickling activity (Ouattara *et al*., 2004, 2009) and never for the antiplasmodial activity. There is so a need to know if Fagaronine or others pure compound are presents in the bark of trunc of *Z. zantoxyloïdes.*

To determine the specificity of the antiplasmodial activity of these semi-purified fractions, we tested them for cytotoxicity against K562S cells. The corresponding IC_{50} and selectivity index of fractions 2, 3, 4 and 9 suggest that the observed good antiplasmodial activity might not be due to the toxicity activity of these fractions. Fagaronine displayed an IC_{50} = 2.22 μg mL^{-1} against K562S cells in previous study (Prado *et al*., 2004) seems to be more cytotoxic than our semi purified fractions 2, 3, 4 and 9.

These results justify the use of bark of trunk from *Z. zanthoxyloides* for the treatment of malaria/fever in the province of Comoe and allow us further investigation to purify, isolate molecules and to determine mechanism of action and DMPK profile of leads compounds from semi purified fractions 2, 3, 4 and 9. This is the first time that semi purified fractions from extract from bark of trunk for *Z. zanthoxyloïdes* is tested for antiplasmodial activity against resistant strains of *P. falciparum.*

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