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Evaluation of Bioactive Compounds, Antioxidant Activity, and Anticancer Potential of Wild *Ganoderma lucidum* Extracts from High-Altitude Regions of Nepal

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Abstract: *Ganoderma lucidum*, a medicinal mushroom with a rich ethnobotanical history, was investigated using wild specimens collected from high-altitude regions of Nepal. This study aimed to identify key bioactive compounds and assess the influence of solvent type—water, ethanol, methanol, and acetone—on extraction efficiency and biological activity. Extracts were evaluated for antioxidant potential, cytotoxicity against HeLa cells, and phytochemical composition via gas chromatography–mass spectrometry (GC-MS). Solvent type significantly affected both yield and bioactivity. Acetone yielded the highest crude extract (3.43 mg/g), while ethanol extract exhibited the highest total phenolic (376.50 ± 9.32 mg PGE/g) and flavonoid content (30.33 ± 0.50 mg QEs/g). Methanol extract was richest in lycopene (0.0670 ± 0.001 mg/g) and β-carotene (0.4536 ± 0.000 mg/g). Ethanol extract demonstrated consistently strong DPPH, superoxide, hydroxyl, and nitric oxide radical scavenging activity, along with high reducing power. All extracts showed dose-dependent cytotoxicity against HeLa cells, with ethanol and water extracts showing the greatest inhibition (>65% at 1000 μg/mL). GC-MS profiling identified solvent-specific bioactive compounds including sterols, terpenoids, polyphenols, and fatty acids. Notably, pharmacologically relevant compounds such as hinokione, ferruginol, ergosterol, and geranylgeraniol were detected. These findings demonstrate the therapeutic potential of *G. lucidum*, underscore the importance of solvent selection, and suggest that high-altitude ecological conditions may influence its bioactive metabolite profile.

Keywords: *Ganoderma lucidum*; solvent extraction; antioxidant activity; cytotoxicity; high-altitude fungi; DPPH; MTT assay; taxonomy; GC-MS

1. Introduction

Ganoderma lucidum, commonly known as Lingzhi in China, Reishi in Japan, and Dadu chyau in Nepal, is a polypore mushroom with a long and storied history of use in East Asian medicine for promoting health and longevity [1]. It is often referred to as the "King of Herbs" or the "Mushroom of Immortality." Its medicinal use dates back over 2,000 years, with its effects documented in ancient scripts like the Shen Nong Ben Cao Jing from China's Eastern Han dynasty (25-220 AD) [2]. Traditionally, it has been used to treat a variety of ailments and is believed to enhance stamina, increase brain power, improve circulation, and strengthen the immune system [3].

Modern research has begun to validate these traditional claims, attributing the mushroom's medicinal properties to its rich and varied chemical composition. Bioactive compounds such as polysaccharides, triterpenes, adenosine, organic germanium, phenolic compounds, flavonoids, and ergosterol contribute to its therapeutic effects, which include antioxidant, anti-cancer, anti-inflammatory, and antimicrobial activities [4–8]. For instance, triterpenic acids have demonstrated significant anti-cancer effects, while polysaccharides have shown anti-diabetic and antibiotic

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properties [9–12]. The antioxidant properties of *G. lucidum* are particularly noteworthy, as they neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are implicated in the development of chronic diseases such as cancer, aging, diabetes, cardiovascular disorders, and neurodegeneration [4,13]. Studies have shown that polysaccharides in *G. lucidum* exhibit potent ROS-scavenging activity and can inhibit tumor growth through immunomodulation and the induction of apoptosis [6,9,10]. Furthermore, its anti-inflammatory effects have been observed in animal models, suggesting significant therapeutic potential [14].

Nepal, with its diverse geography ranging from the Terai plains to the high Himalayas, provides a unique environment for a wide variety of mushrooms [15–19]. While approximately 1,300 mushroom species have been reported in Nepal, with 73 identified as having medicinal value, the therapeutic potential of most, including native *G. lucidum*, remains largely unexplored [20,21]. Research on Nepalese mushrooms, initiated by the Nepal Agricultural Research Council (NARC) in 1974, has historically focused more on cultivation and taxonomy rather than on the detailed analysis of their bioactive properties.

The unique environmental conditions of Nepal's high-altitude regions—characterized by lower oxygen levels, intense UV radiation, and distinct soil compositions—may lead to the production of *G. lucidum strains* with enhanced or novel bioactive profiles [22,23]. Despite Nepal's rich biodiversity, research on high-altitude *G. lucidum* is scarce, leaving a gap in our understanding of its full potential. This study, therefore, aims to investigate the bioactive constituents, antioxidant activities, and cytotoxic effects of wild *G. lucidum* collected from the high altitudes of Nepal, utilizing various solvent extracts to comprehensively characterize its mycochemical composition and therapeutic promise.

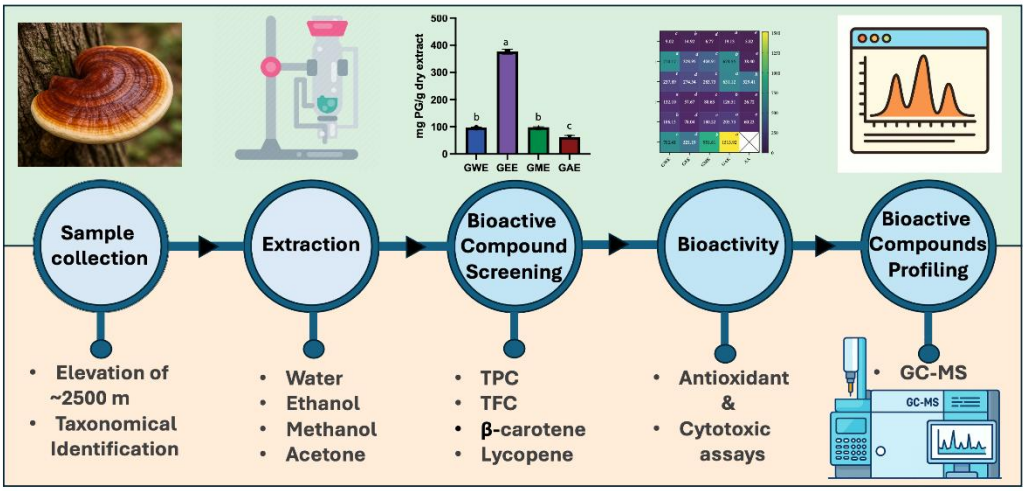

Figure 1. Overview of the study workflow, from *G. lucidum* collection and solvent extraction to bioactive compound screening, biological activity assays, and GC-MS profiling.

