



Cytotoxicity and potential anticancer activities of herbal formulations from cultivated *Ganoderma* species in Nigeria against Human Hepatocellular Carcinoma

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ABSTRACT. Domesticated wild *Ganoderma* species have emerged as a valuable adjunct in the treatment of specific cancers and tumors, offering new avenues for therapeutic intervention. However, many existing treatments for Human Hepatocellular Carcinoma (HepG2), which are mostly costly, are limited by cirrhosis. In contrast, natural compounds in *Ganoderma* have potential synergistic and low-toxicity properties that could enhance therapeutic outcomes. In this study, heavy metal analysis, anti-proliferation screening of extracts and formulations of three cultivated *Ganoderma* species against HepG2 and Vero cells, and cytoplasmic fluorescence imaging of cells tracked with Red CMTPX were conducted. *Ganoderma* isolates were identified using Polymerase chain reactions (PCR) with Internal transcribed spacer sequences (ITS1 and ITS4) using a GenBank BLAST. Heavy metals were at acceptable levels according to the WHO standard. Indeed, the effective anti-proliferative, cell-mediated cytotoxicity of these species was observed. The hot water extract of *Ganoderma sessile*, *Ganoderma oregonense*, and *Ganoderma mbrekobenum* combined formulation (MCF-11) exhibited potent cytotoxicity with an IC₅₀ of 44.7 µg mL⁻¹ against HEPG2 cells, while showing mild toxicity with IC₅₀ of 158.4 µg mL⁻¹ against Vero cells. Mycelia biomass formulation of *G. mbrekobenum* showed a remarkable value of IC₅₀ < 30 µg mL⁻¹. At 100 µg mL⁻¹, the effect of MCF-11 on treated cells was evident, as the cells lost their typical morphology and the cytoplasm appeared shrunken and rounded, with reduced cell volume. However, extracts demonstrated dose-dependent toxicity, indicating anti-HepG2 potential.

Keywords: Anti-proliferative activity; medicinal mushrooms; liver cancer; genetic identification.

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Introduction

Cancer is a disease characterized by the unregulated proliferation of abnormal cells, which are subject to evolution by natural selection within the human body (Brown et al., 2023). The most common type of liver cancer, Hepatocellular carcinoma (HCC), is among the deadliest forms of cancer around the world due to its poor prognosis and limited treatment options (Yang et al. 2019). There is a strong correlation between cirrhosis and liver cancer incidence (Singal et al., 2020). Liver cancer often develops because of liver cirrhosis, a chronic condition marked by irreversible scarring of the liver tissue (El-Serag, 2012). Cirrhosis, commonly caused by hepatitis infection or alcohol abuse, creates a pro-inflammatory environment that promotes genetic mutations and malignant transformation, significantly increasing the risk of hepatocellular carcinoma (Forner et al., 2018). In Nigeria, approximately 8.4 per 100,000 people are infected with HCC, and the incidence rate is almost equal to the reported rate due to delays in diagnosis and treatment (Bray et al., 2018; Sung et al., 2021). HCC cases have risen worldwide, with an exceptionally high number in India and the USA (Venook et al. 2010). The disease is said to develop due to the destruction of hepatocytes, with the contribution of reactive oxygen species and the chronic formation of hepatocarcinogenesis (Owen et al., 2000).

For many years, traditional medicine in various cultures has relied heavily on natural and herbal products for treating infections and malignancies (Tavakoli et al., 2012). A review documented more than twenty

African plants with phytoconstituents, including diterpenes and triterpenes, that exhibit cytotoxicity against HepG2 cell lines and inhibit hepatocarcinogenesis, proliferation, invasion, metastasis, and angiogenesis (Gaobotse et al., 2023). Swetha et al. (2022) reported that the methanol extract of uttroside B from *Solanum nigrum* showed potent chemotherapeutic activity against HepG2 cells. Particular species of *Ganoderma*, such as *G. lucidum* and *G. mbrekobenum*, which are commonly found in Nigeria, have been reported to exhibit potent anticancer and antitumor effects (Nwogu et al., 2022; Ofodile et al., 2025).

Ganoderma lucidum extracts have been shown to mitigate the adverse effects of monosodium glutamate on blood and specific biochemical parameters in Wistar rats (Ofodile et al., 2020a). Although *Ganoderma* is found in the natural habitats of Nigeria, its potential for treating cancer has yet to be fully realized. The incorporation of mushroom metabolites into the standard treatment has been shown to increase the rate of survival from various forms of cancer (Lam et al., 2020).

There are no known poisonous species of *Ganoderma*. Nevertheless, if not stored correctly, they can become contaminated by molds, which is the only health risk (Gill & Rieder, 2008). They possess medicinal properties with no toxicity to humans, and are classified as medicinal mushrooms (Lloyd et al., 2018a). Polluted environments are the source of toxic contaminants like lead (Pb), arsenic (As), mercury (Hg), and cadmium (Cd), among others. Water hyacinth, which was also added to the substrate used to grow the *Ganoderma* species, can be incorporated by mushrooms, which are known to bioaccumulate heavy metals. For that reason, it is crucial to determine the heavy metal levels in cultivated mushrooms to avoid the incidence of toxicity (Wuana & Okieimen, 2011).

