

Antioxidant Response and Selective Cytotoxicity of Methanolic *Ganoderma lucidum* Extract in MCF-7 Breast Cancer Cells Compared to HDFn Fibroblasts

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ABSTRACT

Ammoniacal nitrogen is a pervasive aquatic contaminant that threatens aquatic life, compromises disinfection, and drives *Ganoderma lucidum* also a focus area in biomedical research because extracts contain various biologically active compounds belonging to several classes especially, polysaccharides, triterpenoids, sterols, and phenolic constituents. In the current study, a methanolic extract of *G. lucidum* was evaluated using three combined approaches of preliminary profiling (only by GC-MS), DPPH radical exposure responses, and cytotoxicity against MCF-7 breast cancer cells (relative viability to normal, HDF, fibroblasts), as detailed above. GC-MS application was only a preliminary, chemical screening tool, and all peaks were treated according to library match reliability and likelihood of signal from background). Extract © dip recipe yielded a rising antioxidant response commencing at a ≤ 20 $\mu\text{g/mL}$ concentration, and >100 was a response noted as reaching $64.97 \pm 2.50\%$ scavenging activity (compared to $3.48 \pm 0.02\%$ of the negative DMSO control, but evaluation with ascorbic acid as standard yielded maximum $79.70 \pm 2.10\%$ level for $200 \mu\text{g/mL}$ concentrations). In the MTT assay, the extent to which *G. lucidum* extract lowered the viability of MCF-7 was markedly less than the relative viability lowering seen by HDFn actual viable response so yielded about a selectivity index of about 10.6, confirming preferential cytotoxicity toward the cancer. Extract showed moderate antioxidant activity, with promising selectivity for in vitro cytotoxicity as well, but fractionation, compound confirmation, and mechanical assays are still warranted before being assigned to particular components and/or used in therapy.

Keywords- *Ganoderma lucidum*; methanol extract; GC-MS; DPPH; antioxidant activity; MCF-7; HDFn; cytotoxicity; IC50; selectivity index.

I. INTRODUCTION

Natural products continue to be an important source of diverse compounds for biological screening, especially in biology as related to oxidative stress and cancer [1–4]. Similarly relevant are medicinal mushrooms, producing both polysaccharides, high molecular weight, as well as lower molecular weight metabolites, including triterpenoids, sterols and phenolic derivatives [2–7]. Of the medicinal mushrooms, *G. lucidum* has been extensively investigated, owing to its traditional use, and empirically described antioxidant, immunomodulatory and anticancer-specific activities [1–9].

Chemical characterisation can be useful when biological activity is subsequently assessed in a crude extraction. GC-MS data also must be treated cautiously since library-based matching will yield tentative and not absolute structural identifications, particularly in fungal extracts when detected peaks can arise from the analytical background, often silicone containing or column [10,36,37]. For that reason, in the present work GC-MS was used more as a profiling tool rather than as proof of actively constituting species.

Oxidative stress arises when the generation of reactive oxygen species overwhelms the cellular control systems for oxygen. This imbalance can result in damage to lipids, proteins, and DNA, and has been associated with inflammatory and

malignant processes [34,35]. Because it provides simple, reproducible estimates of radical scavenging capacity that can be compared with those for reference antioxidants such as ascorbic acid, the DPPH assay is widely used as an initial screen [19–24].

Breast cancer continues to be a challenge worldwide, and MCF-7 cells are frequently used as an estrogen receptor positive breast cancer model [29–31]. To test a compound or extract sole against cancer cells gives an incomplete picture of biological information, as a candidate with a prospect should, in theory demonstrate more toxicity against cancer cells as opposed to normal cells. As such, HDN fibroblasts were included as a normal cell comparison.

Use of methanol as a extracting solvent, as it dissolves large classes of polar and moderately polar bioactive including, but not limited to, phenolic constituents and other secondary metabolites that may contribute to the antioxidant and cytotoxic response [10,18,19,24]. GC–MS profiling included as preliminary chemical screening methodology, to give insight into the detectable volatile and semi-volatile constituents of the extract [10], but used chemical profile only in a supporting role (due to GC–MS library identification being tentative) and therefore biological interpretation rather than “confirming” active compounds [36,37].

