Afr. J. Trad. CAM (2007) **4 (2):** 135 - 139

Research Paper

Afr. J. Traditional,
Complementary and Alternative Medicines
www.africanethnomedicines.net

ISSN 0189-6016©2007

THE ANTIPLASMODIAL AGENTS OF THE STEM BARK OF *ENTANDROPHRAGMA ANGOLENSE* (MELIACEAE).

Jean Bickii[a*], Guy Raymond Feuya Tchouya[b], Jean Claude Tchouankeu[b], Etienne Tsamo[b]

[a]Institut de Recherches Médicales et d'Etudes des Plantes Médicinales (I.M.P.M.), Laboratoire de Recherche sur le Paludisme, Yaoundé, Cameroon., [b]Faculty of Science, University of Yaoundé 1, P. O. Box 812 Yaoundé, Cameroon
***E-mail:** jbickii@yahoo.fr , **Tel:** +237 989 20 30

Abstract

In the search of active principles from the stem bark of *Entandrophragma angolense*, we submitted the compounds isolated from the dichloromethane – methanol (1:1) extract of the stem bark to antimalarial test against chloroquine resistant strain W2 of *Plasmodium falciparum* malaria parasite. Only 7α-obacunyl acetate and a cycloartane derivative exhibited a good activity, with IC_{50}s of 2 and 5.4 µg/ml respectively. Other compounds were moderately active.

Key words: *Plasmodium falciparum*; Meliaceae; *Entandrophragma angolense*; Limonoids; Fatty acids; Triterpenoids; 7α-obacunyl acetate; 24-methylenecycloartenol; anti-plasmodial activity.

Introduction

Malaria is one of the most prevalent infections in the world. Despite more than a century of efforts to eradicate or control malaria, the disease remains a major and growing threat to the public health and economic development of countries in the tropical and subtropical regions of the world. In the search for new, safe and effective antimalarial drugs, screening of extracts from plants used in traditional medicine was indicated.

The plant family Meliaceae has been the subject of study as one of the most promising source of compounds with antiplasmodial properties. Some species belonging to this family (*Azadirachta indica, Entandrophragma angolense, Entandrophragma candollei, Entandrophragma utile, Khaya grandifoliola*) are widely used as antimalarials or antipyretics in traditional medicine (Irvine, 1961; Obih et al., 1986; Bray et al., 1990; Weenen et al., 1990a).

The genus *Entandrophragma* includes fourteen species distributed throughout Africa (Aubreville, 1950) among which, five have been identified in Cameroon: *E. angolense* Welwitsch C. D. C., *E. candollei* Harms, *E. congoense* H. Chev, *E. cylindricum* and *E. utile* Sprague (Letouzey, 1985).

During the investigation of the plant species, *Entandrophragma angolense* Welwitsch C. D. C. (Meliaceae) we realised that the dichloromethane – methanol (1:1) extract of the stem bark of that plant is highly potent, inhibiting the development of the chloroquine resistant strain W2 of *Plasmodium falciparum* malaria parasite with an IC_{50} of 18.8 µg/ml. This prompted us to go further and isolate compounds from that extract, in order to submit them to antiplasmodial activity against chloroquine resistant strain W2 of *Plasmodium falciparum* malaria parasite.

Material and methods

Plant materials

Stem bark of *E. angolense* Welwitsch C. D. C. was collected in April 1999 from the Awae forest reserve (Cameroon). The plant was identified, and a Voucher specimen (N° 29933) was deposited, by Dr. Achoundong of National Herbarium, Yaoundé (Cameroon).