Currently, treatments for liver cancers include surgery, chemotherapy, and radiation therapy. However, most of the cytotoxic agents are not effective for HCC (Al-Qubaisi et al., 2011). Despite the growing evidence of the medicinal value of *Ganoderma*, few studies have explored its cytotoxicity against HepG2 liver cancer cells, especially among cultivated species of Nigeria (Wu et al., 2024). Additionally, the effects of their herbal combinations remain underinvestigated, limiting their clinical translation and potential for formulation into herbal remedies. To develop an effective, low-toxicity therapy for liver cancer, it is crucial to investigate the anticancer effect of the *Ganoderma* species, which was the main aim of the research.

This paper reports the toxicity of *Ganoderma sessile*, *Ganoderma oregonense*, *Ganoderma mbrekobenum* extracts alone and their formulations with ginger and garlic on the human liver carcinoma HepG2 cell line and normal kidney cells (Vero, CCL-81™) to test whether the mushroom extracts and formulations spare normal, healthy cells or cause general toxicity. Ethically and scientifically, it must be demonstrated that the extracts are not harmful to non-target cells before proceeding with animal models. Kidney cells are considered non-target cells in this experiment (Huang et al., 2024; Pocasap et al., 2021). In addition, the study also highlights the heavy metal content of mushroom extracts to ensure their safety and the bioaccumulation levels of heavy metals from water hyacinth, which was supplemented to the substrates.

Materials and methods

Sample collection

Fresh samples of (YCT-Q, MH2, and MH3) were collected and characterized molecularly. The voucher specimens were kept at the Mushroom Research and Training Laboratory, Yaba College of Technology, Yaba, Lagos. Sawdust of obeche (*Triplochiton scleroxylon* K. Schum.) was collected from a sawmill in the Shomolu area, Lagos, sieved, and preserved for use. Water hyacinth (*Eichhornia crassipes* Mart) was also harvested from Lagos Lagoon in Ikorodu and Oworonshoki, Lagos, and dried using an industrial dryer at 50°C before being shredded to sizes of 40-100 mm. *Sorghum bicolor* (L.) grains, rice bran, and Calcium carbonate (CaCO₃) were purchased from a rice mill at Abeokuta, Ogun State, and a chemical market at Ojota, Lagos, respectively. YCT-MH3 isolated from on the Dead stump of *Terminalia catappa* around the Mushroom Laboratory, N 6° 51' 73.6" E 5° 33' 72.4", MH2, on the dead stump of *Elaeis guineensis*, University of Lagos, Lagoon Front, N 6° 31' 08.2" E 3° 24' 04.6" and YCT-Q culture was purchased from LTC Farm Osogbo, Osun State.

Molecular characterization of samples (DNA extraction, amplification, and sequencing)

Cultures were grown on 2% PDA for seven days. Mycelia were harvested using a sterile spatula, transferred into Eppendorf tubes, freeze-dried, and ground into powder using a plastic pestle (Kontes). DNA was extracted from mycelia and dried fruit bodies by the method used by Lee and Taylor, (1990) and Isikhuemhen et al. (2000). Polymerase chain reactions (PCRs) were performed in 25 µL volumes using ITS1 and ITS4 primers

under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were purified using a PureLink PCR Purification kit (Thermo Fisher Scientific cat# K310001). Cleaned PCR products were submitted for sequencing to Eurofins Genomics (Louisville, Kentucky). The generated sequence was compared against nucleotide sequences in GenBank (BLASTN) to determine the closest putative identity based on percentage similarity with the GenBank sequence (Ofodile et al., 2022).

Mycelia production, extraction, and formulations

The *in vitro* anticancer effects of *Ganoderma* species were investigated using mycelial biomass derived via a method detailed by Anike et al. (2015). In this culture, YCTQ, MH2, and MH3 cultures of *Ganoderma* were applied. In this instance, some culture media (8.5 mm) of mycelia were directly subcultured into the experimental media for spawn biomass preparation. In a liter of stock solution, the media contained: KH_2PO_4 (1 g), NaH_2PO_4 (0.4g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 g), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (74 mg), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (6 mg), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5 mg), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (3.79 mg), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1 mg), thiamine HCl (0.1 mg), pyridoxine HCl (0.1 mg) and nicotinic acid (0.1 mg). The functional media sponge was carbonized using glucose + 30 g L⁻¹ bayam yeast extract powder as the nitrogen source. The media were sterilized at 121°C for 15 minutes and inoculated with a mycelial inoculum at 2% v v⁻¹. Each treatment of (1000 mL) cultures was prepared in Aksu (Erlenmeyer) flasks, shaken at 102 rpm for 7 days under an aseptic environment. As an example, mycelia were collected using microsieves with 63 µm meshes and harvested into a total of three washable segments before being dehydrated to dryness and stored on a 4°C shelf.

In one formulation, *Ganoderma* biomass and fruiting bodies, collectively with ginger and garlic, were used in the ratio of 75% lead, 15% support, and 10% driver. A dehydrator (GRT LT-A08 model) was used to dry the samples at 50°C. Extracts were prepared through a modification of the method described by Hui et al. (2018) and Zhang et al. (2018), namely by boiling water (100°C) for 5 minutes and absolute alcohol. After Dehydration at 50°C, the extracts were filtered and stored in a refrigerator for *in vitro* experiments.

