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IN VITRO AND *IN VIVO* ANTIMICROBIAL EVALUATION OF ALKALOIDAL EXTRACTS OF *ENANTIA CHLORANTHA* STEM BARK AND THEIR FORMULATED OINTMENTS

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Abstract: The *in vitro* and *in vivo* antimicrobial evaluation of the formulated ointment of alkaloidal extract of *Enantia chlorantha* Oliv. (Annonaceae) was the concern of this study. The alkaloidal fraction of the stem bark extract was formulated into simple ointment using British Pharmacopoeia formula for preparation of simple ointment. Agar diffusion and agar dilution methods were used for the *in vitro* antimicrobial studies. Ketoconazole 4000 µg/mL and tioconazole cream 1% were used as reference standards while normal saline was used as control. The fungicidal activity kinetics of the plant extract was carried out using selected concentrations of the plant extract against the most sensitive organism (*Candida albicans*). For the *in vivo* studies, 25 albino rats weighing between 180–200 g were divided into 5 groups, anesthesized (thiopental sodium 50 mg/kg), infected with overnight culture of *Candida albicans* and incubated at 37°C for three days to allow for growth of the microorganisms. Each of the five groups was treated on the third day of incubation with different concentrations of the formulated simple ointment (200 mg/mL, 100 mg/kg, 50 mg/mL), tioconazole cream 1% (reference standard) and normal saline control, respectively. The alkaloidal extract exhibited greater zones of inhibition with *Candida glabrata* and *Trichophyton tonsurans* while *Candida albicans* and *Trichophyton interdigitali* also showed some sensitivity. There was no surviving organism at the end of 240 min at 100 mg/mL concentration with 10^{-4} dilution factor. Treatment of the infected rats with the formulated simple ointments (200, 100 and 50 mg/mL) showed that 50 mg/mL ointment had a better percentage reduction in the fungal loads at the end of the experiment when compared with the 200 mg/mL simple ointment as well as the standard tioconazole 1% cream and normal saline treated rats, respectively. The alkaloidal fraction of *Enantia chlorantha* stem bark as well as the formulated ointment exhibited significant *in vitro* and *in vivo* antifungal activities against different species of *Candida*, dermatophytes and plant fungi.

Keywords: anticandidal, *Candida albican, Enantia chlorantha*, ointment formulation

Current treatment of candidiasis and other infections caused by *Candida* species is becoming very expensive and unaffordable by a large section of the population in developing economies. Many plant extracts have folkloric use in the treatment of infections. *Enantia chlorantha* plant extract has previously been investigated for antifungal activities (1). The plant belongs to the family Annonaceae and is known locally in Yoruba as Awopa, Osopupa or Dokita Igbo. It is an under storey tree of high rainforest. It is also an ornamental tree which may grow up to 30 m high, with dense foliage and spreading crown. The outer bark, which is thin and dark brown, is fissured geometrically while the inner bark is brown above and place cream beneath. The stem is fluted and aromatic while the elliptic leaves are about 0.14–0.15 m long and 0.05–0.14 m broad (2). The leaves display up to 20 pairs of prominent lateral vein and parallel secondary nerves. It is a dense forest tree found in the Western and Southern forest of Cameroon, Southern part of Nigeria, Gabon, Angola and Zaire. It is widely distributed along the coasts of West and Central Africa (2). *Enantia chlorantha* is a medicinal tree used for the treatment of malaria and other ailments of the human body. Gill and Akinwunmi (3) reported the use of infusion of the plant bark for the treatment of cough and wounds. Wafo et al. (4) noted the antiviral activity of extracts from the dried stem bark. *Enantia chlorantha* is also used for the management of stomach problems in the southern forest zone of Cameroon as well as for the treatment of jaundice, tuberculosis, urinary tract infection, malaria, hepatitis and some forms of ulcer. The decoction of the

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root in addition to the root of *Carica papaya* is used for the management of malaria. The decoction of the stem bark of *Enantia chlorantha* is also used for the treatment of leprous spots and liver damage. The stem bark has also been reported to be used as antipyretic and uterus stimulant (5). Palmatine chloride and jatrorrhizine chloride have been identified as the major antimicrobial constituents of the plant extracts (6).