Bickii *et al., Afr. J. Trad. CAM* (2007) **4 (2):** 135 - 139

Extraction and isolation procedures

Dried and finely powdered stem bark (10 kg) was extracted with dichloromethane-methanol (1:1) (22 L x 3 times) at room temperature. After filtration and removal of the solvent under vacuum, 60 g of the dichloromethane-methanol extract obtained (300 g, 3%) was chromatographed in a 70-230 mesh silica gel column (1 kg) with stepwise gradient elution by n-hexane/EtOAc mixtures (100:0; 90:10; 80:10; 70:30; 60:40; 50:50; 30:70; 20:80; 0:100). Ninety column fractions, each containing 300 ml were collected and combined according to their TLC profiles on precoated Kiesegel 60 F_{254} plates developed with n-hexane/EtOAc mixtures. Seven groups of fractions A (1-22), B (23-33), C (34-47), D (48-56), E (57-67) F (68-72) and G (73-90), respectively, were eluted. Fraction A contained only oils. Fraction B was subjected to column chromatography over Si gel (70-230 mesh) eluting with n-hexane-ethyl acetate gradient of increasing polarity resulting in the isolation of 22-hydroxyhopan-3-one **5** (150 mg), sitosterol (20 mg), 24-methylenecycloartenol **6** (80 mg) (structure to be confirmed), tricosanoic acid **4** (10 mg). Fraction C upon recrystallisation from n-hexane/EtOAc (70:30) yielded methylangolensate **3** (600 mg). The mother liquors, after concentration and chromatography over Si gel eluting with n-hexane /EtOAc (85:15) afforded methyl oleanate (8 mg) and betulinic acid (20 mg). Fractions D and F afforded 7α- acetoxydihydronomilin **1** (500 mg) and 7α- obacunylacetate **2** (120 mg) upon respective recrystallisation from 70:30 and 80:20 n-hexane/EtOAc. Fraction E was rechromatographed on a silica gel column using n-hexane/EtOAc gradient to obtain more 7α- acetoxydihydronomilin **1** (70 mg) and 7α-obacunylacetate **2** (30 mg). Fraction G upon recrystallisation yielded β-sitosterol-3-*O*-β-D-glucopyranoside (20 mg). The isolated compounds were identified by comparing their spectroscopic and physical data with those reported in the literature and by their TLC comparison with authentic samples.

From the chemotaxonomic point of view, it is of interest to note that, although the occurence of 7α-acetoxydihydronomilin **1** and 7α- obacunyl acetate **2** had been reported in *Uncaria* genus (Rubiaceae) (Ahmed et al., 1978) and *Citrus* genus (Rutaceae) (Bennett and Hasegawa, 1982), respectively, this is the first time their isolation is reported from *E. angolense*.

***In vitro* antimalarial assay**
Cultivation of *P. falciparum*

P. falciparum strain W2, which is resistant to chloroquine and other antimalarials (Singh and Rosenthal, 2001), was cultured in sealed flasks at 37°C, in a 3% O_2, 5% CO_2 and 91% N_2 atmosphere in RPMI 1640, 25 mM HEPES, pH 7.4, supplemented with heat inactivated 10% human serum and human erythrocytes to achieve a 2% hematocrit. Parasites were synchronized in the ring stage by serial treatment with 5% sorbitol (Sigma) (Lambros and Vanderberg, 1979) and studied at 1% parasitemia.

Compounds were prepared as 20 mg/ml stock solutions in DMSO, diluted as needed for individual experiments, and tested in triplicate. The stock solutions were diluted in supplemented RPMI 1640 medium so as to have at most 0.2% DMSO in the final reaction medium. An equal volume of 1% parasitemia, 4% hematocrit culture was thereafter added and gently mixed thoroughly. Negative controls contained equal concentrations of DMSO. Positive controls contained 1 µM chloroquine phosphate (Sigma). Cultures were incubated at 37°C for 48 hrs (1 parasite erythrocytic life cycle). Parasites at ring stage were thereafter fixed by replacing the serum medium by an equal volume of 1% formaldehyde in PBS. Aliquots (50 µl) of each culture were then added to 5 ml round-bottom polystyrene tubes containing 0.5 ml 0.1% Triton X-100 and 1 nM YOYO nuclear dye (Molecular Probes) in PBS. Parasitemias of treated and control cultures were compared using a Becton-Dickinson FACSort flow cytometer to count nucleated (parasitized) erythrocytes. Data acquisition was performed using CellQuest software. These data were normalized to percent control activity and 50% inhibitory concentrations (IC_{50}s) calculated using Prism 3.0 software (GraphPad) with data fitted by non linear regression to the variable slope sigmoidal dose-response formula $y = 100/[\ 1+ 10^{(\log IC50-x)H}]$, where *H* is the hill coefficient or slope factor (Singh and Rosenthal, 2001).