Heavy Metal and mineral element composition analysis

The mineral elements and heavy metal content were measured using the acid digestion technique and the Agilent 240 AA Flame Atomic Absorption Spectrometer (AAS), as described by Nwachukwu et al. (2017) and Ofodile et al. (2020b), with slight modifications. One gram of each blended, air-dried sample was placed in a 250 mL conical flask, and its weight was measured using an Ohaus Adventurer Analytical Balance. Concentrated HNO_3 (10 mL) was then added, and the mixture was heated on a hot plate in a fume hood. The heating was done so that the solution blackened until a clear liquid was achieved. Twenty milliliters (20 mL) of distilled water were then added to the mixture, which was subsequently cooled, and the solution was heated again to facilitate the dissolution of the substances. The cooled solution was filtered through a Whatman 125 mm filter paper into a 100 mL volumetric flask. Distilled water was added up to the mark and was transferred to round bottles. All samples and standard lamps (Cu, Cr, Cd, Co, Ni, Pb, Zn, Mn, and Fe) were analyzed through the air-acetylene flame in triplicate.

Anti-proliferative activity evaluation

Cytotoxicity screening using XTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] assay

The human hepatocellular carcinoma cell lines HepG2 (HB-8065™, liver) and normal kidney cells Vero (CCL-81™) were used in this study. These cell lines were purchased by Professor Leonard L. Williams (Center for Excellence in Post-Harvest Technologies, North Carolina A&T State University, USA) from ATCC, USA. Cell culture procedures were performed according to the manufacturer's protocol. Cultures were initiated in T75 cm² flasks and incubated at 37°C with 5% CO₂ in a humidified incubator. The prepared growth medium was ATCC-formulated Eagle's Minimum Essential Medium (ATCC 30-2003), supplemented with 10% fetal bovine serum (FBS). Subculturing was performed at 80–90% confluence, and cells were harvested by treating them with 2 mL trypsin-EDTA (0.25% Trypsin/0.53 mM EDTA) for 5 minutes, followed by neutralization with 6–8 mL of complete medium. The cell suspension was centrifuged at 25 x 100 rpm for 5 minutes, and the cell pellet was resuspended in 8 mL of complete medium. Cell viability and count were determined using the NanoEntek EVE™ Automatic Cell Counter, USA.

The anti-proliferative activity of six crude extracts and formulations of *Ganoderma* species, including *G. mbrekobenum*, *G. sessile*, and *G. oregonense*, was assessed using the CyQUANT™ Invitrogen XTT Cell Viability Assay Kit (Thermo Fisher Scientific, USA). The extracts were prepared as follows: *G. mbrekobenum* basidiocarp hot water extract (Gm), *G. sessile* basidiocarp hot water extract (Gs), alcohol extract of combined *G. mbrekobenum*, *G. oregonense*, and *G. sessile* (MCF11b), hot water extract of the same combined species (MCF-11), *G. mbrekobenum* hot water extract of mycelial biomass (10), and alcohol extract of *G. sessile* formulation (3b). Stock solutions (50 mg mL^{-1}) were prepared by adding 30 μL of DMSO to 10 mL of D-PBS solution, filtering the mixture through a 0.2 μm filter, and then diluting to concentrations of 10, 50, 100, and 250 $\mu\text{g mL}^{-1}$. Cisplatin (Sigma-Aldrich, USA) served as a positive control, while vehicles used as diluents for extracts and Cisplatin were negative controls.

Each well of the 96-well plate was uniformly filled with 100 μL of a cell suspension containing 2×10^4 cells per well. To promote cellular attachment, the plates were maintained at 37°C with a concentration of 5% CO_2 for approximately 24 hours. The following steps were involved in removing the medium, and the cells were then washed with Dulbecco's Phosphate-Buffered Solution (DPBS). Afterward, the cells were synchronized and starved by applying 100 μL of complete medium containing 1% FBS, and then they were kept at this stage for an additional 16 hours. Subsequently, the cells were exposed to extracts at concentrations ranging from 10 to 250 $\mu\text{g mL}^{-1}$ and incubated for 24 hours. Thereafter, the cells were rinsed with D-PBS to prevent interference from the extracts used in the absorbance measurement. Approximately 70 μL of mixed A and B XTT reagents was added to each well and allowed to settle undisturbed for 2-4 hours in the incubator. The viability calibration curve was generated, and the cell viability percentage was calculated using the data obtained. BioTek Synergy HTX microplate reader was used to compute the optical density (OD) at a wavelength of 450 nm (660 nm was the reference wavelength used).

Detection of morphological changes by light microscopy

For morphological analysis, HepG2 cells were exposed to 10–250 $\mu\text{g mL}^{-1}$ of each *Ganoderma* extract for 24 hours. Post-treatment, cells were stained with CellTracker Red CMTPX (5 μM) for cytoplasmic labeling to observe cell morphology. Images of morphological changes were captured using an Accu-Scope EXI-310 light microscope (10x magnification), and fluorescence images were obtained using the BZ-X710 All-in-One fluorescent microscope (excitation/emission: 577/602 nm for cytoplasm, 20x magnification).

