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In-vitro evaluation of the antimicrobial activities of *Enantia chlorantha* Oliv. extractives

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Summary

A bioactivity-directed franctionation of the root-and stem-barks of *Enantia chlorantha* resulted in the isolation of palmatine chloride 1 and jatrorrhizine chloride 2 as the major antimicrobial constituents. MIC determinations indicated that these compounds were superior to those of well established broad spectrum antibiotics against some of the organisms used.

Résumé

Une fractionation bioactivement menée de la racine et de l'écorce de l'arbre de la plante *Enantia chlorantha* a donné lieu à l'isolation du chlorure de palmatine 1 et du chlorure de jatrorrhizine 2 comme des composants antimicrobians principaux. Les determinations MIC ont indiqué que ces composes sont bien superieurs aux autres antibiotiques quand il s'agit de certains organismes contre lesquels ils ont été testes.

Introduction

Enantia chlorantha Oliv. (Family : Annonaceae) is an understorey tree of high rain-forests, reputed in traditional medicine in West Africa for its anti-infective properties[1].

The stem-bark is used as a powder on sores in Nigeria, as scrapings on ulcers in Gabon, and to wash leprosy blotches and other skin infections in Liberia. The Guerre of Ivory Coast often incorporate the bark extract of a related species, *Enantia polycarpa* into arrow poison[1,2].

Although there is evidence for the activity of the *E. chlorantha* alkaloids against *Staphylococcus aureus* [3], information on the activity of the crude extracts and constituents on other pathogenic micro-organisms particularly the gram-negative organisms is scanty. In the present study, a bioactivity-directed extraction and fractionation of the stem and root-barks using fungi as well as gram-negative and gram-positive bacteria is undertaken with a view to further provide justification for the folkloric usages of this plant in the management of several infection in West African communities.

Experimental details

Plant materials

The plant materials were obtained from Sapoba Forest Reserve, Bendel State of Nigeria in Nov. 1987 and authenticated by Dr. Zac Gbile (Forestry Research Institute of Nigeria, voucher specimen IB/97/241).

Extraction and isolation

The stem-bark (0.7 kg) and root-bark (0.6 kg) were separately successively and exhaustively extracted with solvents of increasing polarity using hexane, chloroform, methanol and 70% aqueous alcohol. The extracts obtained were separately evaporated to dryness in vacuo to give the yields shown on Table 1. 20 g of the crude chloroform extract of the stem-bark was dissolved in chloroform:methanol (1:1) and purified by passing through a short column packed with cellulose MN 100. Elution under pressure with 200 mls of the same solvent afforded 3.1 g crude alkaloidal residue on evaporation in vacuo. Preparative thin-layer chromatography (Sil gel PF, 1 mm thick) was performed, using dichloromethane: methanol (5:3) as the developing solvent system.

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Table 1: Yields of *Enantia chlorantha* stem-bark (0.7 kg) and root-bark (0.6 kg) extractives

Solvent	Root extract Weight (% Yield)	Stem extract Weight (% Yield)
Hexane	3.5 g (0.6%)	16.5 g (2.3%)
Chloroform	11.1 g (1.8%)	61.7 g (8.8%)
Methanol	17.5 g (2.3%)	53.3 g (7.6%)
Ag. Ethanol	9.0 g (1.5%)	Liquid (n.d.)

* n.d. = not determined

Micro-organisms

The micro-organisms used in this study include *Staphylococcus aureus* (NCTC 4163), *Bacillus subtilis* (NCTC 823), *Pseudomonas aeruginosa* (NCTC 6750), *Escherichia coli*-type I, *Candida albicans* (NCPF 3179), and *Aspergillus niger* (ATCC 16404). Fresly prepared bacterial cell suspensions containing approximately 0.6 x 10^6 colony forming units (CFU) per ml and fungal spore suspensions of about 2 x 10^6 CFU/ml were used in the experiments.

