

Antiplasmodial activity of the n-hexane extract from *Pleurotus ostreatus* (Jacq. Ex. Fr) P. Kumm.

Abstract

Introduction: Several mushrooms species have been reported to be nematophagous and anti-protozoan. This study reported the antiplasmodial and cytotoxic properties of the n-hexane extract from the edible mushroom *Pleurotus ostreatus* and the isolation of a sterol from the extract.

Materials and Methods: Anti-plasmodial and cytotoxicity assays were done *in vitro* using the plasmodium lactate dehydrogenase (pLDH) assay and human HeLa cervical cell lines respectively. The structure of the isolated compound from the n-hexane extract was elucidated using spectroscopic techniques.

Results: The n-hexane extract (yield: 0.93 % w/w) showed a dose dependent antiplasmodial activity with the trend in parasite inhibition of: chloroquine ($IC_{50} = 0.016 \mu\text{g/ml}$) > n-hexane extract ($IC_{50} = 25.18 \mu\text{g/ml}$). It also showed mild cytotoxicity ($IC_{50} > 100 \mu\text{g/ml}$; selectivity index >4) compared to the reference drug emetine ($IC_{50} = 0.013 \mu\text{g/ml}$). The known sterol: ergosterol was isolated and characterized from the extract.

Conclusion: This study reporting for the first time the anti-plasmodial activity of *P. ostreatus* revealed its nutraceutical potential in the management of malaria.

Keywords: *Pleurotus ostreatus*, nutraceuticals, malaria, cytotoxicity, ergosterol.

INTRODUCTION

The scourge of malaria infections has continued to be a global health burden with countries in Sub-Saharan Africa contributing about ninety percent¹. Children and expectant mothers are the worst hit with attendant high mortality if not treated promptly. Malaria remains a threat to the poor people living in endemic regions, where access to quality health facilities is limited and cost of orthodox drugs is high. More worrisome is the high occurrence of drug resistant *Plasmodium falciparum* strains of the causative parasite. These obstacles to receiving effective treatment for malaria have led to the continued search for new anti-malarial agents that are relatively non-toxic. Bioactive metabolites from nature's flora and fauna are veritable leads in drug development.

Mushrooms are basidiomycetous fungi and the edible ones are popular not only for their nutritive value but also as functional foods in the treatment of various diseases. The anti-parasitic properties of some mushrooms and closely related fungi species have been reported. Some of which includes: the anti-malarial properties of cordyceps species², and *Bulgaria inquinans*³, the amoebicidal⁴ and anti-trypanosomiasis⁵ properties of *Pleurotus ostreatus*. Other reported biological activities include: nematocidal⁶, anti-inflammatory and immunomodulatory⁷ and anticancer⁸⁻⁹ properties among others. As a follow up to earlier reports on the scientific validation of the health benefits and the characterization of bioactive secondary metabolites from indigenous edible mushrooms in Nigeria¹⁰⁻¹³, this present study aimed to determine the nutraceutical potentials of the n-hexane extract of the fruiting bodies of the edible mushroom *Pleurotus ostreatus* in the management of malaria infections.

EXPERIMENTALS

Collection of mushroom sample

Pleurotus ostreatus (Fresh fruiting bodies) were collected from the Dilomat farm, Rivers State University of Science and Technology (RSUST), Port Harcourt, Rivers State and identified by a Mycologist in the Department of Crop and Soil Sciences, Faculty of Agriculture, University of Port Harcourt, Port Harcourt, Rivers State. After due authentication, a voucher specimen (UPH/C/075) was deposited at the herbarium of the Department of Plant Science and Biotechnology of the same University. The fresh fruiting bodies of *Pleurotus ostreatus* were chopped into small pieces after which they were dried under a current of air in a de-humidified environment. The dried samples were pulverized using an electric blender.