Statistics analysis

Statistical analysis of all experiments was performed using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Cytotoxicity was determined by IC_{50} using the log (inhibitor) vs. normalized response method, with a 95% confidence interval. The viability assay was performed in triplicate, and the experiment was repeated three times. Heavy metal analysis was also done in triplicate. Data were expressed as mean $\pm$ SD ($n = 3$) and analyzed using two-way analysis of variance (ANOVA) followed by the Tukey post-hoc test. Differences at $p < 0.01$ were considered significant for cytotoxicity experiments, and $p < 0.05$ for the heavy metal and mineral element composition analysis of the extracts of *Ganoderma* species.

Results

Molecular identification of *Ganoderma* samples

Two different fruiting bodies were collected from dead trees in the wild: YCT-Q from a dead log of a mango tree (*Mangifera indica*) and MH 3 from a dead log of a *Terminalia catappa*. YCT-Q was a commercial product purchased from LTC Farms, Osogbo, Osun State. They were sequenced for the ITS1 and ITS4 regions of ribosomal DNA and found to be closely related to other *Ganoderma* species reported from Africa and the United States. The species of *Ganoderma* were submitted to NCBI GenBank and assigned accession numbers: PQ578285 for *Ganoderma mbrekobenum*, PQ578286 for *Ganoderma oregonense*, and PQ578284 for *Ganoderma sessile* (Table 1).

Table 1. Molecular characterization of *Ganoderma* samples.

S/N	Sequence code	% relatedness	NCBI BLAST relative	Accession Number	Query coverage
1	YCT- Q	100	<i>Ganoderma sessile</i> Murrill, 1902	PQ578284	100%
2	MH3	100	<i>Ganoderma mbrekobenum</i> MIN 850481	PQ578285	100%
3	MH2	100	<i>Ganoderma oregonense</i>	PQ578286	100%

Heavy Metals Composition of the extracts of the *Ganoderma* species and formulations

Copper content of Gs Dried (0.45 ± 0.02 mg mL⁻¹), Gs (0.46 ± 0.02 mg mL⁻¹), and Gs Myc. (0.47 ± 0.06 mg mL⁻¹) were similar while Gs form (0.22 ± 0.02 mg mL⁻¹) and hot water extract of the combination of the three species of *Ganoderma*, MCF-11 (0.22 ± 0.02 mg mL⁻¹) were the same which was significantly the lowest at ($p < 0.05$). The extracts Gs Dried (0.03 ± 0.00 mg mL⁻¹), Gs Form (0.02 ± 0.00 mg mL⁻¹), and MCF-11 (0.02 ± 0.00 mg mL⁻¹) had a significantly lower cadmium content. Gs Dried and Gs were lead free, while Gm Dried and Go Dried were chromium free ($p < 0.05$) Table 2.

MCF-11 had a significantly elevated level of iron (479.91 ± 17.09 mg mL⁻¹) at ($p < 0.05$) while Go Dried had lower levels of iron (92.33 ± 0.81 mg mL⁻¹). Gm myc (245.28 ± 1.73 mg mL⁻¹) had the highest level of manganese (Mn), while Go Dried (3.63 ± 0.23 mg mL⁻¹) had the lowest level at a significantly different level ($p < 0.05$) Table 2.

Table 2. Heavy metal and mineral composition (mg mL⁻¹) of *Ganoderma* samples and formulations.

Samples	Zn	Cu	Cd	Pb	Cr	Fe	Mn
Gm Dried	$0.31^A \pm 0.00$	$0.63^P \pm 0.01$	$0.06^U \pm 0.00$	$0.01^W \pm 0.00$	$0.00^Y \pm 0.00$	$308.17^E \pm 8.09$	$10.97^N \pm 0.85$
Gs Dried	$0.36^B \pm 0.01$	$0.45^L \pm 0.02$	$0.03^S \pm 0.01$	$0.00^V \pm 0.00$	$0.01^Z \pm 0.00$	$308.17^E \pm 8.09$	$10.97^N \pm 0.85$
Go Dried	$0.31^A \pm 0.00$	$0.62^P \pm 0.01$	$0.06^U \pm 0.00$	$0.01^W \pm 0.00$	$0.00^Y \pm 0.00$	$92.33^C \pm 0.81$	$3.63^K \pm 0.23$
Gm Form	$0.35^B \pm 0.01$	$0.53^M \pm 0.01$	$0.05^U \pm 0.00$	$0.02^W \pm 0.01$	$0.01^Z \pm 0.00$	$201.45^D \pm 1.53$	$112.19^T \pm 0.73$
Gs Form	$0.53^D \pm 0.01$	$0.22^K \pm 0.02$	$0.02^S \pm 0.00$	$0.02^W \pm 0.00$	$0.02^A \pm 0.00$	$101.42^C \pm 1.10$	$60.92^Q \pm 0.80$
Gm	$0.35^B \pm 0.00$	$0.63^P \pm 0.01$	$0.06^U \pm 0.00$	$0.02^W \pm 0.00$	$0.01^Z \pm 0.00$	$101.45^C \pm 1.54$	$56.9^P \pm 0.47$
Gs	$0.38^C \pm 0.00$	$0.46^L \pm 0.02$	$0.04^T \pm 0.00$	$0.00^V \pm 0.00$	$0.01^Z \pm 0.00$	$308.17^E \pm 8.09$	$10.97^N \pm 0.85$
Gm Myc	$0.36^B \pm 0.00$	$0.64^P \pm 0.00$	$0.06^U \pm 0.00$	$0.02^W \pm 0.00$	$0.01^Z \pm 0.00$	$430.57^H \pm 1.54$	$245.28^V \pm 1.73$
MCF-11	$0.54^D \pm 0.01$	$0.22^K \pm 0.02$	$0.02^S \pm 0.00$	$0.02^W \pm 0.00$	$0.02^A \pm 0.00$	$479.91^G \pm 17.09$	$88.48^S \pm 1.10$

