Antiprotozoal, antiviral and cytotoxic properties of the Nigerian Mushroom, Hypoxylon fuscum Pers. Fr. (Xylariaceae)

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ABSTRACT

In Nigeria, mushrooms are utilised for the treatment of several ailments. Although the biological activities of some Nigerian mushrooms have been reported, there is dearth of information on the therapeutic potentials of *Hypoxylon fuscum*. The aim of this study is to investigate the antileishmanial, antiplasmodial, antitrypanosomal, cytotoxic and antiviral activities of *H. fuscum*. The antiplasmodial studies revealed that the extract was active against chloroquine-sensitive D6 and chloroquine-resistant W2 strains of *Plasmodium falciparum* (IC_{50} of 6.98 and 8.33 µg/mL, respectively). The extract showed antitrypanosomal activity on *Trypanosoma brucei brucei* but lacked inhibitory activity against *Leishmania donovani*. The extract displayed cytotoxicity on *Artemia salina* larvae and rhabdomyosarcoma cell line, with CC_{50} value of 3.33 and 8.60 µg/mL, respectively, and also displayed antiviral activity on echoviruses (E7 and E19). This study demonstrated that *Hypoxylon fuscum* possess several pharmacological activities and may provide a drug lead for the development of effective chemotherapeutic agents.

Keywords: Antiprotozoal activity; Echoviruses; *Hypoxylon fuscum*; MTT assay; Mushroom

INTRODUCTION

In today's world, the ever-increasing global demand for natural products as a credible source of pharmaceutical products, has placed intense pressure on several angiosperms. In recent times, efforts are now being channelled toward the exploitation of lower organisms such as bacteria and fungi[1]. Mushrooms are fungi, that lack chlorophyll, and thus derive their nutrients from the metabolism

of non-living organic matters, such as putrefying leaves and tree trunks. Of the 140,000 known species of mushrooms that exist globally, about 10% have been properly identified and only approximately 5% have been investigated for their pharmacological activities[2]. Mushrooms with medicinal properties (medicinal mushrooms; MMs) have been reported to possess more than 100 medicinal functions, with the notable ones being antineoplastic, antidiabetic, antiviral, antibacterial, antifungal, immunomodulatory and antiparasitic properties[3]. MMs are also used in the food industry as dietary foods and supplements; the cosmetic industry as cosmeceuticals; and in the agriculture sector as pesticides, herbicides and insecticides. Various pharmaceuticals with secondary metabolites obtained from MMs have made it to several clinical trials. For instance, the polysaccharide extract of Maitake mushroom, *Grifola frondosa,* showed excellent Phase II clinical trial results in breast cancer patients[4]. Krestin, a polysaccharide isolated from the fruiting body of *Trametes versicolor*, was reported to inhibit tumor growth in animal models and human clinical trials[5,6]. Other notable pharmaceuticals formulated from MMs available in global drug industry include lentinane, copsin and schyzophillan, which is used as antitumor, antimicrobial and immunomodulatory agent, respectively[7].

In Nigeria, mushrooms are utilised for the treatment of several ailments[8-10]. For example, the extracts of *Ganoderma lucidum* and *Ganoderma appalanatum* are used for the management of arthritis, diabetes and liver diseases in the southern part of Nigeria[11, 12]. *Pleurotus tuberregium* is utilised for the treatment of headache, fever, cold and constipation[13]. In addition, *Lycoperdon umbrinum* (puffball) is used in southwestern Nigeria for treating wounds, inflammation, diarrhoea and other ailments[14].

Hypoxylon fuscum Pers. Fr., like several other members of the Xylariaceae family, grows on dead trees, especially on the trunk. Xylariaceous fungi, especially the genus *Hypoxylon*, have been shown to contain diverse bioactive secondary metabolites[15], including antimicrobial azaphilones from *H. multiforme*[16], carneic acids A and B from *H. carneum*[17], sclerin and its diacid from *H. fragiforme*[18]. While there have been several reports of biological activities of other *Hypoxylon species*[19-21], a thorough search of available literature revealed that the therapeutic potentials of *H. fuscum* remains largely unexplored. Therefore, this work was carried out to investigate the phytochemical constituents, as well as, the antiplasmodial, antileishmanial, antitrypanosomal, antiviral and cytotoxic activities of the methanol extracts of *H. fuscum* in several in vitro models.

