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Antifungal Activity of Crude Extract and Metabolites of Leaves of *Enantia chlorantha* Oliv. (African Yellow Wood)

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ABSTRACT

An ethanol extract of leaves of *Enantia chlorantha* (LE) was treated with $NaHCO_3$, HCl and chloroform to obtain acidic metabolite (ALE), basic metabolites (BLE) and neutral metabolite (NLE) respectively. Phytochemical investigation revealed the presence of flavonoids and aldehydes/ketones in all the samples; alkaloids in LE, ALE and NLE; saponins in LE, ALE and BLE; carbohydrates in ALE; steroids in LE; terpenoids in ALE and BLE, cardiac glycosides in LE and BLE and phenols in BLE and NLE. Soluble extractives of the plant sample were also studied using four different solvents in both cold and hot extraction. Solvents used were petroleum ether, chloroform, ethanol and water. Highest soluble extractive values were found with water and ethanol (21.84%/23.78% and 22.28%/30.78%) while the least extractive values were found in chloroform and petroleum ether (10.50%/10.66% and 7.00%/10.93%) for cold and hot extraction respectively. Antifungal activity of LE, ALE, BLE, NLE and Ketoconazole (standard) showed that all the fungal pathogens were resistant to ALE and BLE. LE and NLE exhibited reasonable activity against the pathogens which were comparable with the activity of the standard. NLE showed higher zones of inhibition than LE in almost all the pathogens tested.

1. Introduction

Apart from the primary metabolites (carbohydrates, proteins, lipids, nucleic acids) plants also accumulate metabolites called "secondary" whose physiological function is not always obvious. These secondary metabolites are an important source of molecules used by man as drugs, food etc., [1]. Medicinal plants are a reservoir of natural compounds with beneficial effects. Certain compounds identified in the extracts of leaves, seeds, stem and root such as phenolic compounds are endowed with extremely important biological activities [2]. Several researchers [3, 4] demonstrated that polyphenols are the main antimicrobial plant compounds having different modes of action: (inhibitory and lethal) towards many microorganisms.

Enantia chlorantha Oliv. (African yellow wood) is a medicinal plant that has been used traditionally in Southwestern, Nigeria. It belongs to the family Annonaceae and has diverse uses. One of those medicinal plants that have been used in many localities in Nigeria to manage fever is *Enantia chlorantha* [5]. *E. chlorantha* Oliv (family-Annonaceae) locally known as Oniong (Ibibio), Awogba, Oso pupa or Dokita igbo (Yoruba), Osomolu (Ikale), Kakerim (Boki) and Erenba vbogo (Bini), is widely distributed along the coasts of west and Central Africa. It is also very common in the forest regions of Nigeria [5]. It may grow up to 30 m high with dense foliage and spreading crown [6]. The outer bark which is thin and dark brown is fissured geometrically, while the inner bark is brown above and pale cream beneath [7]. 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 [8]. The stem bark is used for treating leprosy spots, as haemostatic agent and uterus stimulant [9]. The possible use of the plant in conditions such as rickettsia fever, typhoid fever, infective hepatitis or jaundice has also been reported [10]. Studies have shown that the stem bark of *E. chlorantha* has wide spectrum antimicrobial activity [5], antimalarial and antipyretic properties. In Cameroon, stem bark extract is used to treat jaundice and urinary tract infections [11].

The more current and most effective antibiotics are very expensive and out of reach of many Africans, majority of whom reside in the rural areas. These antibiotics are also associated with some serious side-effects. A medicinal plant, such as *E. chlorantha*, is readily available and affordable. Recently, the side effects associated with the use of orthodox medicine have resulted in an increased demand for the phytopharmaceutical products of traditional medicine [12]. The present study was undertaken specifically to investigate the phytochemicals present in ethanol extract of *E. chlorantha* leaves and its metabolites and also to assess their antimicrobial potency against some human pathogenic fungi.