Although previous studies have shown anticancer-related effects of *G. lucidum* extracts and isolated constituents [11–17], the biological impact of a crude extract may depend on the extraction solvent, tested concentration range and chemical composition as well as cell model used, hence we aimed at the evaluation of the methanolic extract of *G. lucidum* through three linked endpoints: GC-MS chemical profiling, DPPH antioxidant activity and selective cytotoxicity against MCF-7 breast cancer cells compared to a normal human fibroblasts.

II. MATERIALS AND METHODS

2.1 Chemicals

Eagle’s Minimum Essential Medium (EMEM), fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich, Germany; MTT assay kit from AbbeXa Ltd., United Kingdom; while other reagents used during the work were of analytical grade.

2.2. Preparation of *Ganoderma lucidum* methanol extract

The dried *G. lucidum* substance was cleaned and ground into powdered form [6,7,10]. A total of 50 grams of the powder was soaked in 300 mL 80% methanol and kept shaking for 24 h at room temperature. The mixture was filtered using whatman No. 1 filter paper. The extracted solution was evaporated to dryness under reduced pressure at 70 °C using a rotary evaporator; the resultant crude extract was then stored in a tightly closed container under refrigeration until analysis. The solution was filtered through a 0.22 µm membrane filter before use in biological assay. The same batch of extract was used, for GC-MS profiling, DPPH and cytotoxicity assay in order to maintain stability among the results.

2.3. GC-MS chemical profiling

The methanolic extract was analysed by GCMS to show what might be visible with the speedy analysis of volatile and semi-volatile substances. The GC output was processed with ChemStation integrated processing; the masses were crosscompared with the NIST14 library. Peaks were tabulated that, detecting those visibly present, according to retention time, relative area, and, where possible, CAS number, and also with respect to library quality. As this method is tentatively identifying, low and medium qualities were not considered as structures present. Peaks relating to siloxanes were present, but caution should be exercised, as cyclic siloxanes and linear siloxanes might come from septa, vials, inlet components, column bleeds, or other sources of silicone within the GCMS workflow [36,37].

2.4. DPPH radical scavenging assay

Antioxidant activity of the extract was determined by using DPPH radical scavenging method [19–24]. Extraction with concentrations of 12.5, 25, 50, 100 and 200 µg/mL were made and ascorbic acid was evaluated friedly as reference antioxidant. After incubation with DPPH solution in dark, absorbance was measured”, using a spectrophotometer or a microplate reader.” Radical scavenging activity was calculated using the following formula: scavenging activity (%) = $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$. The measurements were done in triplicate and expressed as the mean ± standard deviation.

2.5. Cell culture

Cytotoxicity and selectivity were evaluated in viability assays with MCF-7 human breast cancer cells and HDFn normal human dermal fibroblasts [29]. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in EMEM, containing fetal bovine serum and antibiotics. Only healthy, actively dividing cells were assayed for viability.

2.6. MTT Cytotoxicity assay

Cell viability was determined through the MTT colourimetric assay, which estimates the metabolic activity of living cells [25–27]. The MCF-7 and HDFn were seeded onto 96-well plates and allowed to adhere to the bottom of the plates before being treated. They were then exposed to an increasing number of serial concentrations of *G. lucidum* methanolic extraction: 25, 50, 100, 200, and 400 µg/mL. The DMSO based extract was used in these experiments and a solvent control was used to ensure that DMSO did not affect viability independently. Following incubation, MTT reagent was used according to kit directions, the resultant formazan product was dissolved, and absorption was read on a microplate reader. Untreated

cells were used as the control. Cell viability = cell viability (%) = $A_{treated} / A_{control} \times 100$. IC50 values were estimated by nonlinear regression of the dose-response curves.

2.7. Determination of IC50 and selectivity index

Overall dose response curves for each assay were generated independently for MCF-7 cells and HDFn using a four-parameter nonlinear regression model. IC50 was defined as the extract concentration that achieved 50% cell kill [28]. A selection activity index was determined by comparing cut-off point IC50 ratios to assess the relative favorability of cytotoxicity towards the cancer cell line.