Preparation of extract

The dried pulverized fruiting body (362.1 g) was cold macerated for 72 hours with n-hexane with fresh replacement of solvent at 24 hours interval to obtain the n-hexane extract (NHE). The NHE was concentrated using a rotary evaporator (Model RE52A, Labscience made in India for England) and used for this study.

Phytochemical Methods

Confirmatory phytochemical tests were carried out on the extract using standard phytochemical screening reagents¹⁴⁻¹⁵

Isolation and purification of compound **1**

The bioactive n-hexane extract NHE (1g) was dissolved in n-hexane and pre-adsorbed on silica gel in the ratio of 1:1 w/w to form a homogenous paste which was allowed to air dry in a fume cupboard. The mixture was loaded on a chromatography column (internal diameter 4.1 cm and packed with normal phase silica gel mesh 200-400 to a height of 27 cm). The column was eluted with gradient of increasing order of polarity: n-hexane (100 %, 500 mL), n-hexane: dichloromethane (1:1, 500 mL); and dichloromethane (100 %, 500 mL). After Thin Layer Chromatography examination of the eluates, they were pooled into 3 sub-fractions F1-F3. The F2 eluted with n-hexane: dichloromethane (1:1) yielded a white solid compound **1** after re-crystallising with acetone. Its purity was determined using Thin layer chromatography (TLC) performed on plates pre-coated with silica gel 60 HF₂₅₄ (Merck, TLC grade, with gypsum binder). The TLC bands were visualized by exposure to iodine and by spraying with concentrated H₂SO₄ using spray gun. Complementary purity confirmation by melting points determination was recorded on an electrothermal melting point apparatus and are uncorrected.

The ¹H and ¹³C NMR spectra of **compound 1** was recorded at 300 MHz (75 MHz for ¹³C NMR analysis) on a Bruker Avance spectrometer in deuterated CDCl₃. Chemical shifts are expressed in parts per million (ppm) downfield of Trimethylsilane (TMS) as internal reference for ¹H resonances, and referenced to the central peak of the appropriate deuterated solvent's resonances. Infra red (IR) spectra were recorded on 1600 ATI Matson Genesis series FTIR™ spectrometer. Mass spectra were recorded on a FINNIGAN MAT 12 spectrometer. Unambiguous assignment of the positions was done using two-dimensional NMR (2D-NMR) experiments like: Heteronuclear Multiple Bond Correlation(HMBC), Heteronuclear Single Quantum Correlation (HSQC) and proton-proton correlation spectroscopy (H-H-COSY).

Açıklama [AM1]: RESPONSE TO REVIEWER 2 COMMENT: The wrong word "Compounds" is now corrected to "compound"

Cell viability assay

Briefly mammalian HeLa cells were plated in 96-well plates at 2×10^4 cell per well in 150 μ L the culture medium. The culture medium was prepared from Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5 mM L-glutamine, 10% (v/v) fetal bovine serum and antibiotics (penicillin/streptomycin/amphotericin B). After an overnight incubation in a 5% CO₂ humidified incubator, the various concentrations (0.006104 -100 μ g/mL) of the test samples prepared following a 10-fold serial dilutions approach in 96-well plates were added to the cultures (duplicate wells; 200 μ L final culture volume) and incubation continued for an additional 48 hours. The viability of cells in individual wells was assessed by adding 20 μ L of resazurin toxicology reagent (Sigma-Aldrich) per well and measuring fluorescence intensity (exc. 560 nm/em. 590 nm) in a Spectramax M3 plate reader after an incubation of 2 hours. Fluorescence readings in experimental wells were converted to % cell viability relative to control wells containing untreated cells and used to obtain the dose-response plots of mean % cell viability against log (test sample concentration) using the non-linear regression function of Microsoft Excel 2007 software with the median inhibition concentration IC₅₀ values derived from the plot by extrapolation. Emetine various concentrations (0.00000325-32.5 μ g/mL) prepared following a 10-fold serial dilutions approach in 96-well plates was used as standard drug for comparison.