Different dilutions of the compounds were incubated at 37°C with cultured W2 strain *P. falciparum* parasites for 48 hours. Parasites were thereafter fixed and stained, and parasitemias of treated and control cultures were determined. Results are means, compared to untreated controls, from 3 experiments. Error bars represent standard deviations of results.

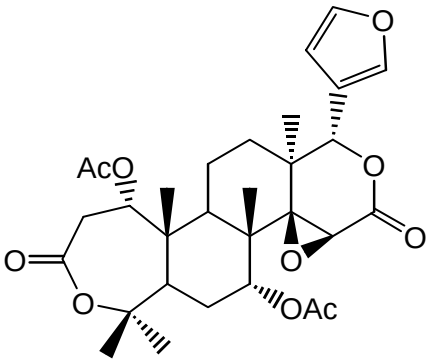
Results

The phytochemical investigation of the stem bark of *E. angolense* (Meliaceae) led to the isolation of known limonoids 7α- acetoxydihydronomilin **1,** 7α- obacunylacetate **2**, methylangolensate **3**, together with

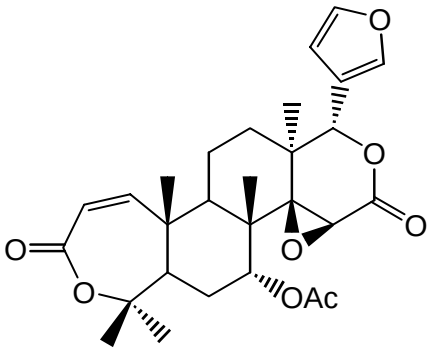
Table 1: IC_{50} values of isolated compounds against *Plasmodium falciparum* strain W2.

Compounds	Stocks (mg/ml)	IC_{50} (µg/ml)* (Mean ± SD)
1	20	19.5 ± 1.3
2	20	**2 ± 0.6**
3	20	22.9 ± 0.7
4	0.6	83.5 ± 1.5
5	0.6	137.8 ± 1.8
6	20	**5.4 ± 0.8**

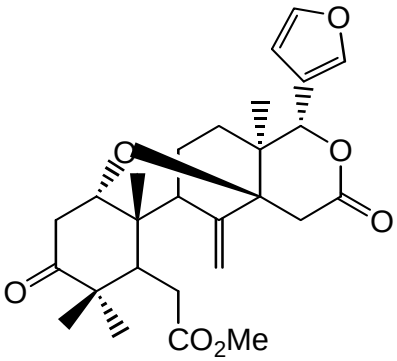
(*) The results are expressed as geometric means ± SD from triplicate separate experiments.