2.8. Statistical analysis

All experiments were performed in triplicate and results were presented as mean±standard deviation. Statistical analysis was conducted with GraphPad Prism or equivalent software and comparisons in the antioxidant assay were made using Sidak’s multiple comparisons test where appropriate, with p values <0.05 considered statistically significant. Nonlinear regression was used to estimate IC50 values and fit quality was evaluated by R2 values.

III. RESULTS AND DISCUSSION

3.1. GC-MS chemical profiling of *G. lucidum* methanol extract

GC-MS analysis offered a tentative chemical profile of the volatile and semi-volatile compounds that the methanolic extract revealed. Four peaks dominated between approximately 14 to 22 min. One peak at 14.065 min was tentatively assigned 2-methoxy-4-vinylphenol (relative area 12.48%, library quality 90). This assignment is pertinent because phenolic structures may potentially contribute to the observed radical scavenging behavior by donating hydrogen/electron [18,19,24]. The two major peaks from the two larger peak areas were assigned to cyclohexasiloxane, dodecamethyl- at m/z 15.159 min and pentasiloxane, dodecamethyl- at 18.807 min, relative area of 36.95 and 33.67% respectively. We believe that these signals should be treated with caution and not, overall, overassessed as fungal bioactive metabolite hits. Siloxanes are not uncommonly seen as GC-MS background [36,37]. One further peak, 22.076 min, was tentatively attributed as 3,4-dihydroxyphenylglycol, 4TMS derivative, relative area esdtrace 16.91%, library quality 55 (less than the preferred hit area of >70 confirmatory)

Table 1. Major GC-MS peaks detected in the methanol extraction of *G. lucidum*.

Peak	Retention time (min)	Tentative identification	CAS No.	Area (%)	Library quality / comment
1	14.065	2-Methoxy-4-vinylphenol	007786-61-0	12.48	Quality 90; phenolic constituent
2	15.159	Cyclohexasiloxane, dodecamethyl-	000540-97-6	36.95	Quality 93; possible siloxane background
3	18.807	Pentasiloxane, dodecamethyl-	000141-63-9	33.67	Quality 52; possible siloxane background
4	22.076	3,4-Dihydroxyphenylglycol, 4TMS derivative	056114-62-6	16.91	Quality 55; tentative

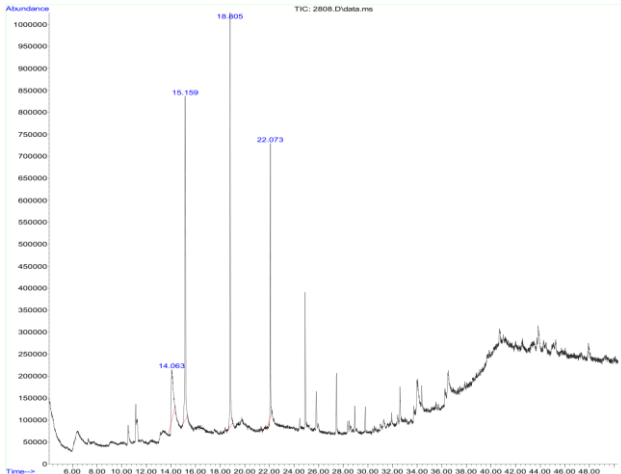


Figure 1. GC-MS total ion chromatogram of the *G. lucidum* methanol extract showing the main peaks detected between approximately 14 and 22 min.

3.2. Antioxidant activity of *G. lucidum* methanol extract

The same extract gave a more concentration-dependent DPPH radical scavenging response (Table 2 and Figure 2). At the highest concentration of 200 µg/mL, an extract of $64.97 \pm 2.50\%$ scavenging activity was recorded, while at the same concentration, ascorbic acid attained $79.70 \pm 2.10\%$. The extract, at 100 µg/mL, gave a $54.24 \pm 2.13\%$ activity compared to $74.07 \pm 1.01\%$ for ascorbic acid. The activity declined to $(28.12 \pm 3.59\%)$ at 12.5 µg/mL.

Table 2. DPPH root scavering activity of *G. lucidum* methanol extract compared with ascorbic acid.