Key: Extracts of the following-Gm dried: *Ganoderma mbrekobenum*, Go dried: *G. oregonense*, Gs dried: *G. sessile*, Gm Form.: *G. mbrekobenum* formulation, Go Form: *G. oregonense* formulation, Gs Form: *G. sessile* formulation, Gm: water extract of *G. mbrekobenum*, Gs: water extract of *G. sessile*, Gm Myc: mycelia of *G. mbrekobenum*, Gs Myc: water extract of *G. sessile*, Gano MCF: *G. sessile*, *G. oregonense* and *G. mbrekobenum* combination formulation 5% ($p < 0.05$). Not cited in the text. Figures and tables must be placed after they are cited in the text.

Cytotoxicity effect of the extracts of *Ganoderma* species and formulation on HEPG2 cells and Vero cells

Results in Figure 1 (a-g) and Table 3 show the effect of extracts from *Ganoderma* species and their formulations on the viability of HEPG2 cells, as well as the Inhibitory Concentration 50 (IC₅₀) of the extracts, respectively.

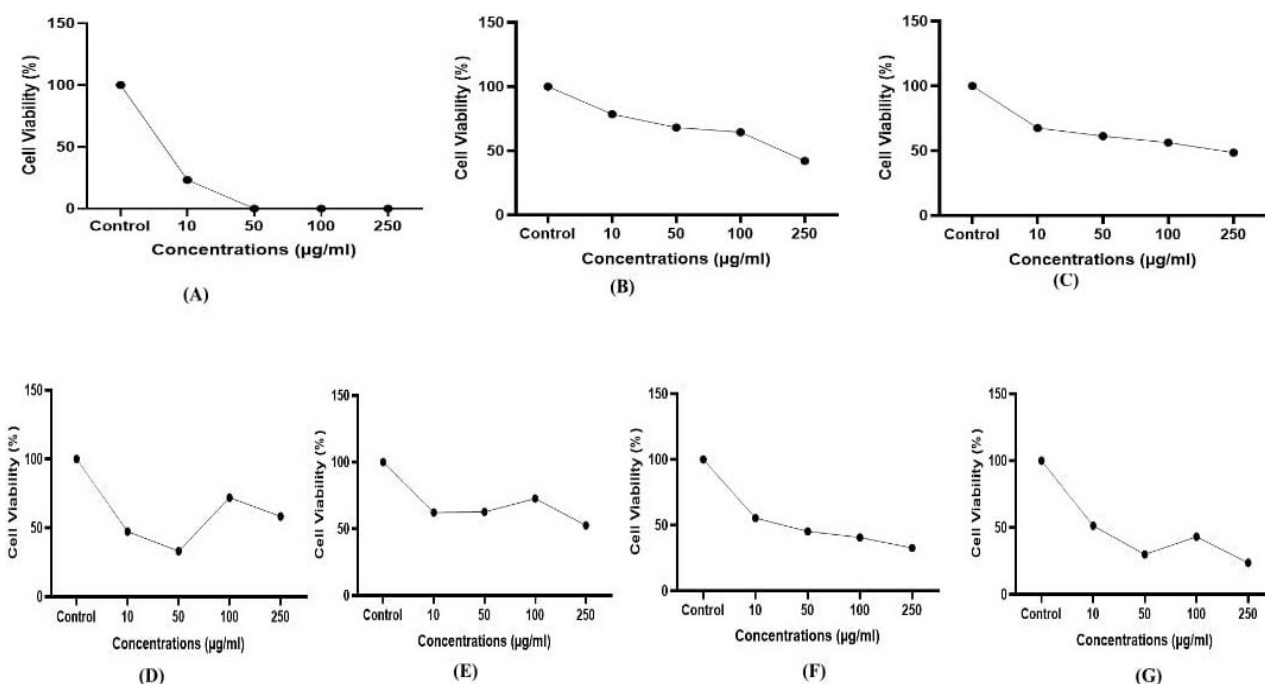


Figure 1. Cell viability of HEPG2 cells treated with extracts of *Ganoderma* species and formulation by XTT assay (a) Effect of Cisplatin on HEPG2 Cell Viability, (b) Effect of Gm on HEPG2 Cell Viability, (c) Effect of Gs on HEPG2 Cell Viability, (d) Effect of MCF 11b on HEPG2 Cell Viability, (e) Effect of 3b on HEPG2 Cell Viability, (f) Effect of MCF 11 on HEPG2 Cell Viability, (g) Effect of 10 on HEPG2 Cell Viability.