Plasmodium falciparum growth inhibition assay

Briefly, the *P. falciparum* (3D7 strain) parasites were maintained in medium composed of RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM Hepes (buffered between a pH of 7.2 and 7.4), 5%(w/v) Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 μ g/mL gentamicin sulfate and 2-4% (v/v) human red blood cells, in an atmosphere containing a mixture of O₂, CO₂, N₂ (5:5:90 v/v/v). For the growth inhibition assays, parasite cultures were adjusted to 2% parasitaemia and 1% haematocrit (final) and incubated for 48 hours, after addition of the test samples (final test concentrations range of 0.006104 -100 μ g/mL prepared in duplicate following a 4-fold serial dilutions approach in 96-well plates (200 μ L culture/well; two wells per test sample dilution). After the incubation period, the levels of parasite were determined by colorimetric

determination of parasite lactate dehydrogenase activity¹⁶. Chloroquine (eight final test concentrations within the range 0.00000516129 – 51.6129 µg/mL) prepared following a 10-fold serial dilution was used as standard anti-malarial drug for comparison. At 620nm the absorbance values in the wells containing test samples and standard drug (chloroquine) were converted to percentage parasite viability relative to the wells containing untreated parasite cultures. The median pLDH inhibition concentration (IC₅₀) values were derived from graphs of mean % parasite viability against Log (test sample concentration) using the non-linear regression function of Microsoft Excel 2007 software.

RESULTS

Phytochemical analysis of the NHE and structural elucidation of compound 1: The n-hexane extract (NHE) was found to contain: isoprenoids (triterpenoid/steroids, cardenolides) and fatty acids as metabolites from phytochemical screening using appropriate standard reagents. Compound **1** was isolated and characterized from the NHE after chromatography separation and spectroscopic analysis respectively.

Structural elucidation/characterisation of isolated Compound 1:

Appearance: white solid,

Melting point: 155-160°C;

Solubility: freely soluble in chloroform, dichloromethane;

Molecular Mass: 396.7 (calculated for C₂₈H₄₄O).

IR spectrum [frequency, V, cm^{-1}]: 3412; 3404 [OH str], 3060 [=CH str], 2951; 2868 [CH₂/CH₃ str], 1655; 1600 [C=C str], 1053/1030 [C-O str], 727 [CH₂ rocking].

EI-Mass spectrum: [m/z (rel. int)]: 396 (62.66) [M⁺], 378 [M⁺ - H₂O], 363 (42.96) [M⁺ - (18+15)], 271 (20.45) [M⁺ - aliphatic chain], 253 (30.95) [M⁺ - H₂O - aliphatic chain], 285 [M⁺ - H₂O - 15-ring A], 69 (100), 55 (80.83), 57 (43.95), 43 (74.37).

¹H-NMR (300 MHz, CDCl₃, δppm): Details of the spectra data are presented in Table 1

¹³C-NMR (75 MHz, CDCl₃, δppm): Details of the spectra data are presented in Table 1

Table 1: Spectral data of compound 1 isolated from the n-hexane extract of *P.ostreatus*