2. Experimental Methods

2.1 Collection and Preparation of Plant Samples

Leaves of *E. chlorantha* plants were collected from Ikot Abia Enin village in Mkpat Enin L.G.A. of Akwa Ibom state. The freshly collected plant sample was air-dried and pulverized into a semi-powdered solid using mortar and manual grinding machine.

2.2 Preparation of Extracts

Freshly collected leaves of *Enantia chlorantha* were air-dried and ground to a semi-powder. About 30 g of each of the semi-powdered sample was extracted with 250 mL of ethanol for 12 hrs in a soxhlet extractor equipped with a reflux condenser. The ethanol was removed from the extract using rotary evaporator to give a gel-like solid, which was dissolved in ethanol/water mixture (4:1) and filtered using vacuum pump. The filtrate of each of the sample from the plant was used for preparation of acidic, basic and neutral metabolites and for preliminary phytochemical test and antimicrobial experiment.

2.3 Extractive Values

2.3.1 Cold Extractive Values

The air-dried powdered plant sample (2 g) was macerated separately with solvents (petroleum ether, chloroform, methanol and water) of volume 100 mL in a closed flask for 24 hours, shaken frequently at six hours interval. It was filtered rapidly, taking precaution against loss of solvent, the filtrate was evaporated to dryness in a tarred flat bottom dish and dried on water bath to constant weight and weighed [13].

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2.3.2 Hot Extraction Values

The powdered material of the plant samples (10 g) was packed in a soxhlet apparatus separately for each solvent like petroleum ether, chloroform and ethanol but in case of water extract, the extract was prepared by decoction method. Each extract was evaporated to dryness and constant extractive value recorded.

2.4 Preparation of the Metabolites

2.4.1 Basic Metabolites

The filtrate obtained above was treated with 30 mL of conc. HCl and 30 mL of chloroform and shaken vigorously. The chloroform layer was removed using a separating funnel. The HCl layer was then treated with dilute $NaHCO_3$ solution to adjust pH to about 7. The mixture was evaporated completely using rotary evaporator to produce a crystal which was dissolved in 95% ethanol and filtered. This filtrate was used, without further purification for phytochemical and antifungal investigations.

2.4.2 Neutral Metabolites

The chloroform layer obtained during preparation of basic metabolite above was placed in a separating funnel, treated with 30 mL of dilute $NaHCO_3$ solution, shaken vigorously and allowed to stay under laboratory conditions for at least 3 hours. After equilibrating, the chloroform layer was separated and evaporated completely to produce a gel using rotary evaporator. The gel of neutral metabolites was dissolved completely in chloroform. The dissolved gel of neutral metabolite was used without further purification for phytochemical and antifungal investigations.

2.4.3 Acidic Metabolites

The aqueous layer obtained above during preparation of neutral metabolite was treated with dilute HCl and tested with litmus paper until the mixture attained a pH of about 7. The mixture was evaporated completely using rotary evaporator to produce crystals which was dissolved in 95% ethanol. The ethanol solutions were used without further purification for phytochemical and antifungal investigations.

2.5 Test Microorganisms

Clinical isolates of the fungal pathogens used were obtained as stock culture from the department of microbiology, federal medical centre, Owerri, Imo State, Nigeria. They are *Candida albicans*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Rhizopus oligosporus*, *Aspergillus flavus*, *Aspergillus fumigates*, *Aspergillus niger* and *Fusarium equiseti*.

2.6 Preparation of Inoculums

Active cultures for experiments were prepared by transferring a loopfull of cells from the stock cultures to test tubes of nutrient broth and the tubes were incubated in shaker at 37 °C for 24 hrs and were used as the inoculums.