2. Materials and Methods

2.1. Collection and Identification

Fresh fruiting bodies of wild *G. lucidum* were collected from a dead trunk of *Quercus lanata* on Chandragiri Hill, Nepal (elevation 7482 ft; latitude 27.67402; longitude 85.19874) (Figure. 2). The specimens were identified based on detailed macro- and micro-morphological characteristics (Table 1).

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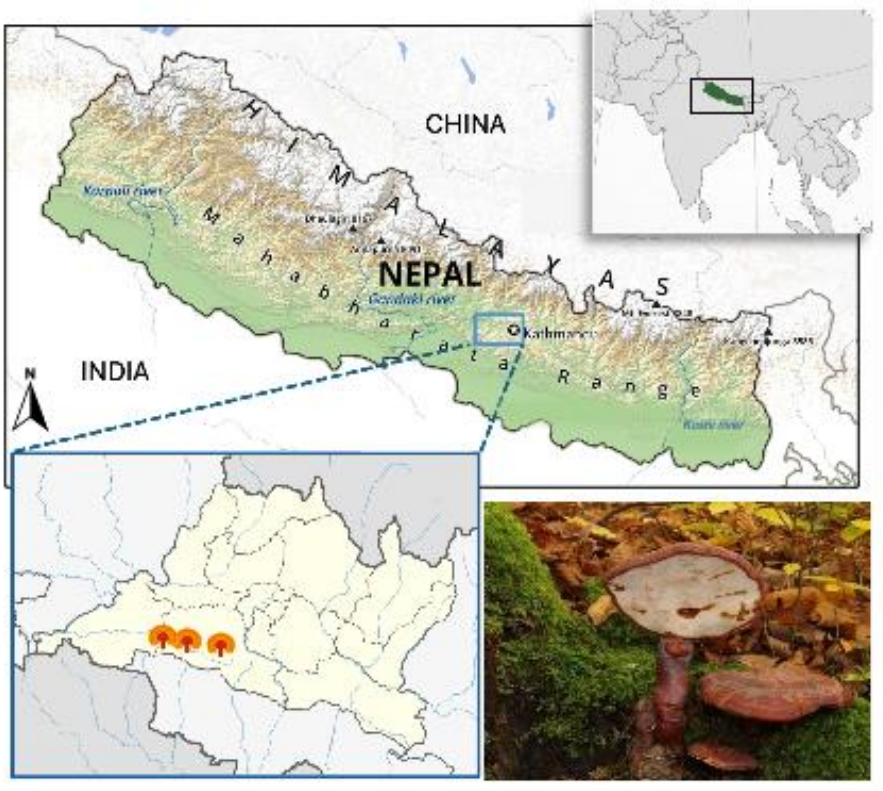

Figure 2. Map of Nepal in a global context (A), *G. lucidum* collection site (B) and its fruiting body growing on *Quercus lanata* (C).

Table 1. Summary of the ecological, morphological, anatomical, and taxonomic characteristics of *G. lucidum*.

Parameter	Description
Collection month	September–October
Location	Chandragiri Hill, Kathmandu, Nepal
Elevation	7482 ft (2280 m) above sea level
Coordinates	Latitude: 27.67402° N; Longitude: 85.19874° E
Ecosystem type	Solitary
Substrate	Wood, stump, log, stick, base of tree, bark
Host tree	*Quercus lanata*
Rot type	White-rot
Surrounding trees (20 ft radius)	Predominantly hardwoods
Basidiocarp size	7–12 × 11–19 × 1.5 cm
Texture	Woody to corky
Stipe	Sub-sessile to laterally stipitate, 2–3 cm
Pileus shape	Reniform
Upper surface	Laccate, dark reddish to purplish, yellowish at margins; brittle, soft
Margin	Blunt, rounded, brown-white
Pore surface	Creamy to milky coffee; ~5 pores/mm
Tube layer	2–9 mm long, white turning brown when brushed or aged
Context	9 mm thick, brown, without horny deposition
Cutis type	Thick-walled claviform with diverticula; 35–42 × 6–8.5 µm
Hyphal system	Trimitic: Generative (3.3 µm, hyaline, thin-walled, with clamp); Skeletal (5.8–7.5 µm, brown, thick); Binding (5–7.5 µm, brown)
Basidiospores	8.3–10 × 6.6 µm; yellowish-brown
Identification authority	Prof. Mahesh Kumar Adhikari, Dept. of Plant Resources, Kathmandu

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2.2. Sample Preparation and Extraction

The samples were cleaned and oven-dried gradually from 45 °C to 60 °C for 3 days until a constant weight was attained. The dried mushroom was milled into a fine powder and stored in airtight containers for future use. 10 gm of *G. lucidum* were subjected for extraction. Solvent extraction was performed for 10 hrs in a Soxhlet apparatus with 250 mL each of water (GWE, 100 °C), 70% ethanol (GEE, 60 °C), 80% methanol (GME, 70 °C), and 50% acetone (GAE, 50 °C). Extracts were concentrated under vacuum in a rotary evaporator (50 °C) and stored in dark vials at 4 °C. The calculation of the % yield was done for each solvent using the formula,

$$\% \text{ Yield} = \frac{\text{weight of the G. lucidum powder before extraction (gm)}}{\text{weight of the obtained extract (gm)}} \text{ X } 100$$

2.3. Estimation of Total Phenolic, Flavonoid, β-carotene and Lycopene

2.3.1. Total Phenolic Content

Total phenolic content was determined using the Folin-Ciocalteu method with pyrogallol as the standard [24]. A calibration curve was established by measuring the absorbance of pyrogallol standards (10-100 µg/mL) at 760 nm. Sample solutions (1 mL) were reacted with 5 mL of 10% Folin-Ciocalteu reagent for 5 minutes at room temperature. Subsequently, 4 mL of Na_2CO_3 solution was added, and samples were vigorously mixed and incubated in the dark for 2 hours at room temperature. Absorbance was read at 760 nm, and results were expressed as pyrogallol equivalents (mg/g dry extract).