S/No	<u>1</u> δ_C ppm	DEPT- 135	Published ¹⁷ δ_C ppm	<u>1</u> HSQC (δ_H ppm)	<u>1</u> H-multiplicity	<u>1</u> H-H-COSY	<u>1</u> HMBC
1	38.4	CH ₂	38.4	2.08	2Hm	H-2	C-3,
2	32.0	CH ₂	32.0	1.52, 1.91	2Hm	H-3, H _{1a}	
3	70.5	CH	70.4	3.65	1Hm	H-2a	
3-OH	-	-	-	-	-	-	-
4	40.8	CH ₂	40.8	2.32	2Hm	H-3	C-3
5	139.8	C	139.8	-	-	-	-
6	119.6	CH	119.6	5.60	1Hd	H-7	C-5, C-10
7	116.3	CH	116.3	5.42	1Hd	H-6	
8	141.4	C	141.3	-	-	-	-
9	46.3	CH	46.2	1.99	1Hm		
10	37.0	C	37.0	-	-	-	-
11	21.1	CH ₂	21.1	1.65, 1.70	2Hm		
12	39.1	CH ₂	39.1	2.05	2Hm		
13	42.8	C	42.8	-	-	-	-
14	54.6	CH	54.6	1.92	1Hm		
15	23.0	CH ₂	23.0	1.40, 1.70	2Hm		
16	28.3	CH ₂	28.3	1.38, 1.80	2Hm		
17	55.8	CH	55.7	1.30	1Hm		
18	12.0	CH ₃	12.0	0.65	3Hs		
19	17.6	CH ₃	17.6	0.93	3Hs		
20	40.4	CH	40.4	2.48	1Hm		
21	21.1	CH ₃	21.1	1.05	3Hd (J = 6 MHz)		
22	135.6	CH	135.6	5.25	1Hdd		C-20, C ₂₃
23	132.0	CH	132.1	5.20	1Hdd		C-20
24	42.8	CH	42.8	1.90	1Hd		
25	33.1	CH	33.1	1.52	1Hm	H-26, H-24, H- 27	
26	19.9	CH ₃	19.8	0.86	3Hd (J = 6 MHz)	H-25	
27	19.6	CH ₃	19.6	0.84	3Hd(J = 6 MHz)	H-25	
28	16.3	CH ₃	17.8	0.97	3Hd(J = 6 MHz)		

s: singlet, d: doublet, dd: doublet of doublet, m: complex multiplet

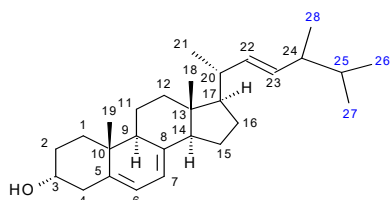


Figure 1: Compound 1: Ergostan-5, 6, 22-trien-3-ol (ergosterol)

Cytotoxicity activity: A marked onset of cytotoxicity for the n-hexane extract NHE (Figure 2 and Table 2) was observed at highest screened concentration of 100 $\mu\text{g/mL}$ (\approx 75% cell viability translating to \approx 25 % cell death and a selectivity index > 4). This is indicative of low cytotoxicity ($\text{IC}_{50} > 100 \mu\text{g/mL}$) compared to the reference drug emetine with $\text{IC}_{50} = 0.013 \mu\text{g/mL}$).

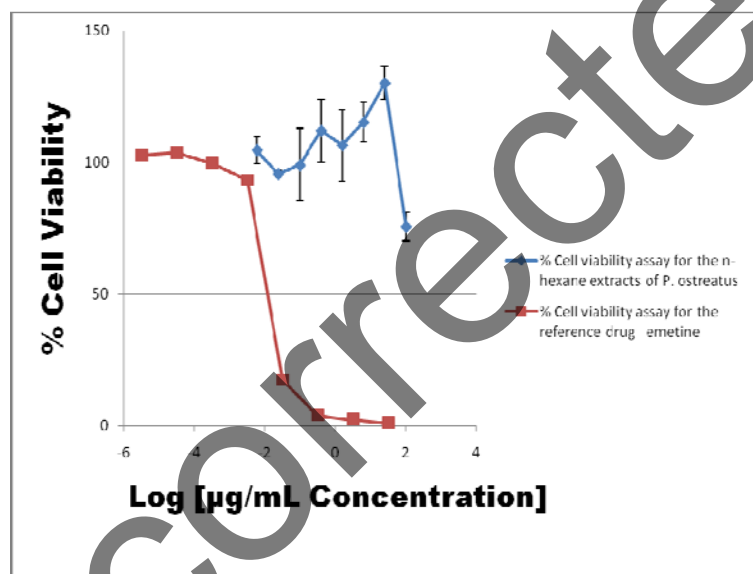


Figure 2 : Dose dependent mammalian HeLa cell viability profile of the n-hexane extracts of *P. ostreatus*

Anti-plasmodial activity

The NHE inhibited *Plasmodium* parasite lactate dehydrogenase activity in a dose dependent manner *in vitro* (Figure 3 and Table 2) with a median inhibition concentration (IC_{50}) of 25.18 $\mu\text{g/mL}$. It was however significantly ($p = 0.02, < 0.05$) less active compared to the standard drug chloroquine diphosphate ($IC_{50} = 0.016 \mu\text{g/mL}$ see Figure 3 and Table 2).