Table 3. Cytotoxicity of the extracts of *Ganoderma* species and formulations

S/N	Samples	IC ₅₀ of HEPG2 cells (µg mL ⁻¹)	IC ₅₀ Vero cells (µg mL ⁻¹)
1	Gm	150.3	116.3
2	Gs	123.0	14.57
3	3b	184.4	48.85
4	MCF -11	44.7	158.4
5	MCF-11b	106.3	394.5
6	10	23.76	29.20
7	Cis	4.578	101.8

Key: Gm: Hot water extract of *Ganoderma mbrekobenum*, Gs: Hot water extract of *G. sessile*, 3b: Gs formulation extract, 10: Gm mycelia biomass formulation extract, MCF-11: *G. sessile*, *G. oregonense*, and *G. mbrekobenum* combined formulation (water extract). MCF-11b: *G. sessile*, *G. oregonense*, and *G. mbrekobenum* combined formulation (ethanol extract). CIS: cisplatin.

The results showed that all the extracts expressed concentration-dependent cytotoxic effects against the treated cell lines to a reasonable extent. HEPG2 cells treated with the extract of *Ganoderma sessile* formulation (3b) showed the highest viability, followed by the hot water extract of *Ganoderma mbrekobenum* (Gm), with IC₅₀ values of 184.4 µg mL⁻¹ and 150.4 µg mL⁻¹, respectively ($p < 0.001$). On the other hand, the toxicity of 3b against Vero cells was substantial, whereas that of Gm was mild, with IC₅₀ values of 48.85 µg mL⁻¹ and 116.3 µg mL⁻¹, respectively. Liver tumor cells (HEPG2) tested with the hot water extract of the mycelia biomass formulation of *G. mbrekobenum* (10) showed the least HEPG2 cell viability with an IC₅₀ of 23.76 µg mL⁻¹. Still, they were also significantly toxic to normal kidney cells, Vero, CCL-81 (kidney), with an IC₅₀ of 29.20 µg mL⁻¹.

Fluorescence images were taken. Changes in morphology were observed in HepG2 treated with or without MCF 11 extract (Hot water extract of *G. sessile*, *G. oregonense*, and *G. mbrekobenum* combined formulation). Results showed that the MCF-11 extract at different concentrations exhibited morphological alterations after 24 h of exposure under a phase-contrast inverted microscope and a fluorescence microscope. The cells were stained with cell trackers to observe the fluorescence characteristics. The control group shows normal morphology. The characteristics of the group that was treated with MCF-11 extract 50 - 250 µg mL⁻¹ revealed abnormal morphology of the HepG2 cells, including shrunken cytoplasmic cells and reduced cell volume (compared to the control). Abnormal changes in morphology were observed in an extract concentration-dependent manner, with IC₅₀ values of 44.7 µL mL⁻¹ and 158.4 µL mL⁻¹ for the HepG2 and Vero cell lines, respectively. At 100 µg mL⁻¹, the effect of MCF 11 on treated cells was evident, as the cells lost their typical morphology and the cytoplasm appeared shrunken and rounded, with reduced cell volume.

Discussion

Molecular identification

In nature, over 450 *Ganoderma* species are widely distributed, where they degrade dead logs and stumps as saprophytes and are parasitic on various live trees, including palm trees, tea, and rubber (Ofodile et al., 2022). Two isolates, MH3 and MH2, produced sequences with BLAST coverage of 100% and 93%, respectively, for YCTQ. The MH3 sample showed 95.57% similarity with *G. mbrekobenum* (KX000898.1, NR_147647.1, and OK324049) from Ghana (Otto et al., 2016), 95.29% identity with *G. mbrekobenum* (ON876020.1) from the Benin Republic (Olou et al., 2023) and Egypt (PP741637.1). Isolate MH2 was 100% identical to *G. oregonense* MH277958.1 from the United States. The YCT-Q sample was 96.81% similar to *G. sessile* (KF605640.1) and 96.45% to *G. sessile* MG654256, MG654258.1 from the United States of America (Lloyd et al., 2018b).

Heavy metals composition

The growth substrate is one of the factors that can highly affect the quality of edible mushrooms (Rai et al., 2015). Given their saprophytic characteristics, mushrooms obtain their nutrients by absorbing dissolved organic matter from dead wood and other decayed materials. Demková et al. (2021) reported that mushrooms can accumulate heavy metals in large concentrations, such as mercury (Hg), lead (Pb) (Dilna et al., 2014), arsenic (As) (Lalotra et al., 2016; Seyfferth et al., 2016), cadmium (Cd) (Lalotra et al., 2016; Seyfferth et al., 2016), manganese (Mn), copper (Cu), iron (Fe), and zinc (Zn). Although some heavy metals, such as Zn, Fe, Mn, and Cu, are essential mineral elements in mushroom fruit bodies, others, including Hg, Pb, As, and Cd, pose health hazards (Khani et al., 2017). Furthermore, most elements can be bioaccumulated by mushrooms, especially from soil and substrates (Saba et al., 2016). In the present investigation, although the mushroom fruit bodies were cultivated with water hyacinth as a growth

supplement, the metals were at acceptable levels within the WHO limits; therefore, they do not pose any health risk to consumers.

The permissible limits set by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) for heavy metals in herbs are: arsenic and lead, 10 mg kg⁻¹; cadmium, 0.3 mg kg⁻¹; and zinc, 50 mg kg⁻¹ (World Health Organization, 2007). The content of these metals was at acceptable levels in the tested samples. The regulatory limits for iron, copper, magnesium, manganese, and chromium in medicinal herbs have not been established (World Health Organization, 2007).