2.3.2. Total Flavonoid Content

Total flavonoid content was determined using the aluminum chloride colorimetric method, adapted from Shraim et al., 2021[25]. Quercetin standard curve was prepared by diluting a 10 mg quercetin stock in 50% methanol to concentrations ranging from 10-100 µg/mL, with absorbance measured at 415 nm. Sample aliquots (1.0 mL) were mixed with 0.5 mL of 1.2% aluminum chloride, 0.5 mL of 120 mM potassium acetate, and 1 mL of distilled water. After 30 minutes of incubation at room temperature, absorbance was read at 415 nm. Results were expressed as mg quercetin equivalents per gram of dry extract (mg QE/g dry weight).

2.3.3. Estimation of β-Carotene and Lycopene Content

β-carotene and lycopene were determined as per Prakash et al., 2016 [26]. Dried extracts (100 mg) were extracted with 10 mL of acetone-hexane (4:6) for 1 min, then filtered. Filtrate absorbance was measured at 453, 505, 645, and 663 nm. Carotenoid concentrations (mg/100 mL) were calculated using the following equations:

$$\text{Lycopene} = (-0.0458 \times A_{663}) + (0.372 \times A_{505}) + (0.0806 \times A_{453})$$

$$\beta\text{-carotene} = (0.216 \times A_{663}) - (0.304 \times A_{505}) + (0.452 \times A_{453})$$

Results are presented as mg carotenoid per gram of dry extract (mg carotenoid/g dry extract)

2.4. Determination of In Vitro Antioxidant Activities

2.4.1. DPPH (2, 2-diphenyl-1-picryl-hydrazyl) Assay

Different concentrations of extracts (20-100 µg/mL) were prepared. To 1 mL of each extract concentration, 2 mL of ice-cold 0.1 mM DPPH solution was added. The mixtures were incubated in the dark at room temperature for 30 min. Absorbance was then measured at 517 nm against a methanol blank [27]. A 3 mL DPPH solution was used as the control. Percentage inhibition was calculated using the formula:

$$\% \text{ inhibition} = [(A_0\text{-}A_1)/(A_0)] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

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2.4.2. Nitric Oxide Radical Scavenging Assay

Nitric oxide radical scavenging activity was determined with slight modification from Alam et al., 2013 [28]. Samples or ascorbic acid (20-100 µg/mL, 1 mL) were mixed with 2 mL of 10 mM sodium nitroprusside in phosphate buffer and incubated at 25°C for 2.5 hours. To 3 mL of the incubated solution, 3 mL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2% H3PO3) was added. Absorbance of the pink color was measured at 540 nm against a blank. Ascorbic acid served as a positive control. Percentage inhibition was calculated using:

$$\% \text{ inhibition} = [(A_0-A_1)/(A_0)] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.4.3. Hydroxyl Radical Scavenging Assay

Hydroxyl radical scavenging activity was assessed by measuring the inhibition of salicylic acid hydroxylation [29]. The 7 mL reaction mixture contained 1 mL of sample/standard (100-500 µg/mL), 2 mL of 6 mM $FeSO_4$, 2 mL of 6 mM H_2O_2, and 2 mL of 6 mM salicylic acid. After incubation at 37 °C for 1 hour, absorbance was measured at 510 nm due to the color change of salicylic acid. Scavenging activity was calculated as follows:

$$\% \text{ inhibition} = [(A_0-A_1)/(A_0)] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.4.4. Superoxide Radical Scavenging Assay

Superoxide radical scavenging activity was assessed by a modified pyrogallol auto-oxidation method [29]. The reaction mixture contained 4.5 mL of 50 mM Tris-HCl buffer (pH 8.2), 0.4 mL of 25 mM pyrogallol, and 1 mL of sample (0.1-0.5 mg/mL). After 5 min incubation at 25°C, the reaction was terminated by adding 1 mL of 8 mM HCl. Absorbance was measured at 420 nm. Ascorbic acid served as the positive control. Superoxide radical scavenging activity was calculated using the formula:

$$\% \text{ inhibition} = [(A_0-A_1)/(A_0)] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.4.5. Reducing Power Assay

The reducing power of samples was assessed via the ferric reducing antioxidant power (FRAP) assay [30]. One mL of sample or standard (20-100 µg/mL) was combined with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) $K_3Fe(CN)_6$. After 20 min incubation at 50 °C in a water bath, 2.5 mL of 10% (w/v) trichloroacetic acid was added to terminate the reaction. The mixture was centrifuged at 3000 rpm for 10 min. A 2.5 mL aliquot of the supernatant was then mixed with 2.5 mL distilled water and 0.5 mL of 0.1% (w/v) $FeCl_3$. Absorbance was measured at 700 nm against a blank.

2.5. Cytotoxicity Assay

HeLa cells were seeded in a 96-well plate. After 24 hours, cells were treated with dimethylsulfoxide or various extract concentrations for 48 hrs. Post-treatment, media were removed and replaced with 100 µL fresh medium containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (final concentration 0.4 mg/mL). Plates were incubated at 37 °C for 3 hrs, during which intracellular purple formazan was observed. Next, 100 µL of solubilization solution (4 mM HCl, 0.1% NP40 in isopropanol) was added, and plates were kept in the dark for 15 minutes at room temperature. Absorbance was measured at 570 nm using a microplate reader [31].

2.6. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

GC-MS analysis of *G. lucidum* extracts was conducted using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) equipped with an Rtx-5 M5 capillary column (30 m × 0.02 mm i.d., 0.25 µm film

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thickness; Restek, Bellefonte, PA, USA). The operating conditions, including solvent cut-off, temperature program, and MS scan parameters, were identical to those described by Tiwari et al. 2023 [32]. Compounds were identified using NIST libraries (NIST 14, Gaithersburg, MD, USA).

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Experiments were conducted in triplicate, expressed as mean ± SD. One-way ANOVA with Tukey's post-hoc test compared extracts ($p < 0.05$).

3. Results

3.1. Extraction Yield

Extraction efficiency is affected by the chemical nature of bioactive compounds, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances [33,34]. The yield of extraction depends on the solvent with varying polarity, temperature, pH, extraction time, and composition of the sample [33,34]. Extraction efficiency (% yield) varied significantly ($p < 0.05$) among solvents, with acetone yielding the highest crude extract (GAE; 5.01%), followed by ethanol (GEE; 3.43%), methanol (GME; 2.98%), and water (GWE; 2.29%) (Table 2).