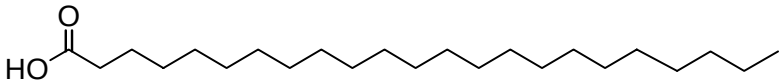
1



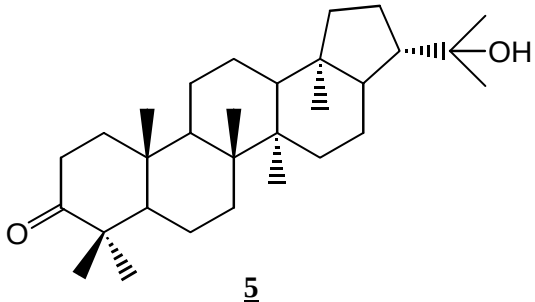
2



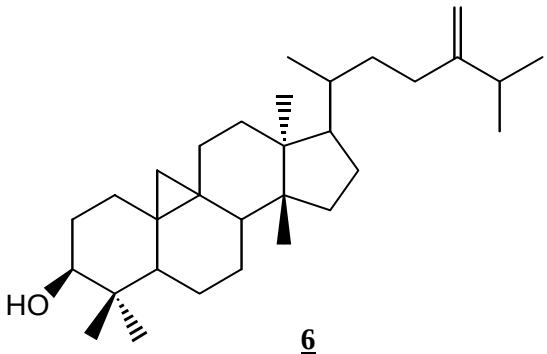
3



4



5



6

Figure 1. Structures of the compounds: 7α- acetoxydihydronomilin **1**, 7α- obacunylacetate **2**, methylangolensate **3**, tricosanoic acid **4**, 22-hydroxyhopan-3-one **5**, 24-methylene cycloartenol **6**.

tricosanoic acid **4**, 22-hydroxyhopan-3-one **5**, and 24-methylene cycloartenol **6** (the structure is not yet confirmed).

The antiplasmodial activity of the isolated compounds have been evaluated against *Plasmodium falciparum* W2 strain. The IC_{50} value of chloroquine for the clone W2 was 133 nM. The IC_{50} values of the isolated compounds are shown in Table 1. From the results obtained, 7α- obacunyl acetate **2** and 24-methylencycloartenol **6** were found to be significantly active *in vitro* against *P. falciparum* parasites, with IC_{50}s of 2 and 5.4 µg/ml respectively. 7α- acetoxydihydronomilin **1** and methylangolensate **3** showed moderate activity while tricosanoic acid **4** and 22-hydroxyhopan-3-one **5** were devoid of activity.

Discussion

Our work on the dichloromethane-methanol extract of the stem bark of *E. angolense* has resulted in the isolation of ten known compounds: 7α- acetoxydihydronomilin **1** (Ahmed et al., 1978), 7α- obacunylacetate **2** (Bennett and Hasegawa, 1982), methylangolensate **3** (Connolly, 1983), tricosanoic acid **4** (Francis, 1939), 22-hydroxyhopan-3-one **5** (Mahato and Kundu, 1994), 24-methylenecycloartenol **6** (Ferreira et al., 2000) (structure to be confirmed), methyloleanate (Glen et al., 1967), betulinic acid (Mahato and Kundu, 1994), β-sitosterol (Furuya et al., 1987) and β-sitosterol-3-*O*-β-D-glucoglucopyranoside (Sakakibara et al., 1983).

Six of the isolated compounds were submitted to antiplasmodial testing against *Plasmodium falciparum* malaria parasite, among which: 3 limonoids (**1**, **2** and **3**), a pentacyclic triterpene (**4**), a fatty acid (**5**) and a tetracyclic triterpene (**6**). From the results obtained (Table 1), compound **2** and compound (**6**) were found to be significantly active against *P. falciparum* parasites with IC_{50}s of 2.0 µg/ml and 5.4 µg/ml respectively.

The antimalarial activities of the limonoids in the present study are comparable to those of other limonoids and purified compounds obtained from other plants family (Noster and Kraus, 1990; Koumaglo et al., 1990; Bray et al., 1990, Weenen et al., 1990b, Nkunya, 1991; Bickii et al., 2000), the highest activity being observed with the 7α-obacunylacetate (**2**).

