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 ($IC_{50}=0.016 \text{ μg/mL}$) > *n*-hexane extract ($IC_{50}=25.18 \text{ μg/mL}$). It also showed mild cytotoxicity ($IC_{50}>100 \text{ μg/mL}$; selectivity index >4) compared to the reference drug emetine ($IC_{50}=0.013 \text{ μg/mL}$). The known sterol, erostan-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 araştırılmış ve ekstreden bir sterol izolasyonu yapılmıştır.

Gereç ve Yöntemler: Antiplazmodiyal ve sitotoksikite deneyleri, sırasıyla, plasmodium lactate dehydrogenase analizi ve insan HeLa servikal hücre hatları kullanılarak *in vitro* gerçekleştirilmişdir. *n*-Hekzan ekstresi izolasyonu *in vitro* gerçekleştirilmişdir. *n*-Hekzan ekstresinden izole edilen bileşikin yapısı, spektroskopik teknikler kullanılarak ayardanıtılmıştır.

Bulgular: *n*-Hekzan ekstresi (verim: %0.93 a/a) parasit inhibisyonunda doza bağlı antiplazmodiyal aktivite gösterdi: klorokin ($IC_{50}=0.016 \text{ μg/mL}$) > *n*-hekzan ekstresi ($IC_{50}=25.18 \text{ μg/mL}$). Ayrıca referans ilaç emetine kıyaslada ($IC_{50}=0.013 \text{ μg/mL}$) hafif sitotoksikite ($IC_{50}>100 \text{ μg/mL}$; 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, malaria, sitotoksikite, ergosterol

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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. 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 and closely related fungi species have been reported. Some of these include: the antimalarial properties of Cordyceps species and Bulgaria inquinans, and the amoebicidal and anti-trypanosomiasis properties of Pleurotus ostreatus. Other reported biological activities include nematicidal, anti-inflammatory and immunomodulatory, and anticancer 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, 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 Dilotmat 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.

Isolation and purification of compound 1

The bioactive NHE (1 g) 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:dichloromethane (1:1) yielded a white solid compound 1 (Figure 1) after re-crystallization 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 H2SO4 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 13C-NMR spectra of compound 1 (Figure 1) were 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 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 2×104 cell per well in 150 μL of 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% CO2 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 toxicity 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. | δ ppm | DEPT-135 | Published^T | 1HSQC (δ ppm) | 1H-multiplicity | 1H-H-COSY | 1HMBC |
|-------|-------|----------|-------------|---------------|----------------|------------|-------|
| 1     | 38.4  | CH₂      | 38.4        | 2.08          | 2 Hm           | H₂         | C₃    |
| 2     | 32.0  | CH₂      | 32.0        | 1.52, 1.91    | 2 Hm           | H₂, H₃     |       |
| 3     | 70.5  | CH       | 70.4        | 3.65          | 1 Hm           | H₂,       |       |
| 3-OH  | -     | -        |             |               |                |            |       |
| 4     | 40.8  | CH₃      | 40.8        | 2.32          | 2 Hm           | H₂,        | C₃    |
| 5     | 139.8 | C        | 139.8       | -             |                |            |       |
| 6     | 119.6 | CH       | 119.6       | 5.60          | 1 Hd           | H₂,        | C₅, C₁₀ |
| 7     | 116.3 | CH       | 116.3       | 5.42          | 1 Hd           | H₃,        |       |
| 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  | CH₂      | 21.1        | 1.65, 1.70    | 2 Hm           |            |       |
| 12    | 39.1  | CH₂      | 39.1        | 2.05          | 2 Hm           |            |       |
| 13    | 42.8  | CH₂      | 42.8        |               |                |            |       |
| 14    | 54.6  | CH       | 54.6        | 1.92          | 1 Hm           |            |       |
| 15    | 23.0  | CH₂      | 23.0        | 1.40, 1.70    | 2 Hm           |            |       |
| 16    | 28.3  | CH₂      | 28.3        | 1.38, 1.80    | 2 Hm           |            |       |
| 17    | 55.8  | CH       | 55.7        | 1.30          | 1 Hm           |            |       |
| 18    | 12.0  | CH₁      | 12.0        | 0.65          | 3 Hs           |            |       |
| 19    | 17.6  | CH₁      | 17.6        | 0.93          | 3 Hs           |            |       |
| 20    | 40.4  | CH       | 40.4        | 2.48          | 1 Hm           |            |       |
| 21    | 21.1  | CH₁      | 21.1        | 1.05          | 3 Hd (J=6 MHz) |            |       |
| 22    | 135.6 | CH       | 135.6       | 5.25          | 1 Hdd          | C₂₀, C₂₃  |       |
| 23    | 132.0 | CH       | 132.1       | 5.20          | 1 Hdd          | C₂₀       |       |
| 24    | 42.8  | CH       | 42.8        | 1.90          | 1 Hd           |            |       |
| 25    | 33.1  | CH       | 33.1        | 1.52          | 1 Hm           | H₂, H₃, H₇ |       |
| 26    | 19.9  | CH₁      | 19.8        | 0.86          | 3 Hd (J=6 MHz) | H₂₅       |       |
| 27    | 19.6  | CH₁      | 19.6        | 0.84          | 3 Hd (J=6 MHz) | H₂₅       |       |
| 28    | 16.3  | CH₁      | 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. 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 (IC50) 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.

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 (IC50 >100 μg/mL) compared to the reference drug emetine with IC50=0.013 μg/mL.

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 (IC50) of 25.18 μg/mL. It was, however, significantly (p=0.02, <0.05) less active compared to the standard drug chloroquine diphosphate (IC50=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.
spectra data (Table 2) are evident with the 1H and 13C chemical reagents confirming it to have a steroidal nucleus. The NMR gave positive Liebermann and Salkowski phytochemical test 1 be the known compound, ergostan-5,7,22-trien-3-ol. Compound 2D), and fourier transform infrared spectroscopic techniques to using mass spectrometry, nuclear magnetic resonance (1D and the NHE, compound 22 The observed dose-dependent Plasmodium lactate dehydrogenase (pLDH) inhibition by could be due to 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 IC50 20 μg/mL or lower as being highly cytotoxic. Those with IC50 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 spectroscopic screening using appropriate standard reagents corroborated our earlier report about the presence of these metabolites in a closely related species Pleurotus tuber regium.31 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.32,33 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-5,6,22-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.

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.

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