Hence, this study is concerned with the formulation and antimicrobial evaluation of purified alkaloidal extracts obtained from the stem bark of *Enantia chlorantha* Oliv. (Annonaceae) found to be effective against *Candida albicans* and other fungal strains.

MATERIALS AND METHODS

Plant material

Enantia chlorantha (Annonaceae) stem bark was obtained from herb sellers in Bode Market in Ibadan, Nigeria and identified at the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Nigeria. A voucher sample was deposited at the Department and compared with previously collected sample (IB 197/241).

Preparation of extract

One kilogram (1 kg) of the sun dried chopped *Enantia chlorantha* stem bark was pulverized with a hammer-mill to obtain a coarse powder and macerated in 70% ethanol for 72 h. After decantation, maceration was for 48 h to ensure thorough extraction. The combined filtered ethanolic extract was concentrated to dryness and the resulting residue weighed and refrigerated until use.

Preparation of alkaloidal fraction (AF)

Forty grams (40 g) of the crude ethanolic extract was acidified with 5% HCl (500 mL) and filtered through kieselghur under vacuum. The filterate was lyophilized and the dried AF mixture was obtained.

Formulation of ointment

The dried alkaloid fraction was incorporated into Simple Ointment BP (7) to produce formulations yielding 50, 100, and 200 mg/mL *Enantia chlorantha* ointments by levigation.

Antimicrobial evaluation

The agar-well diffusion bioassay method (7, 8) was used. One hundred mL molten sterile nutrient broth was cooled to 50^{o}C and inoculated with 1 mL of overnight culture of test organism. Twenty mL quantities of the inoculated medium was each poured into a 9 cm agar plates and allowed to set. Equidistant wells of 6 mm were bored into the agar using a sterile cork borer and the wells were filled with appropriate concentrations of the extracts under test.

All extracts were reconstituted in 50% v/v aqueous methanol, which was used as a control while Bertrosil Cream (tioconazole), Trosyd Cream (tioconazole) and ketoconazole (4000 µg/mL) were used as reference standards. The plates were left at room temperature for 45 min and then incubated at 37^{o}C for bacterial strains and 25^{o}C for fungal strains. After an incubation period of 24 and 48 h for bacterial and fungal strains, respectively, the diameters of the zones of inhibition were measured. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) measures were determined by the broth dilution method (9).

Animal studies

Male and female albino rats weighing between 180 and 200 g, respectively, were purchased from the animal house of the University of Ibadan. Twenty five albino rats weighing between 180–200 g were divided into five groups for the *in vivo* anticandidal studies and were fed with standard feeds and water. Thiopental sodium injection was used as an anesthetic agent and administered based on their body weight at a dose 50 mg/kg. The dorsal flank of the albino rats were carefully shaved and the exposed skin part were bruised. An overnight *Candida albicans* culture was applied on the bruised skin parts of the dorsal flank of the albino rats using sterile swab sticks and observed for a period of 3 days. The growth of the *Candida albicans* was established on the third day. Each of the five groups were then treated on daily basis with different concentrations of the *Enantia chlorantha* ointment (200, 100 and 50 mg/mL), tioconazole cream 1% (reference standard) and normal saline control, respectively. The swabs taken on days 0, 3, 7, 10 and 13 were placed on a tryptone soya broth and incubated for 3 days at 37^{o}C. Serial dilutions were carried out and the fungal loads were counted using colony counter.

Statistical analysis

Statistical analysis was carried out using Students' *t*-test and ANOVA. At 95% confidence interval, p value lower or equal to 0.05 was considered the limit of significance (GraphPad Software Incorporation, San Diego, USA).