Concentration (µg/mL)	Ascorbic acid mean (%)	Ascorbic acid SD	<i>G. lucidum</i> extract mean (%)	<i>G. lucidum</i> extract SD
200	79.70	2.10	64.97	2.50
100	74.07	1.01	54.24	2.13
50	64.27	4.15	38.16	6.69
25	54.67	5.77	29.90	1.32
12.5	36.23	4.87	28.12	3.59

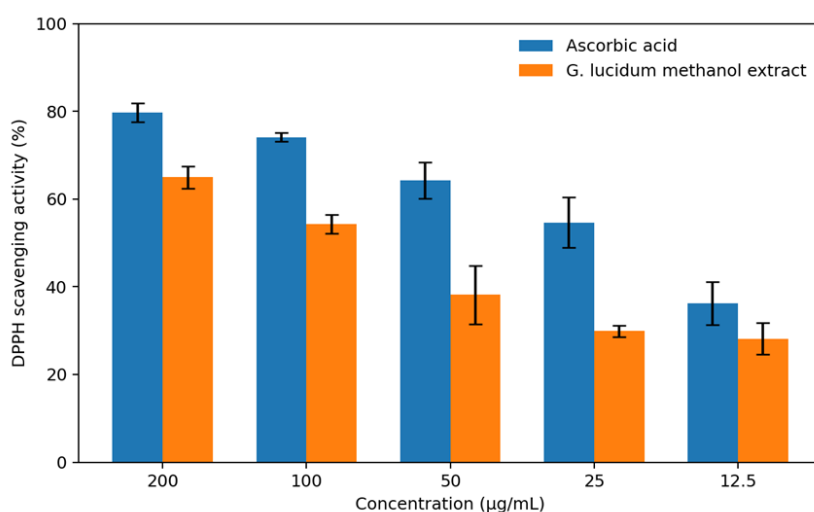


Figure 2. Concentration-dependent DPPH scavenging activity of *G. lucidum* methanol extract and ascorbic acid. Error bars represent SD.

Statistical comparison indicated stronger activity for ascorbic acid at 25, 50, 100, and 200 µg/mL, although the difference at 12.5 µg/mL was not statistically significant. The detection of a phenolic by GC-MS may partially support the antioxidant response seen, since phenolic components can exhibit radical scavenging activity [18,19,24]. However, as the extraction is crude and GC-MS assignments tentative, this activity should probably be viewed as that of several constituents rather than the effect of a single (detected) species [10,36,37]

3.3. Selected cytotoxic activity against MCF-7 and HDFn cells

The extraction decreased the viability of both cell lines, but the MCF-7 cells appeared to be more adversely affected than the HDFn cells for the concentrations tested (Table 3 and Figure 3). At 25 µg/mL, viability was reduced to $78.63 \pm 0.85\%$ in MCF-7 cells while HDFn viability was $94.87 \pm 0.74\%$. At 50 µg/mL viability is decreased to $65.70 \pm 0.33\%$ in MCF-7 cells while HDFn viability is $93.02 \pm 0.29\%$. At 400 µg/mL, viability is decreased to $37.69 \pm 0.88\%$ in MCF-7 cells and $71.61 \pm 0.79\%$ in the HDFn cells.

Table 3. Concentration-dependent DPPH scavenging activity of *G. lucidum* methanol extract and ascorbic acid. Error bars represent SD.

Concentration (µg/mL)	HDFn viability mean (%)	HDFn SD	MCF-7 viability mean (%)	MCF-7 SD
25	94.87	0.74	78.63	0.85
50	93.02	0.29	65.70	0.33
100	88.81	1.69	53.51	2.00
200	81.94	0.95	43.04	0.85
400	71.61	0.79	37.69	0.88