METHODOLOGY

Extraction of mushroom

Fresh samples of the mushroom, *Hypoxylon fuscum*, were collected from its natural habitat (dead branches of several trees) in the premises of the University of Ibadan, Ibadan, Nigeria, between June and September 2017, and identified by Mrs Jumoke Morounfolu of the Department of Botany, University of Ibadan, Nigeria. The air-dried and pulverised mushroom (1.1 kg) was extracted into MeOH (4 L) at room temperature (25-32 °C) for a 72-h period. The crude extract obtained was concentrated using a rotary evaporator at 40 °C and stored in a refrigerator at 4 °C, prior to use.

Phytochemical screening

Following standard procedures described in earlier literature[22,23], the phytochemical analysis of the mushroom extract was carried out to determine the presence of several secondary metabolites, including alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, sterols and tannins.

Antiplasmodial assay

Antiplasmodial assay was carried out at the National Centre for Natural Products Research, University of Mississippi, USA. In this assay, the parasitic lactate dehydrogenase (pLDH) activity was estimated as a measure of the antiplasmodial potential of the extract[24,25]. Briefly, erythrocytes infected with chloroquine-sensitive (D6) or chloroquine-resistant (W2) strains of *Plasmodium falciparum* (2% parasitemia and 2% hematocrit) were prepared in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% human serum and 60 µg/mL amikacin. 200 µL of the preparation was added into each well of a 96-well microplate containing 10 µL of various concentrations of the extract. The positive controls included artemisinin and chloroquine (obtained from Sigma Chemicals Co.), while DMSO contained medium served as the negative control. For the primary antiplasmodial assay, the extract was tested at a single concentration of 15.9 µg/mL, while test concentrations ranging from 5.3 to 47.6 µg/mL was used for the secondary antiplasmodial assay. The experiment was carried out in triplicate and the IC_{50} values calculated from a dose-response curve obtained from the GraphPad software.

Antileishmanial assay

The alamar blue assay was performed on a culture of *Leishmania donovani* promastigotes and axenic amastigotes to determine the antileishmanial potential of the extract[26]. Optimum growth conditions were ensured for the parasites before

treatment was commenced. The promastigotes were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and maintained at pH 7.4 in a humidified atmosphere of 5% CO2 at 26 °C. The axenic amastigotes were cultured in RPMI medium supplemented with 4-morpholineethanesulfonic acid (MES) (4.88 g/L), L-glutamine (298.2 mg/L), adenosine (26.7 mg/L), folic acid (10.1 mg/L), BME vitamin mix, sodium bicarbonate (352.8 mg/L) and 10% FBS and maintained at 37 °C and 5% CO2, with the pH of the medium fixed at 5.5. In the primary assay, the parasites were treated with a single concentration (20 μg/mL) of the extract and incubated for 72 h at 26 °C and 37 °C, respectively, for promastigotes and axenic amastigotes. No further secondary assay was carried out as the extract displayed little or no activity in the primary assay.

Antitrypanosomal assay

For this test, a modification of a method described recently[27] was used. In brief, two days culture of *Trypanosoma brucei brucei* was diluted in a 96-well microplates, containing Iscove's Modified Dulbecco's medium (IMDM) to obtain 5000 parasites/mL. The culture was maintained in a humidified atmosphere of 5% CO2 at 37 °C. For the primary antitrypanosomal assay, 4 μL of the extract (at a single concentration of 20 μg/mL) was added into each well of the microplate containing 196 μL of the IMDM-parasite culture to make a final culture volume of 200 μL. The plates were incubated at 37 °C in 5% CO2 for 48 h. Thereafter, 10 μL of alamar blue (AbD Serotec) was incorporated into each well and the plates were further incubated for 24 h. At the end of the incubation period, the fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation and 590 nm emission. For the secondary screening, the procedure described above was repeated, only that the test concentration ranged from 10 to 0.4 μg/mL. The IC_{50} and IC_{90} values were estimated from the dose-response analysis curve obtained from XLfit version 5.2.2.