RESULTS AND DISCUSSION

The investigation of the antifungal activity of the alkaloidal fraction of the stem bark of *Enantia chlorantha* used ethno-medicinally for the management of various infectious diseases was carried out. The plant extract was tested against different species of *Candida*, dermatophytes and plant fungi, respectively. The fungal species used in these studies were *Candida valida, Candida pseudotropicalis, Candida tropicalis, Candida glabrata, Candida krusei, Candida albicans, Trichophyton rubrum, Trichophyton interdigitalis, Trichophyton tonsurans, Epidermophyton flocossum, Coletotrichum gloesporoides, Trichoderma asperelum, and Fusarium* spp. The bacteria species used in these studies were *Staphylococcus aureus* and *Escherichia coli*.

The minimum inhibitory concentration of the alkaloidal fraction of the crude ethanolic extract of stem bark of *Enantia chlorantha* are shown in Tables 1 and 2, respectively. The results show that the alkaloidal fraction of the stem bark of *Enantia chlorantha* was active at various concentrations (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563 and 0.781 mg/mL) on test organisms. It was at the following order of decreasing sensitivity; *Candida albicans* >

Table 1. Minimum inhibitory concentration of the alkaloidal fraction of the stem bark of *Enantia chlorantha* using agar diffusion method.

	Conc. mg/mL	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	1	3	9	SA	EC
1	200	16	18	17	15	16	20	18	23	19	19	18	20	24	32	20
2	100	12	15	14	13	13	18	16	20	16	16	15	16	19	26	17
3	50	10	13	11	10	11	15	12	17	12	14	13	13	17	23	16
4	25	–	–	–	–	–	14	–	–	10	12	11	–	13	21	13
5	12.5	–	–	–	–	–	13	–	–	–	10	10	–	10	19	10
6	6.25	–	–	–	–	–	10	–	–	–	–	–	–	–	17	–
7	-ve	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	BC	–	–	–	–	–	–	–	–	–	–	–	–	–	27	–
9	TC	–	–	–	–	–	–	–	–	–	–	13	–	15	–	–
10	KE	–	–	–	25	–	12	–	12	20	–	–	–	–	–	–

Keys: -ve: Negative control (methanol), BC: Bertrosil Cream (Tioconazole cream by Drugfield Pharmaceuticals), TC: Trosyd Cream (Tioconazole cream by Pfizer), KE (4000 µg/mL) Ketoconazole, C1: *Candida valida*, C2: *Candida pseudotropicalis*, C3: *Candida tropicalis*, C4: *Candida glabrata*, C5: *Candida krusei*, C6: *Candida albicans*, D1: *Trichophyton rubrum*, D2: *Trichophyton interdigitalis*, D3: *Trichophyton tonsurans*, D4: *Epidermophyton flocossum*, 1: *Colletotrichum gloesporoides* from yam blight, 3: *Triochoderma asperelum* from banana fruit, 9: *Fusarium* spp from yam, SA: *Staphylococcus aureus*, EC: *E. coli*

Table 2. Agar dilution method used to determine the minimum inhibitory concentration of the alkaloidal fraction of the stem bark of *Enantia chlorantia*.

Conc. mg/mL	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	1	3	9	SA	EC
50	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
25	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
12.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.563	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.781	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Keys: – Absence of growth, + Presence of growth, C1: *Candida valida,* C2: *Candida pseudotropicalis*, C3: *Candida tropicalis*, C4: *Candida glabrata*, C5: *Candida krusei*, C6: *Candida albicans*, D1: *Trichophyton rubrum*, D2: *Trichophyton interdigitalis*, D3: *Trichophyton tonsurans*, D4: *Epidermophyton flocossum*, 1: *Colletotrichum gloesporoides* from yam blight, 3: *Triochoderma asperelum* from banana fruit, 9: *Fusarium* spp from yam, SA: *Staphylococcus aureus*, EC: *E. coli*

