Turk J Pharm Sci 2019;16(1):37-42
DOI: 10.4274/tjps.18894

ORIGINAL ARTICLE

Antiplasmodial Activity of the *n*-Hexane Extract from *Pleurotus ostreatus* (Jacq. ex. Fr) P. Kumm.

Pleurotus ostreatus (Jacq. ex. Fr) P. Kumm. *n*-Hekzan Ekstresinin Antiplazmodiyal Etkisi

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ABSTRACT

Objectives: Several mushrooms species have been reported to be nematophagous and antiprotozoan. 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: Antiplasmodial and cytotoxicity assays were done *in vitro* using the plasmodium lactate dehydrogenase 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 dose dependent antiplasmodial activity with the trend in parasite inhibition of: chloroquine (IC_{50}=0.016 µg/mL) > *n*-hexane extract (IC_{50}=25.18 µg/mL). It also showed mild cytotoxicity (IC_{50}>100 µg/mL; selectivity index >4) compared to the reference drug emetine (IC_{50}=0.013 µg/mL). The known sterol, ergostan-5,7,22-trien-3-ol, was isolated and characterized from the extract.

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

Key words: *Pleurotus ostreatus*, nutraceuticals, malaria, cytotoxicity, ergosterol

ÖZ

Amaç: Bazı mantar türlerinin nematofagöz ve antiprotozoan olduğu bildirilmiştir. Bu çalışmada, yenilebilir mantar *Pleurotus ostreatus n*-hekzan ekstresinin antiplazmodiyal ve sitotoksik etkileri araştılmış ve ekstreden bir sterol izolasyonu yapılmıştır.

Gereç ve Yöntemler: Antiplazmodiyal ve sitotoksisite deneyleri, sırasıyla, plasmodium laktat dehidrojenaz analizi ve insan HeLa servikal hücre hatları kullanılarak *in vitro* gerçekleştirilmiştir. *n*-Hekzan ekstresinden izole edilen bileşiğin yapısı, spektroskopik teknikler kullanılarak aydınlatılmıştır.

Bulgular: *n*-Hekzan ekstresi (verim: %0.93 a/a) parazit inhibisyonunda doza bağlı antiplazmodiyal aktivite gösterdi: klorokin (IC_{50}=0.016 µg/mL) > *n*-hekzan ekstresi (IC_{50}=25.18 µg/mL). Ayrıca referans ilaç emetine kıyasla (IC_{50}=0.013 µg/mL) hafif sitotoksisite (IC_{50}>100 µg/mL; seçicilik indeksi >4) gösterdi. Bilinen sterol bileşiği ergostan-5,7,22-trien-3-ol izole edildi ve yapısı tayin edildi.

Sonuç: *P. ostreatus*'un antiplazmodiyal aktivitesini ilk kez rapor eden bu çalışma, sıtma tedavisindeki nutrasötik potansiyelini ortaya koymuştur.

Anahtar kelimeler: *Pleurotus ostreatus*, nutrasötik, malarya, sitotoksisite, ergosterol

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Received: 03.12.2017, **Accepted:** 18.01.2018
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INTRODUCTION

The scourge of malaria infections has continued to be a global health burden, with countries in Sub-Saharan Africa contributing about ninety percent.[1] 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 the 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 nontoxic. 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 antiparasitic properties of some mushrooms and closely related fungi species have been reported. Some of these include: the antimalarial properties of *Cordyceps* species[2] and *Bulgaria inquinans*,[3] and the amoebicidal[4] and anti-trypanosomiasis[5] properties of *Pleurotus ostreatus.* Other reported biological activities include nematicidal,[6] anti-inflammatory and immunomodulatory,[7] and anticancer[8,9] 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 Nigeria,[10-13] the present study aimed to determine the nutraceutical potentials of the *n*-hexane extract (NHE) of the fruiting bodies of the edible mushroom *P. ostreatus* in the management of malaria infections.

EXPERIMENTAL

Collection of mushroom samples

P. ostreatus (fresh fruiting bodies) was collected from the Dilomat farm, Rivers State University of Science and Technology, 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 *P. ostreatus* were chopped into small pieces, after which they were dried under a current of air in a dehumidified 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 h with *n*-hexane with fresh replacement of solvent at 24-h intervals to obtain the 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 reagents.[14,15]

*Isolation and purification of compound **1***

The bioactive NHE (1g) was dissolved in *n*-hexane and pre-adsorbed on silica gel in the ratio of 1:1 w/w to form a homogeneous 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 (TLC) examination of the eluates, they were pooled into 3 subfractions: F1-F3. F2 eluted with *n*-hexane:dichloromethnane (1:1) yielded a white solid compound **1** (Figure 1) after re-crystallizing with acetone. Its purity was determined using TLC performed on plates pre-coated with silica gel 60 HF_{254} (Merck, TLC grade, with gypsum binder). The TLC bands were visualized by exposure to iodine and by spraying with concentrated H_2SO_4 using a spray gun. Complementary purity confirmation by melting point determination was recorded on an electrothermal melting point apparatus and the results are uncorrected.