Table 2. Percentage yield of various solvent extracts.

Extract	Weight of sample before extraction (gm)	Weight obtained after extraction (gm)	% Yield value
Water	10	0.229	2.29 ^{d}
Ethanol	10	0.343	3.43 ^{b}
Methanol	10	0.298	2.98 ^{c}
Acetone	10	0.501	5.01 ^{a}

Values with the same letter (a-d) are not significantly different; different letters indicate significant differences between solvents ($p < 0.05$).

3.1. Estimation of Total Phenolic and Flavonoid Content

Phenolic compounds are recognized as potent chain-breaking antioxidants due to the radical-scavenging capabilities of their hydroxyl groups [35]. The total phenolic content (TPC) exhibited significant variation among the tested solvents (Figure 3A). Ethanol extract (GEE) demonstrated the highest TPC (376.5 ± 9.3 mg PGE/g), which was significantly greater ($p < 0.05$) than that of methanol extract (GME; 97.3 ± 2.8 mg PGE/g), water extract (GWE; 96.6 ± 2.6 mg PGE/g), and acetone extract (GAE; 60.5 ± 7.4 mg PGE/g). Notably, the TPC of GEE exceeded values previously reported for 62 wild mushrooms from Nepal, including other Ganoderma species, and 29 other diverse mushroom species [16,17,36].

Flavonoids were quantified using the aluminum chloride method. GEE and GWE exhibited significantly higher TFCs, with values of 30.33 ± 0.5 mg QEs/g extract and 26.73 ± 0.6 mg QEs/g extract, respectively (Figure 3B). The low flavonoid content was observed in GME; 6.34 ± 0.4 mg QEs/g dry extract and GAE; 7.95 ± 0.23 mg QEs/g extract. Notably, GEE contained approximately 4.7-fold higher total flavonoids compared to GME.

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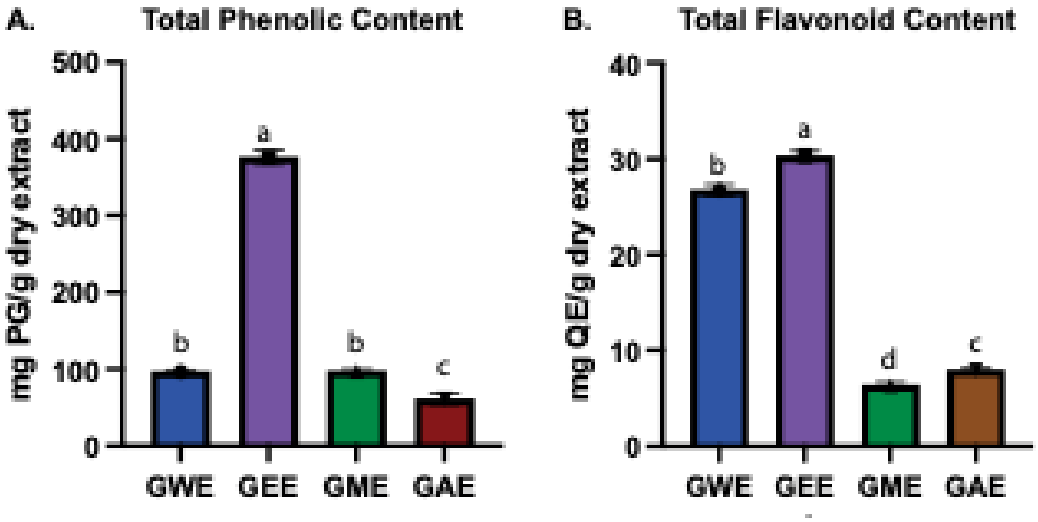

Figure 3. Total phenolic content (A) and Total Flavonoid Content (B) expressed as pyrogallol and quercetin equivalents (mg/g dry extract), respectively. Values with the same letter (a-d) are not significantly different; different letters indicate significant differences between solvents ($p < 0.05$).

3.2. Estimation of β-Carotene and Lycopene

The concentrations of lycopene and β-carotene in G. lucidum extracts were estimated spectrophotometrically. Carotenoid analysis demonstrated limited solvent efficacy. β-carotene content was highest in GME (0.4536 ± 0.000 mg/g), followed by GEE (0.1982 ± 0.006 mg/g), GWE (0.1595 ± 0.001 mg/g), and GAE (0.0944 ± 0.001 mg/g) (Figure 4A).

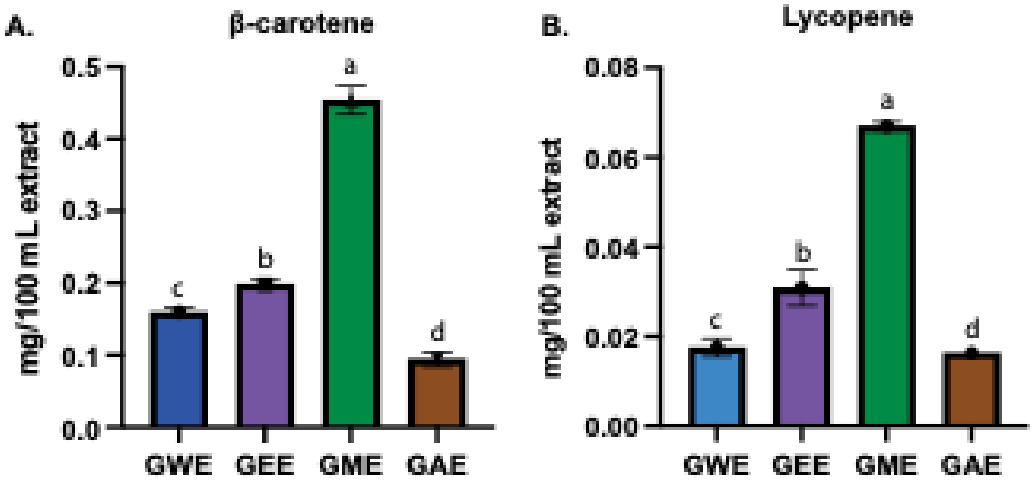

Figure 4. β-carotene (A) and lycopene (B) content as mg/100 mL extract in various solvent extract. Values with the same letter (a-d) are not significantly different; different letters indicate significant differences between solvents ($p < 0.05$).