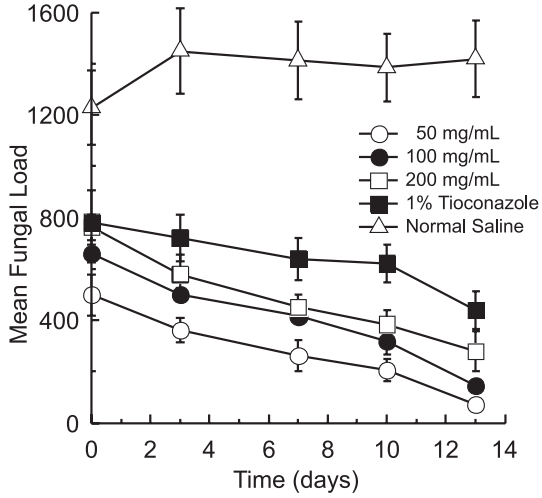

Figure 1. Effect of *Enantia chloranta* ointment on the fungal load in rats

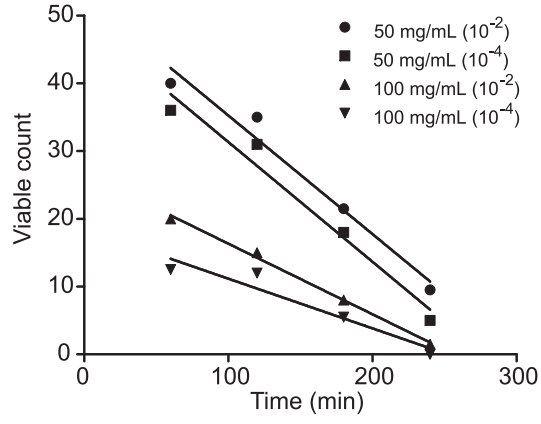

Figure 2. Kinetics of fungicidal activity of alkaloidal fractions of *Enantia chlorantha* against *Candida albicans* at different dilution factors

Candida krusei > *Candida glabrata* > *Candida pseudotropicalis* > *Candida tropicalis* > *Candida valida*. For the dermatophytes, the sensitivity was at the following order of decreasing sensitivity: *Trichophyton interdigitalis* > *Trichophyton rubrum* > *Epidermophyton flocossum* > *Trichophyton tonsurans*. For the plant fungi, the zone of inhibition was in the following order of decreasing sensitivity: *Fusarium* spp > *Trichoderma asperelum,* > *Colletotrichum gloesporoide*, respectively. *Staphylococcus aureus* had a larger zone of inhibition compared to *Escherichia coli*, which were the bacterial species used for the studies.

Results of sensitivity testing for the antifungals used as reference standard (Table 1) revealed that ketoconazole showed larger zones of inhibition against *Candida glabrata* and *Trichophyton tonsurans*. *Candida albicans* and *Trichophyton interdigitalis* showed the same zone of inhibition with the 4000 mg/mL concentration of ketoconazale. Tioconazole cream 1% exhibited better zone of inhibition for the *Fusarium* spp., followed by *Trichophyton rubrum* (Table 1). Methanol, which was used as a negative control, showed no zone of inhibition.

The kill kinetics or the fungicidal activities (Table 3) of the extracts were also investigated using

Table 3. Kinetics of fungicidal concentration of alkaloidal fraction of the stem bark of *Enantia chlorantha* for 50 and 100 mg/mL concentration using 10^{-2} and 10^{-4} dilution factors.

Time (min)	VC (50 mg/mL) 10^{-2}	VC (50mg/mL) 10^{-4}	VC (100 mg/mL) 10^{-2}	VC (100 mg/mL) 10^{-4}
0	Numerous	Numerous	Numerous	Numerous
60	40.0	36.0	20.0	12.0
120	35.0	31.0	15.0	12.5
180	21.5	18.0	8.0	5.5
240	9.5	5.0	1.5	0.0

Key: VC – No. of viable colonies. Numerous – Values of viable colonies or growth above 1000.