The ^1H and ^{13}C-NMR spectra of compound **1** (Figure 1) were recorded at 300 MHz (75 MHz for ^{13}C-NMR analysis) on a Bruker Avance spectrometer in deuterated $CDCl_3$. Chemical shifts are expressed in parts per million (ppm) downfield of trimethylsilane as internal reference for ^1H resonances, and referenced to the central peak of the appropriate deuterated solvent's resonances. Infrared spectra were recorded on a 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 nuclear magnetic resonance (2D-NMR) experiments like heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and proton–proton correlation spectroscopy (H-H-COSY).

Cell viability assay

Briefly mammalian HeLa cells were plated in 96-well plates at 2×10^4 cell per well in 150 µL the culture medium. The culture medium was prepared from Dulbecco's Modified Eagle's Medium supplemented with 5 mM L-glutamine, 10% (v/v) fetal bovine serum, and antibiotics (penicillin/streptomycin/amphotericin B). After overnight incubation in a 5% CO_2 humidified incubator, various concentrations (0.006104-100 µg/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 µL of final culture volume) and incubation continued for an additional 48 h. The viability of cells in individual wells was assessed by adding 20 µL 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 incubation for 2 h. Fluorescence readings in experimental wells were converted to % cell viability relative to control wells containing untreated cells and used to obtain dose-response plots of mean % cell viability against log (test sample concentration) using the nonlinear regression function

of Microsoft Excel 2007 software with the median inhibition concentration IC_{50} values derived from the plot by extrapolation. Emetine of various concentrations (0.00000325-32.5 µg/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 µg/mL gentamicin sulfate, and 2-4% (v/v) human red blood cells, in an atmosphere containing a mixture of O_2, CO_2, and N_2 (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 h, after addition of the test samples (final test concentrations range of 0.006104-100 µg/mL prepared in duplicate following a 4-fold serial dilutions

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

S/No.	1 δ_C ppm	DEPT-135	Published[17] δ_C ppm	1 HSQC (δ_H ppm)	1 H-multiplicity	1 H-H-COSY	1 HMBC
1	38.4	CH_2	38.4	2.08	2 Hm	H_{-2}	C_{-3}
2	32.0	CH_2	32.0	1.52, 1.91	2 Hm	H_{-3}, H_{1a}	
3	70.5	CH	70.4	3.65	1 Hm	H_{-2a}	
3-OH		-	-				
4	40.8	CH_2	40.8	2.32	2 Hm	H_{-3}	C_{-3}
5	139.8	C	139.8	-			
6	119.6	CH	119.6	5.60	1 Hd	H_{-7}	C_{-5}, C_{-10}
7	116.3	CH	116.3	5.42	1 Hd	H_{-6}	
8	141.4	C	141.3	-			
9	46.3	CH	46.2	1.99	1 Hm		
10	37.0	C	37.0				
11	21.1	CH_2	21.1	1.65, 1.70	2 Hm		
12	39.1	CH_2	39.1	2.05	2 Hm		
13	42.8	C	42.8				
14	54.6	CH	54.6	1.92	1 Hm		
15	23.0	CH_2	23.0	1.40, 1.70	2 Hm		
16	28.3	CH_2	28.3	1.38, 1.80	2 Hm		
17	55.8	CH	55.7	1.30	1 Hm		
18	12.0	CH_3	12.0	0.65	3 Hs		
19	17.6	CH_3	17.6	0.93	3 Hs		
20	40.4	CH	40.4	2.48	1 Hm		
21	21.1	CH_3	21.1	1.05	3 Hd (*J*=6 MHz)		
22	135.6	CH	135.6	5.25	1 Hdd		C_{-20}, C_{23}
23	132.0	CH	132.1	5.20	1 Hdd		C_{-20}
24	42.8	CH	42.8	1.90	1 Hd		
25	33.1	CH	33.1	1.52	1 Hm	H_{-26}, H_{-24}, H_{-27}	
26	19.9	CH_3	19.8	0.86	3 Hd (*J*=6 MHz)	H_{-25}	
27	19.6	CH_3	19.6	0.84	3 Hd (*J*=6 MHz)	H_{-25}	
28	16.3	CH_3	17.8	0.97	3 Hd (*J*=6 MHz)		

s: Singlet, d: Doublet, dd: Doublet of doublet, m: Complex multiplet, HSQC: Heteronuclear single quantum correlation, H-H-COSY: Proton–proton correlation spectroscopy, HMBC: Heteronuclear multiple bond correlation

approach in 96-well plates (200 µL 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 activity.[16] Chloroquine (eight final test concentrations within the range 0.00000516129-51.6129 µg/mL) prepared following 10-fold serial dilution was used as standard antimalarial drug for comparison. At 620 nm 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 (IC_{50}) values were derived from graphs of mean % parasite viability against log (test sample concentration) using the nonlinear regression function of Microsoft Excel 2007 software.