2.7 Assay of Antifungal Activity

Antifungal activities of the samples were studied by agar well diffusion method. Suspensions of fungal pathogens were prepared by transferring a loop full of fungi from Sabouraud dextrose agar slants into sabouraud dextrose broth. Lawn cultures of the test pathogens were prepared by swabbing sterile sabouraud dextrose agar plates with the fungal suspensions. Wells were punched with a sterile cork borer (6 mm internal diameter) and 250 mg/mL, 200 mg/mL and 150 mg/mL of the samples were added to the wells. Following incubation at 27 °C for 48 hrs, diameters of the inhibitory zones were measured to the nearest millimeter.

3. Results and Discussion

Phytochemical screening results of the powdered sample of leaves of *E. chlorantha* extracted in ethanol showed the presence of greater number of phytochemicals than the metabolites (Table 1). It is necessary to identify the phytochemical components of local medicinal plants usually employed by herbalists in the treatment of diseases [14]. The presence or absence of certain phytochemicals could be responsible for some of the biological activity of certain plant extracts and metabolites obtained thereof. For example, saponins are special class of glycosides which have soapy characteristics and have been reported to be active antifungal agents [15]. Antimicrobial properties of a number of tannins, flavonoids, alkaloids have been reported. Not only the antimicrobial properties have been ascribed to these plant phytochemicals, but other biological activities including modulation of the immune system have been assigned to these compounds in plants. Phytochemical screening of the ethanol extracts of leaves of *E. chlorantha* revealed the presence of alkaloids, flavanoids, proteins, cardiac glycosides, saponins, tannins, steroids and aldehydes/ketones. While, ALE contains alkaloids, flavanoids, carbohydrates, proteins, triterpenoids, saponins, tannins, and aldehydes/ketones; BLE contains flavanoids, triterpenoids, saponins, cardiac glycosides, phenols and aldehydes/ketones. Alkaloids, flavanoids, triterpenoids, phenols, and aldehydes/ketones were found in NLE. Alkaloids are the largest group of phytochemicals causing toxicity against cells of foreign organisms [16]. Saponin possess antimicrobial property due to its ability to cause leakage of certain enzymes and proteins from the cell [17]. Flavonoids are hydroxylated phenolic substances synthesized by the plants in response to microbial infection and has the ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls [18]. Tannins are known to bind with proline rich proteins and interfere in the protein synthesis [19]. Steroids have been documented to have antibacterial properties; the steroids specifically associate with membrane lipid and exert its action by causing leakages from liposomes [20].

Table 1 Phytochemical investigations of ethanolic crude extract of Enantia chlorantha and its metabolites

Phytochemicals	LE	ALE	BLE	NLE
ALKALOIDS				
Dragendorff's test	+++	++	-	++
Mayer's test	+++	+++	-	++
Wagner's test	+++	++	-	++
FLAVONOIDS				
Alkali test	+++	+	+	+
PROTEIN				
Biuret test	+++	+++	-	-
CARBOHYDRATES				
Molisch's test	-	++	-	-
Fehling's solution test	-	-	-	-
TANNINS				
Hydrolysable tannins	++	+	-	-
Condensed tannins	-	-	-	-
STEROIDS				
Liebermann test	++	-	-	-
Salkowski test	++	-	-	-
Chloroform test	++	-	-	-
TERPENOIDS				
Liebermann test	-	+	+	-
Salkowski test	-	-	-	-
Chloroform test	-	-	++	-
CARDIAC GLYCOSIDES				
Liebermann test	-	-	-	-
Keller Killianic test	+	-	+	-
Bontrager's modification	+	-	-	-
SAPONIN				
Phenol	+++	++	++	-
Ferric Chloride test	-	-	+	+-
Acetic acid test	-	-	-	-
ALDEHYDES/KETONES	+	+	±	++

LE = ethanol extract of leaves of E. chlorantha; ALE = acidic metabolites of leaves of E. chlorantha; BLE = basic metabolites of leaves of E. chlorantha; NLE = neutral metabolites of leaves of chlorantha +++ = high; ++ = moderate; + = low; ± = negligible.