Cytotoxicity effect of the extracts of *Ganoderma* species and formulation

Since the Vero cells (normal kidney epithelial cells from monkeys) were also affected, it indicates that the extract may have non-selective toxicity. Ideally, a good anticancer agent should selectively kill cancer cells without harming normal cells (Chiu et al., 2021). A study evaluating methanol plant extracts on *HeLa* and *Vero* cell lines found that while some extracts showed significant anticancer activity, others caused dose-dependent cytotoxicity in Vero cells, suggesting general toxicity and indicating a need for further investigation into safety and therapeutic options (Artun et al., 2017). A concentration below 30 µg mL⁻¹, which is toxic to both cell lines, may set a benchmark for identifying the maximum safe dosage for future preclinical and clinical studies (Chiu et al., 2021). The hot water extract of the mycelia biomass formulation of *G. oregonense* was not toxic against Vero and HEPG2 cells at concentrations higher than the highest concentration tested. Hot water extract of *G. sessile*, *G. oregonense* and *G. mbrekobenum* combined formulation (MCF-11) had strong cytotoxicity with IC₅₀ of 44.7 µL mL⁻¹ effect on HEPG2 cells with mild toxicity with IC₅₀ of 158.4 µL mL⁻¹ to Vero cells which was comparable to cisplatin effect on Vero cells (Figure 2a and g).

The anti-proliferation effect is the first indication to be assessed when investigating novel antitumor agents. Serrano-Márquez et al. (2021) reported a *G. curtisii* strain that showed significant inhibition against different tumor cell lines with IC₅₀ < 50 µL mL⁻¹, making it a promising candidate for further characterization of its metabolites for anticancer therapies. (Serrano-Márquez et al., 2021). The ethanol extract of *G. sessile*, *G. oregonense*, and *G. mbrekobenum* combined formulation (MCF-11b) was mildly toxic to liver tumor cells but inactive against Vero cells, with an IC₅₀ concentration higher than the highest concentration tested. Liu et al. (2009) reported anti-proliferative activity against tumor lines in some species of the genus *Ganoderma*, attributing the activity to the presence of triterpenoids, sterols, and nucleosides, which induce apoptosis through mitochondrial transmembrane depolarization and cell cycle arrest.

Figure 3(a-c) shows the MCF-11 after 24 hours of exposure. The control group exhibits normal morphology, while the characteristic abnormal morphology of the HEPG2 cells, including shrunken cytoplasmic cells and reduced cell volume (compared to the control), is observed in cells treated with this extract.

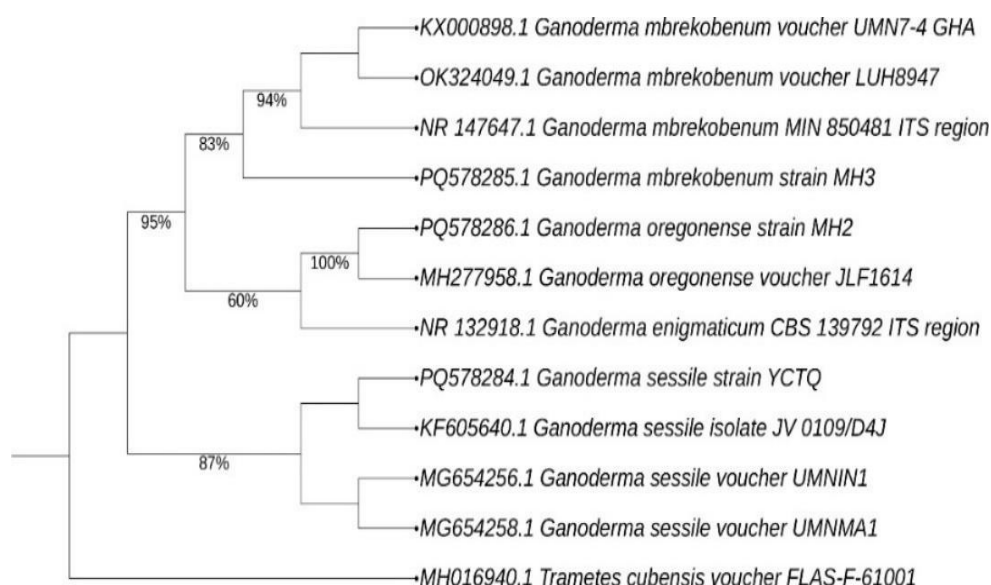


Figure 2. A Dendrogram of the *Ganoderma* species was constructed using the UPGMA method with a 1000 bootstrap replicate value. Evolutionary distances were computed using the Maximum Composite Likelihood method. The strains sequenced in this study are the ones being discussed. *Trametes cubensis* was included as an outgroup. The phylogenetic analysis was performed using the MEGA11 software.