These compounds are quite different in their structures, which may explain the variations observed in their activities. Since the limonoids belong to the tetranortriterpenoid meliacolide class (share the same basic chemical structure), compounds (**1**) and (**2**) belong to this meliacolide class with an opened ring A. The good activity exhibited by compound (**2**) can be explained by the fact that (**2**) possesses an α, β-unsaturated carbonyl moiety at C1/C2 in ring A which was hypothesized to be involved in the Micheal addition reaction with the parasite nucleic acids (Weenen et al., 1990b). In fact, the reduction of the double bond in compounds possessing an α, β-unsaturated carbonyl moiety at C1/C2 in ring A has been shown to decrease the antimalarial activity (Bray et al., 1990). This observation agrees with the report that α, β-unsaturated ketone function is an important feature for enhanced antimalarial activity of quassinoids and terpenes which share similar chemical structures with limonoids (O'Neill et al., 1986; Weenen et al., 1990b; Phillipson and Wright, 1991). The difference of activity between compound (**3**) and the other two limonoids could be explained by the fact that compound (**3**) is a meliacolide with an opened ring B which probably decreases its activity.

The cycloartane derivative, 24-methylenecycloartenol was active against *P. falciparum* parasites with an IC_{50} of 5.4 µg/ml. Although its chemical structure is to be well-confirmed, the difference of activity observed between this compound and compound **2** might be explained by the difference in their chemical structures. So, compound **2** is a tetracyclic triterpene with a furanic lateral chain and a lactonized ring A, while compound (**6**), although being also a tetracyclic triterpene, possesses a non cyclic lateral chain. It is of interest to notice that, the presence of the cyclopropyl ring in the structure of compound (**6**) should be taken into account in the explanation of its activity. Further work based on the study of the structure-activity relationship will permit to elucidate that phenomenon.

Conclusion

Limonoids isolated from the stem bark of *E. angolense* have been shown to exhibit moderate *in vitro* antimalarial activities against *P. falciparum* parasites. Two compounds: 7α-obacunylacetate and 24-methylenecycloartenol were the most active. The results of this study can be correlated to the traditional use of *E. angolense* in the treatment of malaria. Further studies are however necessary to evaluate the toxicity and to elucidate the mechanism of action of the active compounds. Combinations with other antimalarial drugs may be interesting in the goal of fighting drug resistance.

Acknowledgements

This work was supported by the WHO/UNDP/World Bank/TDR Research grant N°99088 (Dr J. BICKII).

Bickii *et al., Afr. J. Trad. CAM* (2007) **4 (2):** 135 - 139

The authors thank Julie E. Lehman at the University of California, San Francisco, and Dr. Achoundong at the National Herbarium, Yaoundé, Cameroon. We are indebted to Prof. Connolly of the Department of Chemistry, the University of Glasgow, Glasgow G12 8QQ U.K., for recording the ^{1}H and ^{13}C NMR spectra.