Antimicrobial susceptibility testing for bacteria and yeasts

Broth dilution, turbidometric and disc diffusion methods were used for the primary screening of the crude extracts against bacteria and yeast, and were performed in accordance with the recommendations of the European Pharmacopoeia[4]. Previous preliminary sensitivity tests in our laboratories have shown that the more volatile solvents were inhibitory to *Staphylococcus aureus, B. subtilis,* and *C. albicans* [5]. Disc diffusion was therefore used for testing the chloroform and hexane extracts against these species.

For the turbidometric method, sterile glass tubes containing 3 ml tryptone soya broth were inoculated with 50 µl of 0.6 x 10^6 CFU/ml suspensions from 18-20 h broth cultures giving rise to cell concentration in the tubes of about 10^4 CFU/ml. For the majority of the assays 50 µl of the test solutions were added to 3 ml inoculated broth. For assays against *Pseudomonas aeruginosa*, 20 µl of hexane and chloroform test solutions were used. Final concentrations of crude extracts for the *Pseudomonas* assays therfore ranged from 33 µg ml^{-1} to 137 µg ml^{-1}. Analogous concentrations used for other test organisms range from 83 µg ml^{-1} to 416 µg ml^{-1}. Control tubes were included in each screen; one containing culture medium only, another containing inoculated medium and a third containing inoculated medium and the appropriate solvent. All tubes were incubated for a period of 18-24 h at 37°C (bacteria) and 30°C (yeast) after which the presence or absence of growth was recorded.

For the disc diffusion method, tryptone soya agar (4 ml) was inoculated at 45°C with 66 µl of a 2.0 x 10^6 CFU/ml bacteria or yeast suspension and poured into the surface of a prepared tryptone soya agar plate. Sterile discs (10 mm diameter) were impregnated with 100 µl test solutions making the discs to contain about 0.5 to 2.5 mg of crude extracts after evaporation of the solvents. The impregnated discs were placed onto the solidified agar surface and incubated for 18 to 20 h at 37°C (bacteria) and 30°C (yeasts). Sterile filter-paper discs impregnated with only the solvents as well as unimpregnated sterile discs were used as controls. In cases where inhibitions of growth had occurred, the diameters of inhibition were measured.

Determination of MIC or pure compounds

The broth dilution method[6] was used to determine the MIC's of the pure isolated compounds as well as the standard antibiotics (ampicillin, streptomycin, gentamicin) and antimalarial agents (chloroquine and trimethoprim) against bacteria and *Candida albicans*. TSB (3 ml) was inoculated with bacterial or fungal suspensions to give a final concentration of 10^4CFU/ml. For the determinations, test compounds were dissolved in water, and various volumes of the solution (up to 50 µl) were added to the inoculated broth. All tubes were incubated for a period of 18-24h at 37° (bacteria) and 30° (yeast). The MIC was determined as the lowest concentration of the test compound which prevented visible growth after the incubation period.

Results and Discussions

In-vitro primary antimicrobial screening of crude extracts of *E. chlorantha* revealed that the methanol extract of the stem-bark is active against all the test organisms used in this study (Table 2) except *Pseudomonas aeruginosas* while the corresponding methanol extract of the root-bark only showed activity against *B. subtilis*. The chloroform extract of the stem-bark also showed very strong activity against all the test organisms except *B. subtilis*. The antifungal activity of the stem-bark against *C. albicans* using the fixed disc method is particularly remarkable (Table 3).

Combination of over-pressure liquid chromatography and thin-layer chromatographic separation of the chloroform extract afforded palmatine 1 (Fig 1A) as the major constituent. The methanol extract afforded both palmatine and jatrorrhizine in the ratio 4:1.

Table 2: Primary screening of crude extracts of *Enantia chlorantha* activity using turbidometry method

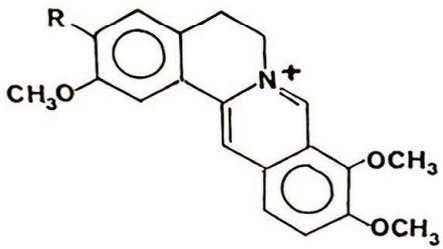
Extract	Organisms				
	I	II	III	IV	V
Stem-bark					
Hexane	—	—	+	NT	NT
Chloroform	+	—	+	NT	NT
Methanol	—	+	+	+	+
Aq. Ethanol	—	+	+	+	+
Root-bark					
Hexane	—	—	+	NT	NT
Chloroform	+	—	+	NT	NT
Methanol	—	+	—	—	—
Aq. Ethanol	—	+	—	+	+
Isolated Compounds					
1	—	—	—	+	+
2	—	—	—	+	+