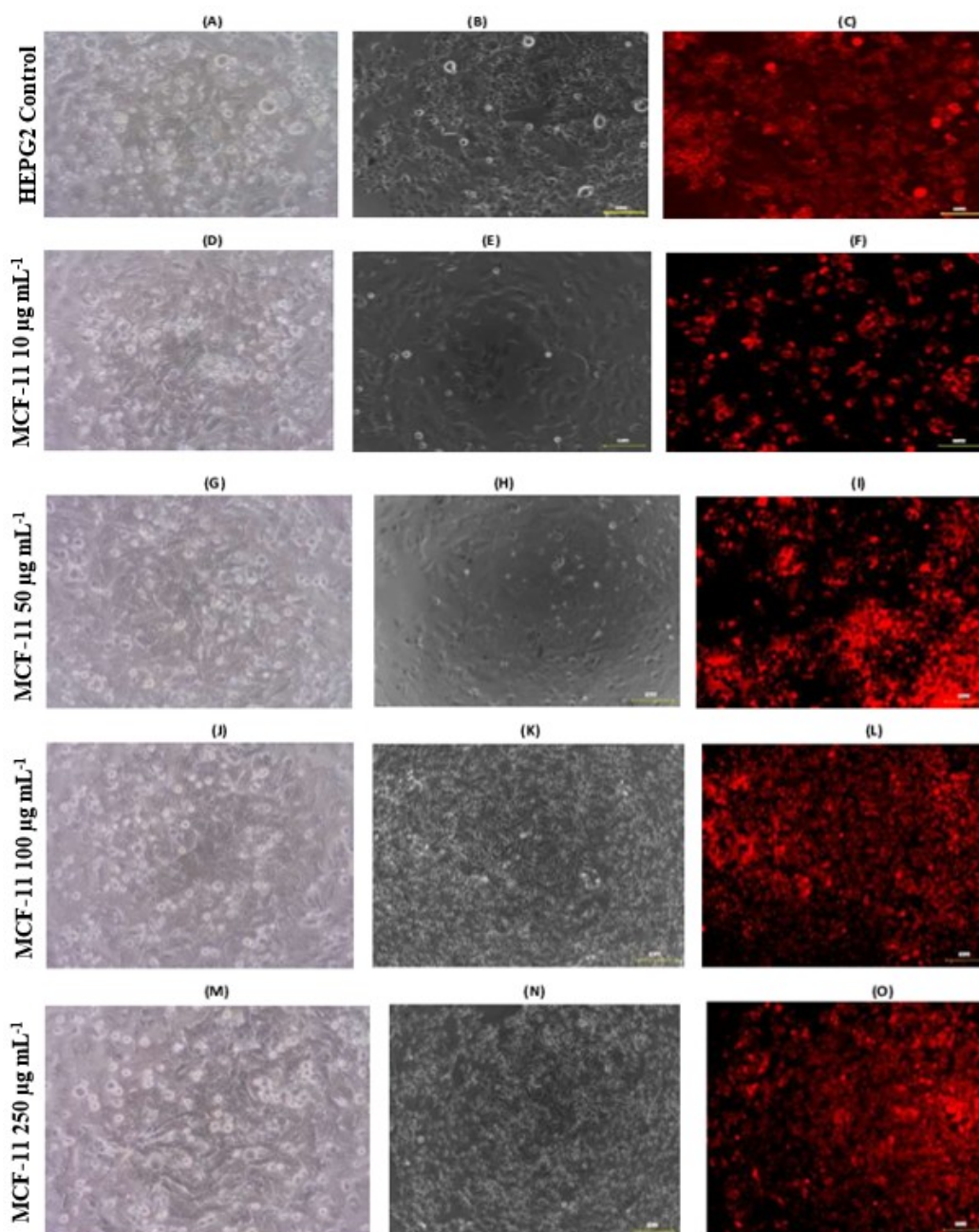


Figure 3. Control group and Test groups of HepG2 cell XTT Assay that received MCF-11 extract treatments (10-250 $\mu\text{g mL}^{-1}$) shown by (A, D, G, J, M) microscopic image of HepG2 Cells After 24 h (10x mag). (B, E, H, K, N) Bright-field fluorescence (C, F, I, L, O) stained-red images were obtained using CMTPIX fluorescence stains. The yellow scale bar shows 100 μm . Fluorescence images were taken.

Changes in morphology

The MCF 11-treated HepG2 cells exhibited vacuolation and more pronounced morphological changes, characterized by extensive small protrusions on their cell membranes, which is often indicative of cell damage or apoptosis (Khalil et al., 2015). However, this may suggest excessive autophagy processes of cell death.

Conclusion

Overall, the best way to evaluate the potential of *Ganoderma* species is to employ molecular characterization, determine their safety regarding heavy metal accumulation, and assess their pharmacological activities. In this case, the focus has been on molecular characterization, phylogenetic analysis, heavy metal and mineral element composition, and antitumor activity against the HEPG2 cell line. The metal analysis of the *Ganoderma* species used in this study reveals acceptable concentrations compared to WHO limits; therefore, they are unlikely to pose any health risk to consumers. The *Ganoderma* species

extracts expressed concentration-dependent cytotoxicity. The mechanism of MCF-11-induced cytotoxicity may involve the inhibition of HepG2 cell viability and possibly induce cell death, which requires further investigation.

However, we suggest studying the apoptosis induction pathway, which could provide more insight into the cytotoxicity and anti-tumor effects of MCF-11 on the human liver. This, in turn, might position it as a potential medicinal agent for the treatment or management of hepatocellular carcinoma.

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