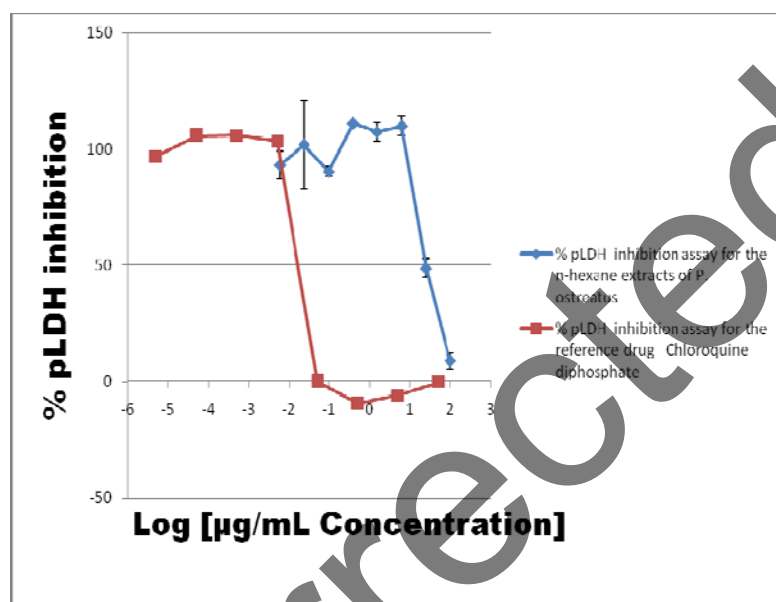


Figure 3: Dose dependent plasmodium parasite growth inhibition profile of the n-hexane extracts of *P. ostreatus*

Table 2: pLDH inhibition and mammalian cell (HeLa) viability assay results for the n-hexane extract (NHE) from *P. ostreatus* fruiting bodies

n-hexane extracts			Reference drug Emetine		Reference drug Chloroquine diphosphate	
Concentration	pLDH inhibition %		Concentration		Concentration	pLDH inhibition %
µg/mL		Cell viability %	µg/mL	Cell viability %	µg/mL	
0.006	93.160±6.067	104.670±5.054	0.00000325	102.571	0.00000516129	96.77495
0.024	101.742±19.000	95.809±1.053	0.0000325	103.607	0.0000516129	105.6567
0.098	90.250±2.164	99.096±13.659	0.000325	99.648	0.000516129	105.9009
0.391	110.896±0.239	111.970±11.730	0.00325	93.383	0.00516129	103.3283
1.563	107.290±4.063	106.438±13.305	0.0325	17.465	0.0516129	0.032861
6.250	109.731±4.222	115.149±7.605	0.325	3.878	0.5161289	-9.46859
25.000	48.751±3.997	130.106±6.337	3.250	2.282	5.161289	-5.91963
100.000	8.830±3.466	75.664±5.530	32.50	1.032	51.6129	-0.2488
IC ₅₀ (µg/mL)	25.179±2.456	>100	0.013		0.016	
Selectivity						
Index	>4	NA		NA		NA