Similarly, lycopene content varied considerably among the extracts, ranging from 0.0163 ± 0.000 to 0.0670 ± 0.001 mg/g of dry extract (Figure 4B). The highest lycopene content was found in the methanolic extract (GME; 0.0670 ± 0.001 mg/g), followed by the ethanolic extract (GEE; 0.0308 ± 0.004 mg/g), water extract (GWE; 0.0175 ± 0.002 mg/g), and acetone extract (GAE; 0.0163 ± 0.000 mg/g). However, overall β-carotene and lycopene contents were comparatively low when contrasted with the phenolic and flavonoid contents of the same extracts.

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3.3. Comparative In-Vitro Antioxidant Activities

Antioxidant activity cannot be definitively concluded from a single assay due to the diverse mechanisms involved and variations between in vitro test models. These diverse mechanims include free radical scavenging, metal ion chelation, reducing power, single electron transfer, and others [37,38]. Therefore, this study employed multiple in vitro antioxidant assays (DPPH radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging, nitric oxide radical scavenging, and reducing power) to comprehensively evaluate and compare the antioxidant potential of G. lucidum solvent extracts.

3.3.1. DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the extracts (at 100 µg/mL) ranged from 86.58 ± 4.65% to 95.59 ± 0.17% (Figure 5). Methanolic extract (GME) exhibited the highest activity (95.59 ± 0.17%), followed by acetone extract (GAE; 92.13 ± 0.34%), ethanolic extract (GEE; 90.91 ± 0.06%), and water extract (GWE; 86.58 ± 4.65%). Ascorbic acid, as a standard, showed 97.26 ± 0.69% inhibition. All extracts demonstrated high antioxidant activity, scavenging over 80% of the DPPH radical even at 80 µg/mL. These findings indicate better scavenging activity compared to some previously reported wild mushrooms from Nepal, including G. lucidum [16,17,36].

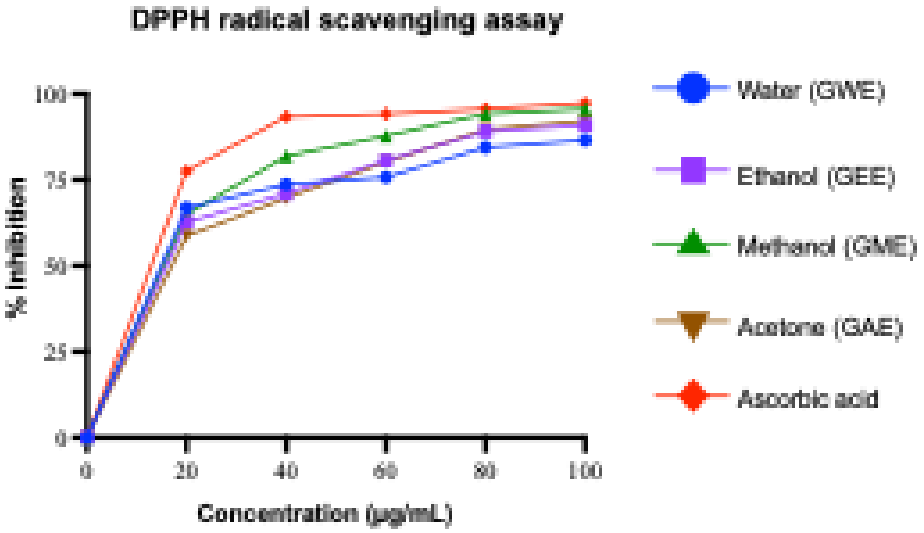

Figure 5. DPPH radical scavenging activity (%) of G. lucidum extracts prepared with four different solvents, compared to the standard antioxidant ascorbic acid.

3.3.2. Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity was positively correlated with increasing extract concentrations ($p < 0.05$) (Figure 6). At 500 µg/mL, GEE exhibited the highest scavenging activity (72.24 ± 0.61%), followed by GME (54.84 ± 1.72%), GAE (39.37 ± 1.70%), and GWE (38.70 ± 0.65%). Ascorbic acid showed 98.80 ± 0.15% scavenging. The observed order of activity was: GEE > GME > GAE > GWE. These results suggest the extracts' ability to scavenge superoxide anion radicals, potentially preventing oxidative damage, likely attributed to the electron-donating capacity of their phenolic hydroxyl groups [39].

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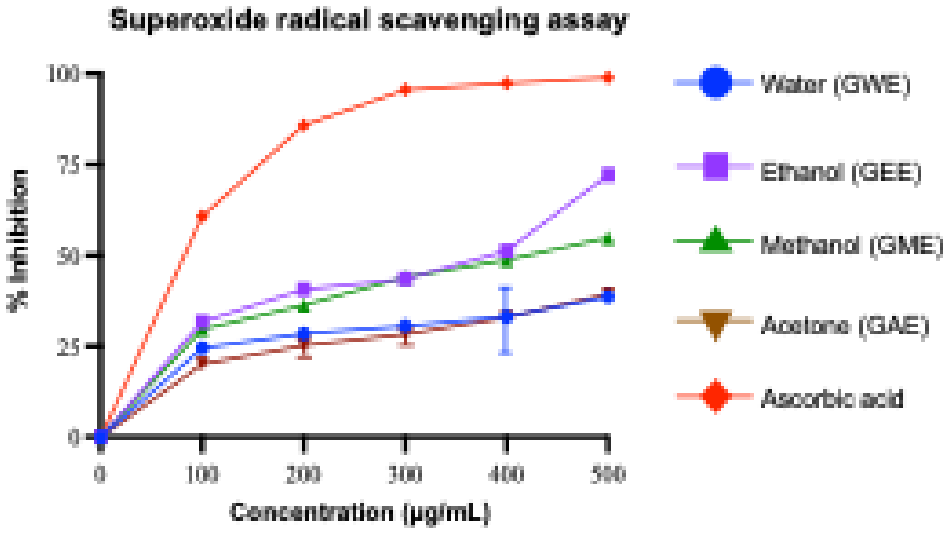

Figure 6. Superoxide radical scavenging activity (%) of G. lucidum extracts prepared with four different solvents, compared to the standard antioxidant ascorbic acid. .