Cytotoxicity screening

Brine Shrimp Lethality Assay (BSLA)

The BSLA is a simple, unsophisticated, bench-top assay used to identify extracts with potential cytotoxic compounds. The protocol described by McLaughlin, 1991, with little modification, was followed in this work[28]. In brief, *Artemia salina* (brine shrimp) eggs were hatched in a vessel filled with natural sea water under constant aeration for 48 h. After hatching, a Pasteur pipette was used to collect ten nauplii into several tubes containing 4.5 mL of brine solution. The extract was serially diluted to obtain working concentrations ranging from 1000 to 1 μg/mL. 0.5 mL of each concentration was added to the vial containing the ten nauplii

and the brine solution and incubated for 24 h at room temperature (25 – 32 °C). The number of dead and live nauplii in each well was counted using a magnifying lens. Cyclophosphamide was used as the positive control while brine solution alone was used as the negative control. The experiment was carried out in triplicate at room temperature (25 – 32 °C). The concentration responsible for killing 50% of the nauplii population (LD_{50}) was calculated using the GraphPad Prism software.

Cell culture and virus

The RD cells, obtained from the Centre for Disease Control, Atlanta, were cultured in T25 flask containing Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics. Cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C. Cells were passaged in ratio 1:3 once it reaches confluency following treatment with 0.05% trypsin. Cells were viewed using an inverted microscope. Three serotypes of echovirus (E7, E13, and E19) isolated from stool at the WHO Polio Laboratory, Department of Virology, University of Ibadan, Nigeria, were used for the antiviral studies. The viruses were stored at −70 °C until use.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) cell viability assay

The MTT assay was carried out to determine the effect of the *H. fuscum* extract on the viability of rhabdomyosarcoma (RD) cells. The method described earlier by Mosmann, 1983, was followed with slight adjustments[29]. Briefly, RD cells suspension (100 µL) were seeded into 96-well plates and maintained in the incubator for 24 h. Subsequently, the cells, which obtained a confluency of about 90%, were treated with serial dilutions (100 µL each) of the mushroom extract, ranging from 1000 – 0.01 µg/mL. The extract itself was previously dissolved in DMSO and diluted with the culture medium, ensuring the maximum concentration of DMSO was not more than 0.05%, a concentration that is not toxic to cancer cells[30]. The treatment was carried out for a period of 72 h in the same environment mentioned above. Following this, the old culture medium was removed, and the cells monolayers were washed with phosphate buffer saline (PBS). Thereafter, 100 µL of MTT solution (500 µg/mL) was added into each well of the 96 well plates and incubated at 37 °C for 2 hours. After the incubation period, the preformed formazan crystals were solubilised using DMSO and the optical density (OD) was measured at 570 nm using a multiplate reader (Thermoscientific, Waltham, MA). Cyclophosphamide was used as the negative control while growth medium was used as the negative medium. The experiments were carried out in triplicate with the OD of the negative control fixed arbitrarily

at 100%, and OD of treatments calculated relative to negative control. The concentration responsible for reducing the viability of the cells by 50% (CC_{50}) was calculated using a non-linear regression curve generated from the GraphPad Prism 5 software (GraphPad, USA).

50% tissue culture infective dose ($TCID_{50}$)

To determine the 50% tissue culture infective concentration ($TCID_{50}$), the Sperman-Karber's method was used to estimate the virus titres that caused cytopathic effect (CPE) in RD cell culture. In brief, 100 µL of E7 were added into a T25 culture flask (Corning®, UK), containing RD cells and incubated for 72 h. This increased the virus stock quantity due to the 100% CPE.

Thereafter, 100 µL RD cells (1 x 106 cells/mL) were seeded into three 96-well plates and incubated for 24 h. The virus stock was diluted serially in ten-fold and 100 µL of each dilution was inoculated into the wells. The cell control used contained RD cells without the virus. The plates were incubated at 37 °C and daily CPE scoring was done till the control wells started dying. The 100 $TCID_{50}$ was used for the antiviral assay and the procedure was repeated for E13 and E19.