DISCUSSION

The *Plasmodium* pLDH is an essential energy-producing enzyme. It is the last enzyme in the parasite glycolytic pathway. It is produced by both the sexual and asexual stages of parasites, as well as the mature gametocytes of all human *Plasmodium species*¹⁸⁻²⁰. The parasite and erythrocytic cells (human host) lack a complete citric acid cycle for mitochondrial ATP production making dependence on anaerobic glucose metabolism an imperative. Thus, the pLDH plays an important role in catalyzing energy production in the parasite^{18, 21}. The activity of enzyme pLDH is reported to be disappeared within 24 hours of effective malaria treatment²¹; the pLDH antigen is considered a specific marker for the presence of viable plasmodium in blood. Report by the United States National Cancer Institute (US-NCI) regards plant extract with cytotoxic IC₅₀ 20 µg/mL or lower as being highly cytotoxic²²⁻²⁴. Those with IC₅₀ greater than 100 µg/mL are regarded to be of low to non toxicity²². The observed low cytotoxicity of the NHE is suggestive that the observed anti-plasmodium activity may not necessarily be due to general cytotoxicity of the extract thus a clue to its potential as a source of non-toxic agents for drug development.

The presence of isoprenoids (triterpenoid/steroids, cardenolides) and fatty acids as metabolites in the n-hexane extract from phytochemical screening using appropriate standard reagents corroborated our earlier report about the presence of these metabolites in a closely related *species* *Pleurotus tuber regium*¹¹. The observed dose-dependent *plasmodium* pLDH inhibition by the NHE from the fruiting bodies of *P. ostreatus* could be due to the presence of these observed metabolites. Similar reports on the anti-malarial activities of edible mushroom and related fungi have been documented^{2-3,25}. After chromatography separation of the NHE, the compound **1** was isolated and its structure elucidated using mass spectrometry, nuclear magnetic resonance (1D and 2D) and fourier transform infra-red spectroscopic techniques to be the known compound: ergostan-5,7,22-trien-3-ol. Compound **1** gave positive Liebermann and Salkowski phytochemical test reagents confirming it to have a steroidal nucleus. The NMR spectra data (Table 2) are evident with the ¹H and ¹³C chemical shift signals for a conjugated di-substituted olefinic bond in ring B and the isolated olefinic

Açıklamalı [AM2]: RESPONSE TO REVIEWER 2 COMMENT: The wrong word "specie" is now corrected to "species"

Açıklamalı [AM3]: RESPONSE TO REVIEWER 2 COMMENT: The wrong word "regium" is now corrected to "regium"

bond in the aliphatic side chain, six methyl (two angular and four at the aliphatic side chain) and one secondary carbinol (CHOH) at position 3 of ring A which were unambiguously assigned as stated in Table 2 using 2D-NMR (HMBC, H-H-COSY, and HSQC) experiments. In all, a total of twenty-eight (28) carbon signals: four quaternary (4 x C), eleven methine (11 x CH) out of which one is the secondary carbinol (CHOH) at position 3 of ring A, seven methylene (7 x CH₂) and six upfield methyl (6 x CH₃) were observed which corresponded to the molecular formula C₂₈H₄₄O corroborating the observed molecular ion peak at m/z 396 from the EI-mass spectrum analysis. These trends in spectra data are characteristics of an unsaturated steroidal alcohol and when compared to literature reports for ergosterol¹⁷ were similar. Owing to solubility limitations, compound **1** was however not evaluated for the reported biological activities. Ergosterol derivatives like: ergosterol endoperoxide isolated from *Pleurotus ostreatus* have been reported to exhibit anti-parasitic properties like: trypanocidal⁵ and amoebicidal⁴ activities.

CONCLUSION

This study showed the first time the nutraceutical potential in the management of malaria infection, of the edible mushroom *Pleurotus ostreatus* cultivated in Nigeria. The isolation and characterization of the known steroid ergostan-5, 6, 22-trien-3-ol (commonly called ergosterol) from the bioactive extracts from spectroscopic analysis was also reported. After further investigation, this edible mushroom species may be recommended in the diet as a prophylaxis against malaria infection.

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CONFLICT OF INTEREST

There is no conflict of interest associated with this work.

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