KEY
+ = activity
— = inactive
NT = not tested
I = *Pseudomonas aeruginosa*
II = *Bacillus subtilis*
III = *Escherichia coli*
IV = *Staphylococcus aureus*
V = *Candida albicans*

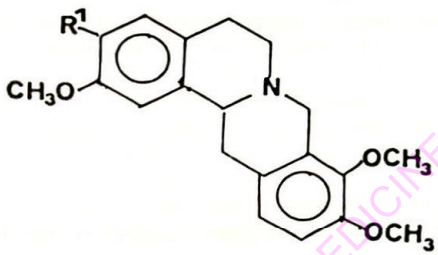
Table 3: Antimicrobial activity of *E. chlorantha* chloroform-soluble crude extracts on *S. aureus* and *C. albicans* using fixed disc method

Extract	Zone of Inhibition*	
	S. aureus	*C. albicans*
Hexane (R)	—	—
Chloroform (R)	19	—
Hexane (S)	—	—
Chloroform (S)	27	32
Chloroform (control)	16	16

* Diameter of disc = 10 mm
R = Root-bark extracts
S = Stem-bark extracts



	R
1	$-OCH_3$
2	$-OH$
5	$-OCOCH_3$



	R^1
3	$-OCH_3$
4	$-OH$

Fig 1a:

None of the *Enatia chlorantha* crude extracts showed any appreciable activity on the fungus *Aspergillus niger*. The isolated compounds, palmatine chloride 1 and jatrorrhizine chloride 2 showed very good activity against *S. aureus* and *C. albicans* (Table 4) on further screening by the broth dilution method to determine MIC values. The MIC values of the compounds range between 3.8 to 45 µg ml^{-1}. Standard antibiotics under the same conditions either showed considerably low activity or no activity at all against these two organisms. Ampicillin was however quite active against *Pseudomonas aeruginosa* and *E. coli* while the isolated compounds were inactive.

Table 4: Minimum inhibitory concentration (MIC) of isolated compounds compared with standard anti-infective agents.

Compound	MIC µg ml^{-1}			
	I	III	IV	V
Ampicillin	1.5	2.5	—	98.3
Streptomycin	165.0	20.0	—	—
Gentamicin	99.2	99.2	—	—
Chloroquine	—	—	—	—
Trimethoprim	—	—	—	—
1	—	—	21.6	45.0
2	—	—	3.8-26.7	38.3

KEY
I = *Pseudomonas aeruginosa*
III = *Escherichia coli*
IV = *Staphylococcus aureus*
V = *Candida albicans*

Several workers have reported antiseptic, antibutyrylcholinesterase, antileukemic and antineoplastic activities for the crude extracts and constituents from *Enantia chlorantha* [7]. The protoberberines have been shown to have antimicrobial activity decreasing in the sequence berberine > palmatine > jatrorrhizine against *Staphylococcus aureus* [3]. In our hands in the present study however, jatrorrhizine was found to show a slightly higher antifungal and antibacterial activity than palmatine against both *Candida albians* and *Staphylococcus aureaus* respectively.

In a separate study, compounds 1 and 2 and derivatives[3-5] have been shown by us[8] to have in-vitro antiplasmodial activities in a comparable range with that of chloroquine (e.g. IC_{50} = 0.51 and 0.36 $\mu g\ ml^{-1}$ for compounds 1 and 2 respectively) against multi-drug resistant *Plasmodium falciparum*.

The moderately strong antimicrobial activities demonstrated by both the crude extracts as well as 1 and 2 in the present study in addition to the already reported antiplasmodial activities[8] would appear to justify the folkloric reputation of *E. chlorantha* stem-bark in the management of parasitic, fungal and bacterial infections.

Acknowledgements

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