RESULTS

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

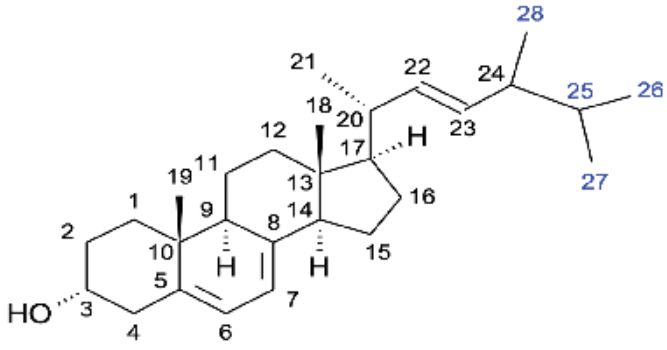

Figure 1. Compound **1**: Ergostan-5,6,22-trien-3-ol (ergosterol)

Structural elucidation/characterization of isolated compound 1:

Appearance: White solid;

Melting point: 155-160˚C;

Solubility: Freely soluble in chloroform, dichloromethane;

Molecular Mass: 396.7 (calculated for $C_{28}H_{44}O$).

IR spectrum: [*frequency, V, cm*$^{-1}$]: 3412; 3404 [OH str], 3060 [=CH str], 2951; 2868 [CH_2/CH_3 str], 1655; 1600 [C=C str], 1053/1030 [C-O str], 727 [CH_2 rocking].

EI-Mass spectrum: [*m/z (rel. int)*]: 396 (62.66) [M$^+$], 378 [M$^+$-H_2O], 363 (42.96) [M$^+$ - (18+15)], 271 (20.45) [M$^+$- aliphatic chain], 253 (30.95) [M$^+$ - H_2O - aliphatic chain], 285 [M$^+$ - H_2O -15-ring A]. 69 (100), 55 (80.83), 57 (43.95), 43 (74.37).

^{1}H-NMR (300 MHz, $CDCl_3$, δppm): Details of the spectra data are presented in Table 1.

^{13}C-NMR (75 MHz, $CDCl_3$, δppm): Details of the spectra data are presented in Table 1.

Cytotoxicity activity: A marked onset of cytotoxicity for the NHE (Figure 2, Table 2) was observed at the highest screened concentration of 100 µg/mL (≈75% cell viability translating to ≈25% cell death and a selectivity index ›4). This is indicative of low cytotoxicity (IC_{50}›100 µg/mL) compared to the reference drug emetine with IC_{50}=0.013 µg/mL).

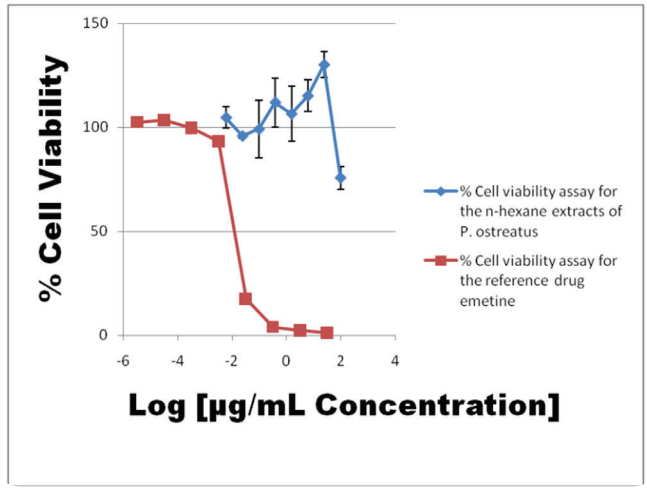

Figure 2. Dose dependent mammalian HeLa cell viability profile of the *n*-hexane extracts of *Pleurotus ostreatus*

Antiplasmodial activity

The NHE inhibited *Plasmodium* parasite lactate dehydrogenase activity in a dose dependent manner *in vitro* (Figure 3, Table 2) with a median inhibition concentration (IC_{50}) of 25.18 µg/