Table 2 Percentage soluble extractive of leaves of *E. chlorantha* with different solvents

Solvents	Cold Extract Values %	Hot Extract Values %
Petroleum ether	7.00 ± 0.20	10.93 ± 0.88
Chloroform	10.50 ± 0.50	10.66 ± 0.30
Ethanol	22.28 ± 0.49	30.78 ± 0.25
Water	21.84 ± 0.16	23.78 ± 0.50

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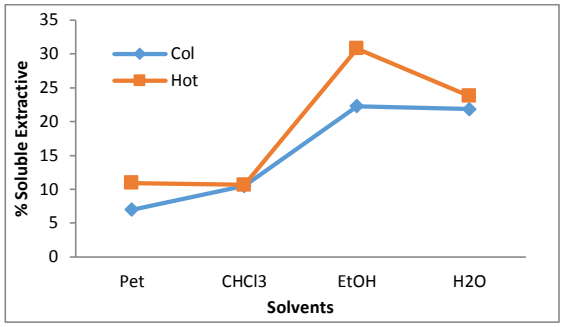

Fig. 1 Percentage extracts of leaves of *E. chlorantha* with different solvents *(Col = Cold extraction, Hot = Hot extraction, Pet = Petroleum ether, $CHCl_3$ = Chloroform, EtOH = Ethanol, H_2O = Water.*

Table 3 Result of antifungal activities of *Enantia chlorantha* and their metabolites

Test organism	Concentration of extracts (mg/mL)	Average zones of inhibition in millimetres				Ketoconazole (10.0 µg/mL)
		LE	ALE	BLE	NLE	
Candida albicans	250	20	-	-	20	28.0
	200	12	-	-	9	
	150	4	-	-	-	
Saccharomyces cerevisiae	250	18	-	-	18	30.0
	200	10	-	-	8	
	150	2	-	-	-	
Rhizopus oligosporus	250	12	-	-	20	30.0
	200	3	-	-	8	
	150	-	-	-	-	
Aspergillus flavus	250	16	-	-	18	32.0
	200	7	-	-	7	
	150	-	-	-	-	
Aspergillus fumigatus	250	15	-	-	15	34.0
	200	7	-	-	6	
	150	-	-	-	-	
Aspergillus niger	250	13	-	-	20	30.0
	200	4	-	-	7	
	150	-	-	-	-	
Fusarium equiseti	250	14	-	-	16	28.0
	200	5	-	-	6	
	150	-	-	-	-	