Antiviral assay

The neutralisation test[31], an assay that measures CPE inhibition in cell culture was used to investigate the antiviral activity of *H. fuscum* extract against echoviruses (E7, E13, and E19). Briefly, 50 µL of 100 $TCID_{50}$ virus suspension were added into a 96-well plate containing a monolayer of confluent RD cells and allowed to stand for 1 h to enable virus adsorption. Subsequently, ten-fold serial dilutions of the extract, starting from the maximum non-toxic concentration (10 µg/mL), was added in triplicate into the wells, except for the negative control and virus control wells that contained only the RD cells and virus, respectively. The plates were incubated at 37 °C in 5% CO_2 humidified incubator for 72 h after which the cell viability was measured using the MTT assay as described above. The 50% inhibition concentration (IC_{50}) was defined as the concentration that reduces CPE by half, with respect to the virus control. The selectivity index (SI) was calculated as the ratio of the extract's concentration that reduces viability of the RD cells by 50% (CC_{50}) to the concentration of the extract that inhibit cytopathic effect to 50% of the control value (IC_{50}). Since there are no antiviral drugs approved for the treatment of enteroviral infections, no drug control was used in this study.

High performance liquid chromatography (HPLC) analysis

The Dionex HPLC system 2695 (Waters) coupled with a Thermoscientific NX 5 µm C18 column (250 x 4.6 mm) and a library of chemical compounds were used

for this analysis. For the experiment, column temperature and variable ultra-violet-visible detector were maintained at 25 °C and 235 nm, repsectively. The binary solvent system comprising of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in methanol (solvent B) was used as the eluent. Reversed-phase analytical HPLC analysis was done as follows; 0-40 min: 30-100% B; 41-50 min: 100% B; 51-60 min: 100-30% B; flow rate, 1 mL/min. Determination of the compounds present in the extract was carried out by comparing the UV of the peaks obtained in the HPLC chromatogram with compounds collection contained in the HPLC library.

RESULTS AND DISCUSSION

The ever-expanding exploitation of medicinal plants as a tool for boiactive compounds discovery have stimulated the interest of natural product scientists in the discovery of potent pharmaceutical prodcuts from medicinal mushroom (MMs). MMs represent a huge but widely unexploited source of new chemotherapeutic agents. In this study, the biological activities of a Nigerian mushroom, *Hypoxylon fuscum*, was investigated. The qualitative analysis of the phytochemical constituents of *H. fuscum* revealed that it contained several secondary metabolites including alkaloids, cardiac glycosides, coumarins, flavonoids and sterols. However, anthraquinones, saponins and tannins were absent in the extract (Table 1).

Table 1. Phytochemical constituents of H. fuscum MeOH extract

Phytochemicals	Results
Alkaloids	++
Anthraquinones	-
Cardiac glycosides	+
Coumarins	++
Flavonoids	++
Saponins	-
Sterols	++
Tannins	-

Key: - = absent; + = present; ++ = abundant

Malaria remains a major threat to global health as the populace of most tropical and subtropical nations of the world are faced with this disease. In addition, recent studies have reported parasitic resistance to currently used antimalarial agents[32,33]. Since natural products have played significant role in the discovery of antimalarial molecules, as exemplified in the discovery of artemisinin from *Artemia annua* and quinine from *Cinchona succirubra*, it is needful to further

explore natural sources to discover new antimalarial leads. The *in vitro* antiplasmodial investigation revealed that *H. fuscum* extract was potent against both chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum* with IC_{50} values of 6.98 and 8.33 μg/mL, respectively, when compared to chloroquine and artemisinin, that displayed IC50 values less than 0.0264 μg/mL against both strains of malaria parasites (Table 2).

Table 2. Antiplasmodial activity of *H. fuscum*

Extract/ Compound	Primary (% inhibition)	Secondary (IC_{50}; μg/mL)		
	D6	W2	D6	W2
H. fuscum	74	68	6.98	8.33
Chloroquine	ND	ND	<0.0264	<0.163
Artemisinin	ND	ND	<0.0264	<0.0264

ND: not determined; IC_{50}: 50% inhibition concentration

Previous reports on antiplasmodial screening of crude extracts have stated that extracts with IC_{50} value less than 10 μg/mL possess high activity, IC_{50} between 10 μg/mL and 50 mg/mL possess moderate activity and those with IC_{50} greater than 50 μg/mL possess no activity[34,35]. It is therefore safe to state that the extracts of *H. fuscum* possess bioactive molecules with potent antiplasmodial activity.