References

1- Ahmed, F. R. and No, A. S., Fallis A. G. (1978). 7α-acetoxydihydronomilin: isolation, spectra, and crystal structure, Can. J. Chem., **56:** 1020–1025.
2- Aubreville, A. (1950). Flore forestière Soudano-guinéenne. Société d'édition géographique, p. 377.
3- Bennett R. D. and Hasegawa S. (1982). 7α-Oxygenated limonoids from the Rutaceae. Phytochemistry, **21(9):** 2349-2354.
4- Bickii, J., Njifutie, N., Ayafor, J. F., Basco, L. K., Ringwald, P. (2000). *In vitro* antimalarial activity of limonoids from *Khaya grandifoliola* C. D. C. (Meliaceae). J. Ethnopharmacol.**, 69:** 27-33.
5- Bray, D. H., Warhurst, D. C., Connolly, J. D., O' Neill, M. J., Phillipson, J. D. (1990). Plants as sources of antimalarial drugs. Part 7. Activity of some species of Meliaceae and their constituent limonoids. Phytother. Res., **4:** 29-35.
6- Connolly, J. D. (1983). The Chemistry and Taxonomy of the Rutales. In: P. G.Waterman and M. F. Grundon (Eds.), Academic Press London, p. 180.
7- Ferreira, J., Floriani, A. E. O., Filho, V. C., Monache, F. D., Yunes, R. A., Calixto, J. B. and Santos, A. R. S. (2000). Antinociceptive properties of the methanolic extract and two triterpenes isolated from *Epidendrum mosenii* stems (Orchidaceae), Life Sciences, **66(9):** 791-802.
8- Francis, F. (1939). The Higher n-Aliphatic Acids and their Methyl and Ethyl Esters. J. Am. Chem. Soc., **61:** 577-581.
9- Furuya, T., Oriharia, Y. and Hayashi, C. (1987). Triterpenoids from *Eucalyptus perriniana* cultured cells*. Phytochemistry, **26:** 715-719.
10- Glen, A. T., Lawrie, W., Mclean, J. and Garby Younes, M. (1967). Terpenoid Constituents of Rose-bay Willow-herb. J. Chem. Soc. (C) 510-515.
11- Irvine, F. R. (1961). Woody plants of Ghana. Oxford Univerity Press. Oxford, p 517.
12- Koumaglo, K., Gbeassor, M., Nikabu, O., De Souza, C., Werner, W. (1991). Effects of three compounds extracted from *Morinda lucida* on *Plasmodium falciparum*. Planta med., **58:** 533-534.
13- Lambros C., Vanderberg J. P. (1979). Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol., **65:** 418-420
14- Letouzey, R. (1985). Carte phytogéographique du Cameroun au 1/500000^{e}. Institut de la Carte Internationale de la Végétation, Toulouse, France, p. 5.
15- Mahato, S. B. and Kundu, A. P. (1994). ^{13}C NMR Spectra of pentacyclic Triterpenoids- A Compilation and some salient features. Phytochemistry, **37:**1517-1575.
16- Nkunya, M. H. H., Weenen, H., Bray, D. H., Mgani Q. A. (1991). Anti-malarial activity of Tanzanian plants and their active constituents: the genus *Uvaria*. Planta med., **57:** 341-343.
17- Noster, S., Kraus, L. J. (1990). *In vitro* activity of *Coutera latiflora* and *Exosreme caribaeum* extracts on *Plasmodium falciparum*. Planta med., **56:** 63-65.
18- Obih, P. O., Makinde, J. M., Laoje, J. (1986). Investigation of various extracts of *Morinda lucida* for antimalarial actions on *Plasmodium berghei* in mice. Afr. J. Med. Sci., **14:** 45-49.
19- O'Neill, M. J., Bray, D. H., Boardman, P., Phillipson, J. D., Warhurst, D. C., Peters, W., Suffness, M. (1986). Plants as source of anti-malarial drugs: *in vitro* antimalarial activities of some quassinoids. Antimicrob. Agents Chemother., **30:** 101-104.
20- Phillipson, J. D., Wright, C. W. (1991). Antiprotozoal agents from plants sources. Planta med., **57 (Suppl 1):** S53-S59.
21- Sakakibara, J., Kaiya, T., Furuda, H. and Ohki, T. (1983). 6- beta (mathemathiq)-hydroxyursolic acid and other tritepenoids of *Enkianthus cernuus*. Phytochemistry, **22:** 2553-2559.
22- Singh A., Rosenthal P. J. (2001). Comparison of efficacies of cysteine protease inhibitors against five strains of *Plasmodium falciparum*. Antimicrob. Agents Chemother., **45:** 949-951.
23- Weenen, H., Nkunya, M. H. H., Bray, D. H., Mwasumbi, L. B., Kinabo, L. S., Kilimali, V. A. E. B. (1990a). Antimalarial activity of Tanzanian medicinal plants. Planta Med., **56:** 368-370.
24- Weenen, H., Nkunya, M. H. H., Bray, D. H., Mwasumbi, L. B., Kinabo, L. S., V. A. E. B. (1990b). Antimalarial compounds containing an α, β-unsaturared carbonyl moiety from Tanzanian medicinal plants. Planta med., **56:** 371-373.