3.3.3. Hydroxyl Radical Scavenging Activity

All samples exhibited significant dose-dependent hydroxyl radical scavenging activity. At tested concentrations, GME showed the highest activity (77.88 ± 0.15%), followed by GEE (76.62 ± 0.28%), and GWE (65.72 ± 0.13%), with GAE showing comparatively lower activity (42.12 ± 1.59%) (Figure 7). Ascorbic acid (47.73 ± 0.13%) served as a standard. Notably, the IC_{50} values for GWE, GEE, and GME were lower than that of ascorbic acid, indicating their superior hydroxyl radical scavenging potential. The potent hydroxyl radical scavenging capacity of G. lucidum extracts suggests a preventive role against lipid peroxidation initiation and protection against DNA damage, mutagenesis, and cytotoxicity [40–43].

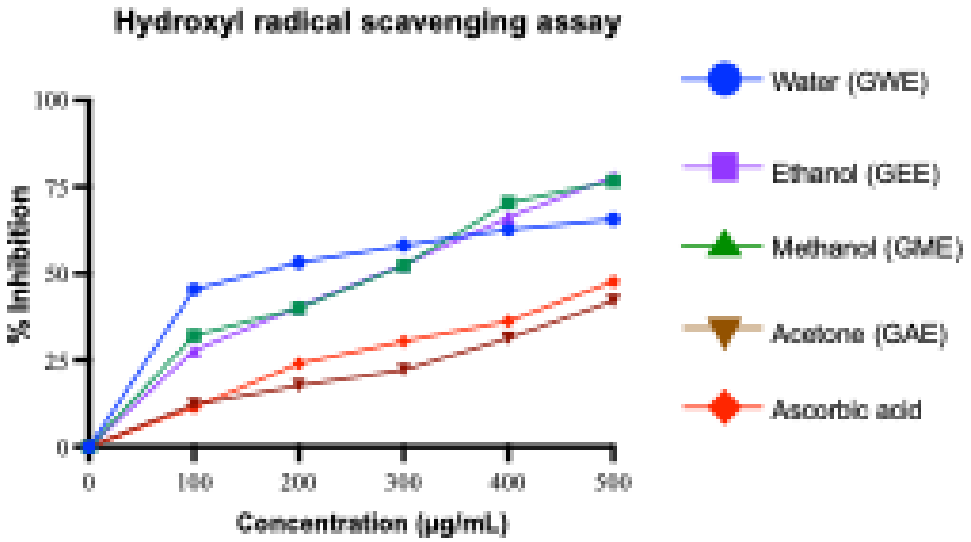

Figure 7. Hydroxyl radical scavenging activity (%) of G. lucidum extracts prepared with four different solvents, compared to the standard antioxidant ascorbic acid.

3.3.4. Nitric Oxide Radical Scavenging Activity

The extracts demonstrated good inhibition of nitric oxide radicals. At 100 µg/mL, GEE exhibited the highest scavenging potential (81.51%), followed by GME (63.81%), GAE (41.88%), and GWE

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(41.62%) (Figire 8). Ascorbic acid showed 97.02% inhibition. The order of activity was GEE > GME > GAE ≈ GWE. The ability of the extracts to scavenge nitric oxide suggests a potential to prevent peroxynitrite formation and a protective role against nitrosamine-mediated carcinogenesis in the digestive tract [44].

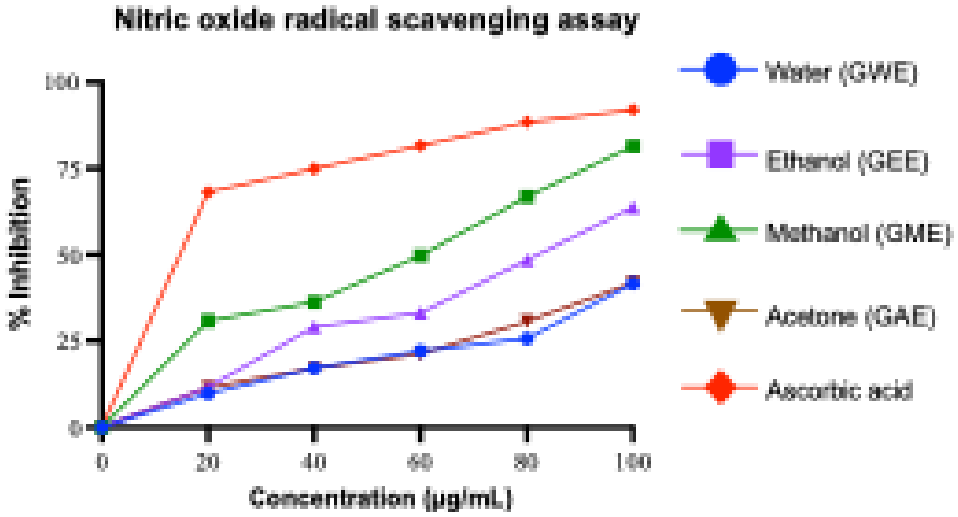

Figure 8. Nitric oxide radical scavenging activity (%) of G. lucidum extracts prepared with four different solvents, compared to the standard antioxidant ascorbic acid. .

3.3.5. Reducing Power Assay

The reducing power assay (electron-donating capacity) further confirmed ethanol's dominance (0.353 ± 0.003 at 100 µg/mL), aligning with radical scavenging trends (Figure 9). The reducing power of the extracts at 100 µg/mL followed the order: GEE (0.353 ± 0.002) > GME (0.176 ± 0.002) > GWE (0.164 ± 0.005) > GAE (0.158 ± 0.001). Standard ascorbic acid showed a reducing power of 0.493 ± 0.001 at the same concentration. The reducing powers of the ethanolic extracts were notably higher than those reported for other G. lucidum, Boletus edulis, and Pleurotus ostreatus [45–48]. This reducing capacity is likely due to their hydrogen-donating ability of the compounds present in the extracts, which can halt peroxide formation and terminate radical chain reactions [49,50].