The extract exerted a significant antitrypanosomal activity on *Trypanosoma brucei brucei* with IC_{50} value of 10.79 μg/mL. *T. brucei brucei* is a pathogenic protozoan that causes human African Trypanosomiasis (HAT; sleeping sickness) and animal trypanosomiasis in humans and animals, respectively[36]. HAT is a neglected tropical disease that is associated with high mortality, especially when mangement is not insituted early. The scarcity of effective chemotherapy for the treatment of this disease and the increase in the resistance of parasite towards available drugs have necessitated the search for the discovery and development of more potent bioactive molecules for the management of HAT[37]. With the potent antitrypanosomal acitivity displayed by the extract of *H. fuscum,* especially its 77% inhibition of the parasite in the primary assay (Table 3), this mushroom could provide a template for the discovery of agents with antitrypanosomiasis activity. However, the extract lacked significant inhibitory activities on the both promastigotes and amastigotes of *L. donovani*, as it displayed IC_{50} greater than 20 μg/mL in the secondary assay.

Table 3. Antileishmanial and antitrypanosomal activity of *H. fuscum*

Parasite	Primary (% inhibition)	Secondary	
		(IC50; µg/mL)	(IC90; µg/mL)
L. donovani promastigotes	0	>20	>20
L. donovani amastigotes	46	>20	>20
T. brucei brucei	77	10.79	> 20

IC_{50}: 50% inhibition concentration; IC_{90}: 90% inhibition concentration

To determine the cytotoxic properties of H. fuscum extract, the brine shrimp lethality assay (BSLA) and the MTT viability assay were used. The BSLA is an assay used in the preliminary screening of plant extracts for the presence of chemotherapeutic molecules. Some studies have reported that plants with toxicity toward Artemia salina larvae may have potentials in anticancer drug discovery[38,39]. The MTT assay is a colorimetric assay that measure cell viability, with respect to the ability of mitochondrial reductase present in viable cells, to reduce water soluble MTT to water insoluble formazan crystals. The quantity of formazan produced at the end of the assay is used to estimate the amount of viable cells in the culture medium[40]. The National Cancer Institute (NCI) has stated that a crude extract should be considered active in a preliminary cell viability studies if it as CC50 < 30 µg/mL after an exposure time of 72 h[41]. In the cytotoxicity assays, the methanol extract of H. fuscum showed remarkable toxicity on both *Artemia salina* larvae (LD_{50} = 3.33 µg/mL) and rhabdomyosarcoma cell (CC_{50} = 8.60 µg/mL) (Table 4), indicating that this extract may possess potent cytotoxic compounds.

Table 4. Cytotoxic acitivty of *H. fuscum*

Extract / compound	BSLA (LD50 µg/mL)	MTT assay (CC50 µg/mL)
H. fuscum	3.33 ± 0.07	8.60 ± 0.02
Cyclophosphamide	0.82 ± 0.02	0.97 ± 0.01

BSLA = brine shrimp lethality assay; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; CC50= 50% cytotoxic concentration

Diseases caused by viruses, especially enteroviruses, are associated with high mortality, as there are no available antiviral agents approved for the management of such infections. The disease burden is also complicated by the lack of good sanitation and hygiene practices in most part of the developing world[42]. Medicinal mushrooms have been reported to display antiviral potentials against several viruses. For instance, triterpenes isolated from *Ganoderma pfeifferi*

displayed potent activity against human immunodeficiency virus type 1 (HIV-1), influenza virus type A and herpes simplex virus type 1[43]. Here, the antiviral activity of the extract of *H. fuscum* was investigated against three serotypes of echoviruses (E7, E13, and E19). With the exception of the E13 strains, *H. fuscum* displayed antiviral activity on the echoviruses under investigation, with an IC50 value of 0.381 and 1.575 µg/mL against the E7 and E19, respectively. In addition, the extract of this mushroom demonstrated good selectivity indexes with better selectivity on E7 (22.57) when compared to E13 (5.46) (Table 5).