Table 4. The percentage reduction of fungal load after 13 days of treatment with formulated ointment, Tioconazole cream 0.10 mg/mL and normal saline.

Sample	Concentration (mg/mL)	Fungal load after 13 days
Formulated ointment of alkaloidal extract	200	63.50
	100	77.97
	50	83.38
Tioconazole cream	0.10	43.93
Normal saline	–	–15.44

the most potent concentration of the plant extracts against the most sensitive organism (*Candida albicans*). The result obtained showed that the number of the viable organisms was reduced with contact time. At the end of 240 min at 100 mg/mL concentration with 10^{-4} dilution factor, there was no surviving organism. The kinetics were found to be linear ($r^2 > 0.9$) for all treatments except for the normal saline control (Fig. 2).

The fungal load for each of the formulated ointment concentrations (200, 100, 50 mg/mL) showed a reduction in the fungal load with respect to the days interval in which the swab were taken as shown in Table 4. Toconazole cream 1% was used as a reference standard in the treatment of *Candida albicans* infected rats. It demonstrated a good reduction of the fungal load as could be seen in Table 4. Normal saline was used as a negative control to treat the *Candida* infected skin of the albino rats used. In this group, there was no significant reduction of the fungal load (Table 4).

The 50 mg/mL ointment formulation produced a higher percentage reduction of fungal load when compared to 200 and 100 mg/mL ointments, tioconazole cream 1% (reference standard) and normal saline control, respectively (Table 4). This significant reduction in fungal loads demonstrated by 50 mg/mL *Enanatia chloranthia* ointment would appear to suggest that there is an optimal concentration of the alkaloidal fraction required to elicit maximal action against *C. albicans*. This observation therefore calls for further probe into the response pattern of *C. albicans* to various concentrations of the individual protoberberine alkaloids in the alkaloidal fraction used in preparing the ointment.

The alkaloidal fraction of the stem bark of *Enantia chlorantha* exhibited antifungal activity against the different strains of *Candida albicans*, dermatophytes and the plant fungi. The result obtained shows that the plant extracts possess antifungal property and can be effective in the treatment of fungal infections since they inhibit the growth of fungal causative agents of infections. This finding buttresses earlier reports of many researchers, that medicinal plants or traditional medicine have a critical role in the provision of health care coverage for over 80% of the world population especially in the developing world (10–13). Tables 1 and 2 show that the alkaloidal fraction of the stem bark of *Enantia chlorantha* have very large zones of inhibition with *Staphylococus aureus* and *E. coli*, respectively (15). Of particular interest in this study, is the resistance of the test organisms to the conventional antifungal drugs but their sensitivity to the alkaloidal fraction

of *Enantia chlorantha* plant extracts. This is an indication that this plant if well harnessed, formulated under standard and good manufacturing practice can compete favorably with some of the existing antifungal drugs. This plant product can therefore be used in the management of *Candida*, dermatophyte and plant fungi infections. Following the result of these findings on different species of *Candida*, dermatophytes, and plant fungi, respectively, it is highly recommended that these plant extracts and constituents be incorporated into antifungal skin creams, causative organism responsible for skin fungal infections and vaginal candidiasis. Other forms of fungal infections like *Candida balanitis*, *Tinea barbae* and oropharyngeal candidiasis can as well be researched into using these same plant extracts.

Further investigations of the activities of these plant extract and constituents against a wider range of fungi, and bacteria should be carried out with a view to develop phytotherapeutic agents for enhanced and cost-effective healthcare delivery in developing economies considering the fact that the plant is safe owing to the toxicological investigation earlier carried out (16, 17), in addition to the relevant chemical constituents of the plant which has already been identified by Moody et al. (1).

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