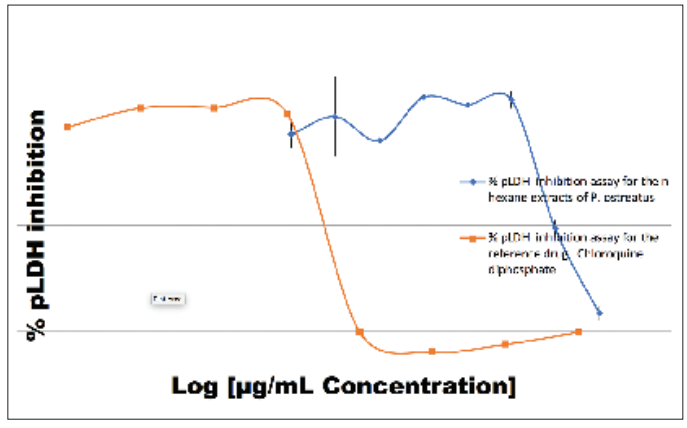

Figure 3. Dose dependent plasmodium parasite growth inhibition profile of the n-hexane extracts of *Pleurotus ostreatus*

mL. It was, however, significantly (p=0.02, ‹0.05) less active compared to the standard drug chloroquine diphosphate (IC_{50}=0.016 µg/mL); see Figure 3 and Table 2.

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

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

n-hexane extracts			Reference drug Emetine		Reference drug	Chloroquine diphosphate
Concentration µg/mL	pLDH inhibition %	Cell viability %	Concentration µg/mL	Cell viability %	Concentration µg/mL	pLDH inhibition %
0.006	93.160±6.067	104.670±5.054	0.00000325	102.571	0.00000516129	9.677.495
0.024	101.742±19.000	95.809±1.053	0.0000325	103.607	0.0000516129	1.056.567
0.098	90.250±2.164	99.096±13.659	0.000325	99.648	0.000516129	1.059.009
0.391	110.896±0.239	111.970±11.730	0.00325	93.383	0.00516129	1.033.283
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	-946.859
25.000	48.751±3.997	130.106±6.337	3.250	2.282	5.161.289	-591.963
100.000	8.830±3.466	75.664±5.530	32.50	1.032	516.129	-0.2488
IC_{50} (µg/mL)	25.179±2.456	>100	0.013		0.016	
Selectivity index	>4	NA		NA		NA

pLDH: Plasmodium lactate dehydrogenase

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 the enzyme pLDH is reported to disappear within 24 h of effective malaria treatment.[21] The pLDH antigen is considered a specific marker for the presence of viable plasmodium in blood. A report by the United States National Cancer Institute regards plant extract with cytotoxic IC_{50} 20 µg/mL or lower as being highly cytotoxic.[22-24] Those with IC_{50} greater than 100 µg/mL are regarded to be of low to zero toxicity.[22] The observed low cytotoxicity of the NHE is suggestive that the observed antiplasmodium activity may not necessarily be due to general cytotoxicity of the extract thus is a clue to its potential as a source of nontoxic agents for drug development.

The presence of isoprenoids (triterpenoid/steroids, cardenolides) and fatty acids as metabolites in the NHE 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 antimalarial activities of edible mushroom and related fungi have been documented.[2,3,25] After chromatography separation of the NHE, compound **1** was isolated and its structure elucidated using mass spectrometry, nuclear magnetic resonance (1D and 2D), and fourier transform infrared 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 ^{1}H and ^{13}C chemical shift signals for a conjugated di-substituted olefinic bond in ring B and the isolated olefinic bond in the aliphatic side chain, and 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 28 carbon signals, i.e., four quaternary (4×C), eleven methine (11×CH) of which one is the CHOH at position 3 of ring A, seven methylene (7×CH_2), and six upfield methyl (6×CH_3), were observed, which corresponded to the molecular formula $C_{28}H_{44}O$, corroborating the observed molecular ion peak at m/z 396 from the EI-mass spectrum analysis. These trends in the spectra data are characteristic of an unsaturated steroidal alcohol and when compared to literature reports for ergosterol[17] were similar. Owing to solubility limitations, compound **1** was, however, not evaluated for the reported biological activities. Ergosterol derivatives like ergosterol endoperoxide isolated from *P. ostreatus* have been reported to exhibit antiparasitic properties like trypanocidal[5] and amoebicidal[4] activities.

CONCLUSIONS

This study showed the first time the nutraceutical potential in the management of malaria infection of the edible mushroom *P. 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.

ACKNOWLEDGEMENTS

AOE gratefully acknowledges financial support from the Royal Society of Chemistry JWT Jones travelling Fellowship award of 28th August 2015 to visit Rhodes University, Grahamstown, South Africa, where the spectroscopy and bioassay components of this project was done. The bioassay component of the project was funded by the South African Medical Research Council

with funds from the National Treasury under its Economic Competitiveness and Support Package. XSN is grateful for a Rhodes University Postdoctoral Fellowship.

Conflict of Interest: No conflict of interest was declared by the authors.

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