Table 5. Antiviral activity of *H. fuscum*

Extract	MNTC (µg/mL)	IC$_{50}$ (µg/mL)			SI	
		E7	E13	E19	E7	E19
H. fuscum	10	0.3811	NA	1.575	22.57	5.46

MNTC: Maximum non-toxic concentration; IC$_{50}$: 50% inhibition concentration; SI: Selectivity index

Meanwhile, the HPLC analysis of this extract revealed that it contained dihydropenicillic acid as its major constituent, as seen in Figure 1 and 2. Several fungi have been reported to produce dihydropenicillinic acid, and its unsaturated analogue, penicillic acid, and these include *Aspergillus flavus, Aspergillus ochraceus, Aspergillus terreus, Penicillium cyclopium and Penicillium griseofulvum*[44]. A previoius study revealed that dihydropenicillinic acid displayed moderate cytotoxicity against several human cancers including human non-small cell lung cancer, human breast cancer, human CNS cancer and human pancreatic cancer at 10 µg/mL[45].
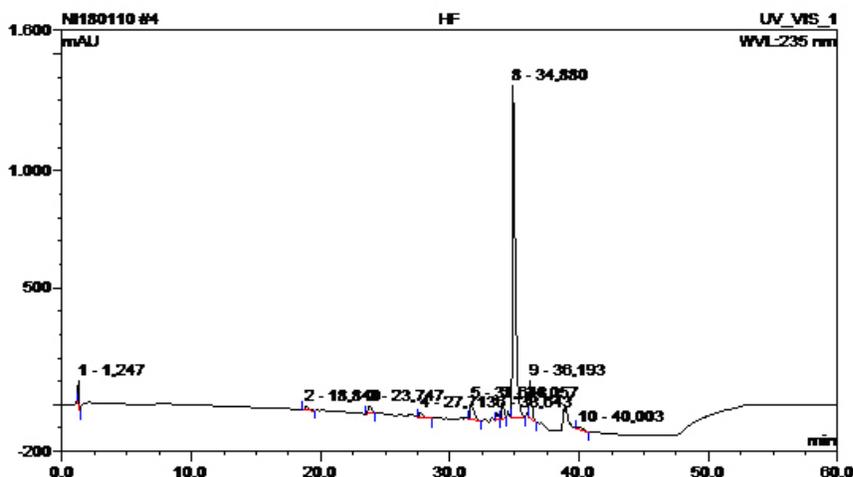

Figure 1 Reverse phase HPLC quantitative chromatogram of the methanol extract of *H. fuscum*
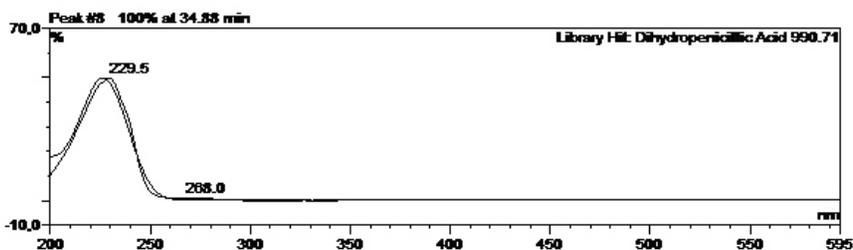
Figure 2 UV spectra assignment of the major peak of the methanol extract of *H. fuscum*

CONCLUSION

This study has demonstrated that extract of the mushroom *Hypoxylum fuscum,* (collected from Nigeria), possesses several pharmacological properties including antiplasmodial, antitrypanosomal, antiviral and cytotoxic properties. To the best of our knowledge, the therapuetic potentials of *H. fuscum* is reported in this work, for the first time. HPLC analysis revealed that dihydropenicillinic acid, which was the major constituent of the extract, may be responsible for the observed biological activities of the mushroom. Considering the diverse biological effects observed in this study, it can be inferred that *Hypoxylum fuscum* may be a source of potential drug lead in the development of effective and affordable chemotherapeutic agents.

ACKOWLEDGEMENTS

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