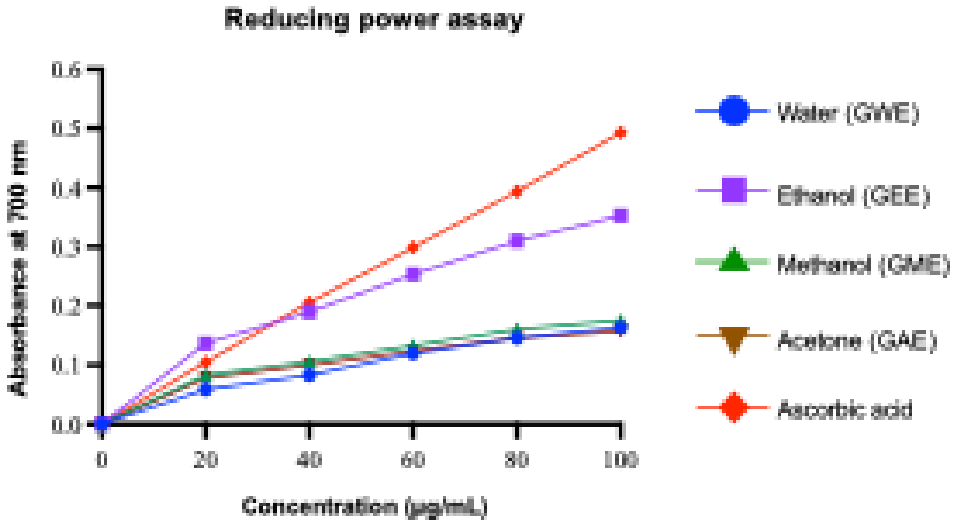

Figure 9. Reducing power assay of G. lucidum extracts prepared with four different solvents, compared to the standard antioxidant ascorbic acid.

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3.4. MTT-Based Cytotoxicity Assay in HeLa Cells

Following the characterization of bioactive compounds and antioxidant activities, the cytotoxic potential of G. lucidum extracts was evaluated against human cervical cancer (HeLa) cells via MTT assay. Extracts were tested at concentrations of 100, 500, and 1000 µg/mL, and results are presented in Figure 10. All extracts exhibited a dose-dependent inhibition of HeLa cell viability. At the highest concentration tested (1000 µg/mL), GEE and GWE extracts demonstrated significantly higher ($p < 0.05$) cytotoxicity, suppressing cell proliferation by >65%. Specifically, GEE achieved 82.53 ± 1.46% inhibition and GWE 67.28 ± 1.39% inhibition. In comparison, GME and GAE showed more moderate inhibition at 1000 µg/mL, with 51.87 ± 2.1% and 34.78 ± 4.69 % inhibition, respectively.

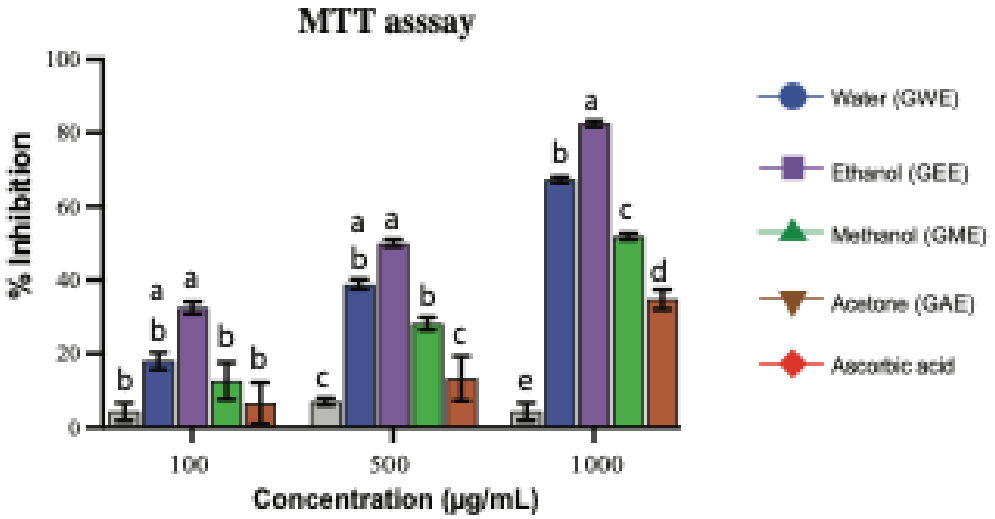

Figure 10. Cytotoxic activity of G. lucidum extracts against HeLa cervical cancer cell lines at varying concentration. For each concentration, values with the same letter (a-e) are not significantly different; different letters indicate significant differences between solvent extracts ($p < 0.05$).

3.5. IC_{50} Comparison of Extraction Solvents for Antioxidant and Cytotoxicity Activities

The extraction efficiency and bioactive potential of G. lucidum were solvent-dependent, with GEE having the highest total phenolic (376.50 ± 9.32 PGE/g) and flavonoid (30.33 ± 0.50 QE/g) content, while GME showed higher carotenoids (lycopene: 0.067 ± 0.001 mg/g; β-carotene: 0.454 ± 0.000 mg/g) (Figure 11). Similarly, antioxidant assays revealed solvent-specific efficacy: GWE, GEE, GME, GAE exhibited an exceptional DPPH radical scavenging comparable to ascorbic acid, IC_{50}: 5.82 µg/mL), whereas GEE dominated in superoxide (IC_{50}: 328.95 µg/mL), nitric oxide (IC_{50}: 57.67 µg/mL), and reducing power (IC_{50}: 78.04 µg/mL) assays (Figure 11). Hydroxyl radical inhibition was strongest in GWE (IC_{50}: 237.89 µg/mL), closely followed by GEE (274.34 µg/mL). Cytotoxicity via MTT assay demonstrated dose-dependent inhibition, with GEE (IC_{50}: 520.19 µg/mL) and GWE (702.41 µg/mL) exhibiting moderate activity (Figure 11). Collectively, ethanol was found to be superior in extracting phenolic and flavonoid-rich fractions with high antioxidant capacity, despite GWE's exceptional DPPH activity, likely due to ethanol extracting polar bioactive compounds with higher antioxidant capacity [51–53]. The correlation between ethanol's high phenolic/flavonoid content and multi-target bioactivity positions it as the optimal solvent for extracting compounds with therapeutic potential against oxidative stress and cancer.