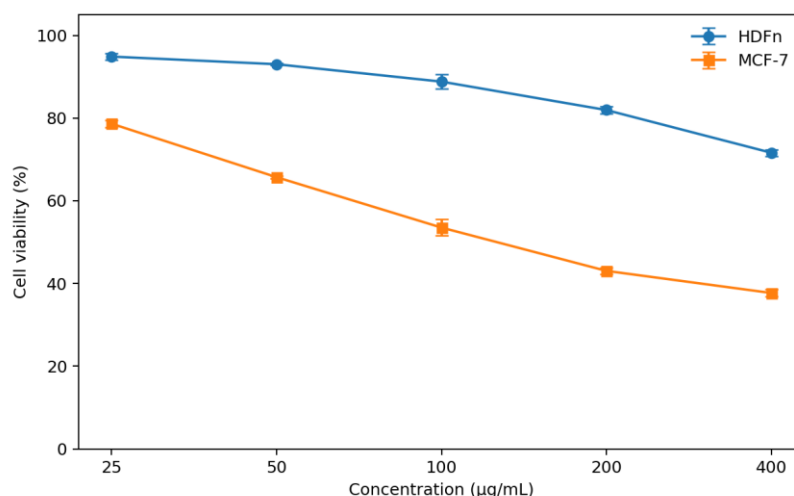


Figure 3. Impact of *G. lucidum* methanol extract on HDF and MCF-7 cell viability. Error bars represent SD.

The expected IC₅₀ value of 51.34 µg/mL for MCF-7 compared to 543.3 µg/mL for HDFn generated a selectivity index of roughly 10.6. The nonlinear dose-response models were high fit values ($R^2 = 0.9959$ for MCF-7, $R^2 = 0.9907$ for HDFn). The difference between the two IC₅₀ values is the most important biological finding of this work, as it indicates preferential cytotoxicity toward MCF-7 cells compared with normal HDFn fibroblasts under the conditions tested.

Table 4. IC₅₀ values and selectivity index of *G. lucidum* methanol extraction.

Cell line	IC ₅₀ (µg/mL)	R ²	Interpretation
MCF-7	51.34	0.9959	Sensitive cancer cell response
HDFn	543.3	0.9907	Lower sensitivity in normal fibroblasts
Selectivity index	10.6	-	IC ₅₀ HDFn / IC ₅₀ MCF-7

3.4. Integrated interpretation

Taken together, the results show two different layers of biological activity - in this case, antioxidant response was moderate since the extract produced a clear concentration dependent effect but remained weaker than ascorbic acid at most concentrations, whilst the cytotoxicity data were more distinguished since the extract yielded a much lower IC₅₀ in MCF-7 cell lines compared to HDFn fibroblasts. This difference fuels the idea that the methanolic extract contains constituents that affect cancer cell viability more strongly than normal cell viability under the assessed conditions.

The analytics should be viewed as ‘backing’ up the results of activity rather than ‘proof’ of the molecules of interest. The signal for 2-methoxy-4-vinylphenol appears tentative and is congruous with the antioxidant result since phenols may be linked to root scavenging activity [18,19,24]. However, the large siloxane-related peaks should be treated with suspicion, perhaps being analytical background rather than true fungi metabolites [36,37]. Bright – but suspect that none are made cleanly as shown here. The biological got to the active site compellingly but cannot be directly assigned to the pieces by GC-MS.

This study has limitations as well. The MTT assay measures for metabolic activity alone and does not show that apoptosis, necrosis, cell-cycle arrest, or a specific molecular pathway occurred by itself [25–27]. More experiments including apoptosis markers, cell-cycle analysis, mitochondrial membrane potential, intracellular ROS measurement, bioactivity-guided fractionation, need to be performed before mechanistic, or theoretical conclusions can be drawn.

IV. CONCLUSION

The methanolic extract of *G. lucidum* indicated moderate concentration-dependent DPPH radical scavenging activity and selective cytotoxicity against MCF-7 breast cancer cells compared to HDFn normal fibroblasts. It achieved maximum effect of $64.97 \pm 2.50\%$ DPPH scavenging activity at 200 µg/mL while the MCF-7 IC₅₀ was 51.34 µg/mL compared with 543.3 µg/mL for HDFn normal fibroblasts yielding a selectivity index of approximately 10.6. The selectivity index indicates preferred cytotoxicity of the extract against the cancer cell line under the assay conditions used. GC-MS profiling provided limited chemical information with siloxane related peaks interpreted to be those associated with analytical background. Overall these allow for the extraction to be investigated further through confirmation of compounds, fractionation and then biological assays to check the mechanisms for functions before implications for therapy can be drawn out.

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