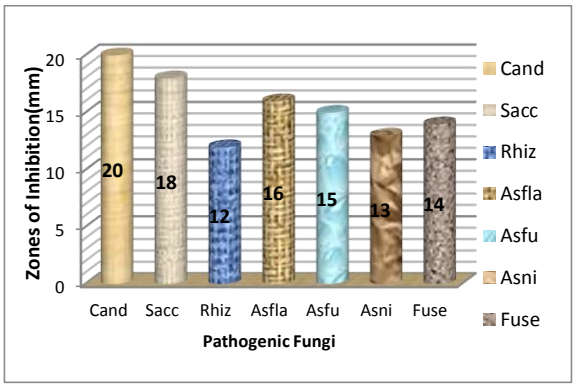

Fig. 2 Chart showing zones of inhibition of fungi by LE at 250 mg/mL

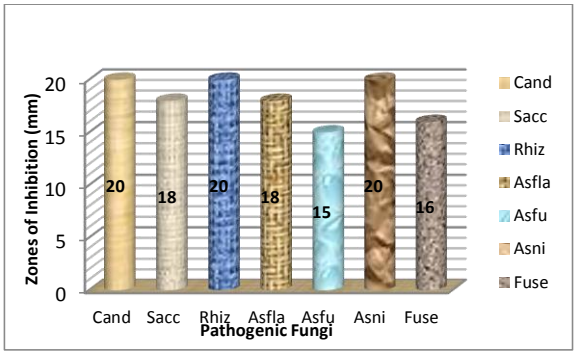

Fig. 3 Chart showing zones of inhibition of fungi by NLE at 250 mg/mL

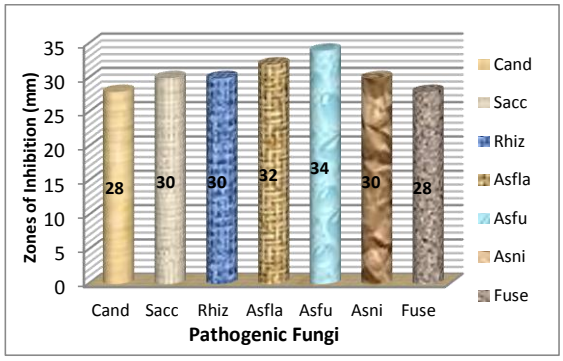

Fig. 4 Chart showing zones of inhibition of fungi by Ketoconazole at 10.0 µg/mL (*Can = Candida albicans, Sacc = Saccharomyces cerevisiae, Rhiz = Rhizopus oligosporus, Asfla = Aspergilus flavus, Asfu = Aspergilus fumigates, Asni = Aspergilus niger, Fuse = Fusarium equiseti)*

Extractive ability of four different solvents were studied using cold and hot extraction. Solvents used were petroleum ether, chloroform, ethanol and water. Highest soluble extractive values were found with water and ethanol while the least extractive values were found in chloroform and petroleum ether. Comparing the two extraction methods using Fig. 1, hot extraction yielded a higher soluble extractive in all the solvent than the cold extraction. Among all the solvents used, the amount of petroleum ether soluble extractive was comparatively lower than the chloroform, ethanol and water soluble extractives. This is consistent with what Momin and Kadam [21] reported from their work. They found that the amount of ether soluble extractive is comparatively lower than the alcohol and water soluble extractives. The percentage range of soluble extractive in both cold and hot extractions was as given in Table 2. Study of extractive values can serve as valuable source of information and provide suitable standards to determine the quality of plant material in future investigation [22].

The present study was carried out to compare the antifungal activity of ethanol extracts of leaves of *E. chlorantha* with those of acidic, basic and neutral metabolites obtained from the extracts are given in Table 3. The results of antifungal potential of ethanol extract of leaves *E. chlorantha* and its metabolites. Figs. 2-4 illustrate the extent at which each of the sample inhibited the tested fungal pathogens. NLE showed greater antifungal activity against *Rhizopus oligosporus, Aspergillus flavus, Aspergillus niger, Fusarium equiseti* than LE but relatively equal activity against *Candida albicans, Saccharomyces cerevisiae, Aspergillus fumigates*. Higher activity possessed by NLE may be due to the absence of proteins, carbohydrates, tannins, steroids, terpenoids, cardiac glycosides and saponins. All the fungi tested were completely resistant to ALE and BLE. On the whole, the standard drug (Ketoconazole 10.0 µg/mL) exhibited higher activity against all the tested pathogenic fungi than the plant samples. Absence of activity against the tested pathogenic fungi may be due the presence of terpenes in ALE and BLE which were not found in LE and NLE.

4. Conclusion

The result of phytochemical analysis showed variations in the presence of different phyto-compounds in LE, ALE, BLE and NLE. The variations in the phyto-constituents of these samples are believed to be responsible for the differences in their antifungal activity against the tested pathogenic fungi. Of all the samples obtained from the leaves of *E. chlorantha*; *Candida albicans, Rhizopus oligosporus, Aspergillus flavus, Aspergillus niger, Fusarium equiseti* were highly susceptible to NLE and were resistant to ALE and BLE. Comparing the two extraction methods used in determining the soluble extractive of the selected solvent, hot extraction yielded a higher soluble extractive in all the solvents than the cold extraction. Among all the solvent used, the amount of petroleum ether soluble extractive was comparatively lower than the chloroform, ethanol and water soluble extractive.

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