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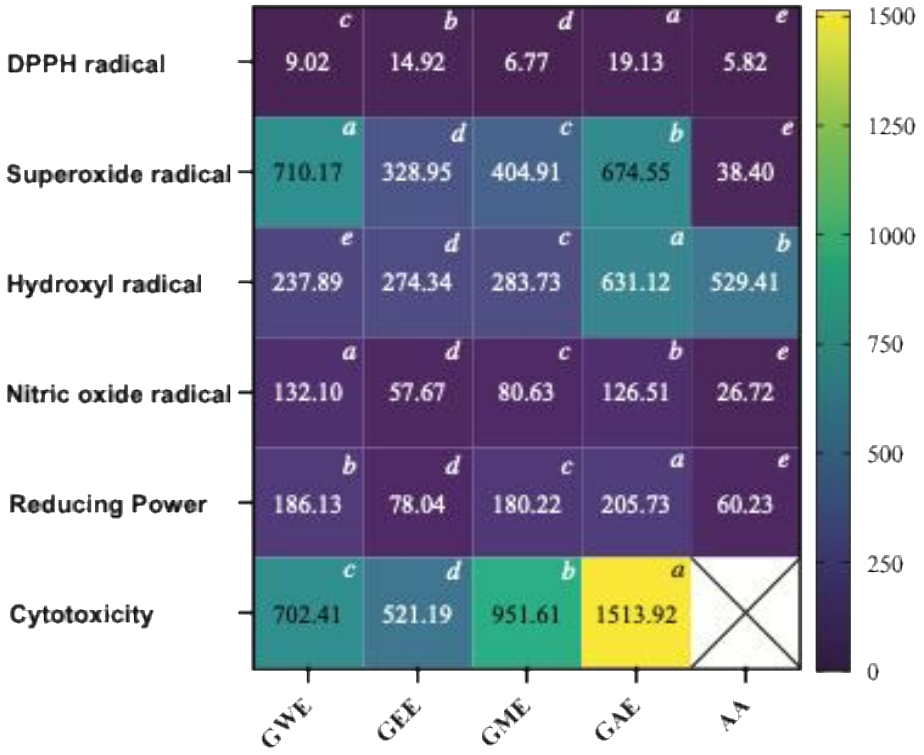

Figure 11. Heatmap of IC_{50} values obtained from various antioxidant and cytotoxic assays. Values are expressed in µg/mL. Within each assay, values with the same letter (a-e) are not significantly different; however, different letters indicate significant differences between solvents ($p < 0.05$). Ascorbic acid: AA.

3.6. Solvent-Dependent Variation in Bioactive Compounds via GC-MS Profiling

GC-MS analysis of G. lucidum extracts showed solvent-dependent profiles of bioactive compounds, including sterols, triterpenoids, terpenoids, and polyphenols (Table 3). Fatty acids dominated ethanol (53.18%) and methanol (48.57%) extracts, with 9,12-octadecadienoic acid (linoleic derivative: 20.60% in ethanol, 14.05% in methanol) and pentadecanoic acid (14.52% in ethanol) as major constituents. Acetone exhibited the lowest fatty acid content (5.64%) but uniquely contained ergosterol (Vitamin D2 precursor) and retinoic acid. Polar protic solvents (ethanol, methanol) efficiently extracted free fatty acids and esters, including (E)-9-octadecenoic acid ethyl ester (oleic derivative: 3.86% in ethanol), while acetone's mid-polarity favored sterols (7,22-ergostadienone, 9(11)-dehydroergosteryl benzoate) and triterpenoids (ergosta-4,6,8(14),22-tetraen-3-one). Similarly, Hinokione, an abietene diterpene, was detected across solvent extracts (0.9% in GAE, 2.9% in GEE, and 5.5% in GME) (Table 3).

Pharmacologically significant compounds included ferruginol (exclusive to ethanol), nerolidol acetate (methanol-specific), geranylgeraniol (anti-inflammatory terpenoid), and Hinokione (anti-inflammatory and anticancer) (Table 3). Ethanol and methanol extracts had the highest polyphenol, diterpenoid, and fatty acid content, whereas acetone had higher sterols and triterpenoids, demonstrating solvent polarity as a critical determinant of bioactive compound selectivity. These findings show G. lucidum's diverse phytochemical composition and the impact of solvent choice in optimizing targeted metabolite extraction.

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Table 3. Summary of key compounds detected by the GC-MS analysis in various solvents and their reported pharmacological relevance.

Compound Name	Solvent Extracts (% area)			Compound class	Key pharmacological relevance	Reference
	GEE	GME	GAE			
7,22-Ergostadienone	3.54	2.90	2.55	Sterol	Antithrombotic activity with cardiovascular benefit; antidiabetic, anticancer, and neuroprotective effects; Pro-inflammatory properties (activating Toll-like receptors, cytokines, and chemokines)	[51,54–57]
9(11)-Dehydroergosteryl 3,5-dinitrobenzoate	2.90	3.13	2.70	Sterol conjugate	Anti-inflammatory; antibacterial (MRSA and S. aureus); and cytotoxic properties	[58,59]
δ-Tocopherol	2.13	3.91	0.75	Tocopherol	Antioxidant; anti-inflammatory (primarily via inhibiting protein kinase C and reducing eicosanoid production); anticancer (both in vitro and in vivo prostate xenograft models); cvardiovascular and neuroprotective	[60,61]
4-[5-(2-bromophenyl)-1,2,4-oxadiazol-3-yl]-1,2,5-oxadiazol-3-amine	-	-	0.35	Synthetic heterocycle	Anticancer (potentially via targeting Topoisomerase II relaxation activity); antibacterial; anti-inflammatory; analgesic properties; antioxidant	[62–66]
Ergosta-tetraenone	3.86	-	1.67	Sterol derivative	Anticancer (via G_2/M arrest and apoptosis induction); nephroprotection (mitigation of renal damage in mouse model); anti-inflammatory	[67–69]
Ergosterol	-	-	73.99	Sterol	Vitamin D2 precursor; lipid soluble antioxidant; anticancer effects (cell cycle arrest and modulates Wnt/β-catenin signaling pathway); antimicrobial; antidiabetic; immunomodulatory effects	[70–72]
Ferruginol	3.18	-	-	Abietane diterpene	Anticancer (apoptosis induction in melanoma, prostate, lung, and ovarian cancer cells); neuroprotective (reduces α-synuclein toxicity and restores LTP in Alzheimer's models); cardioprotective (both invitro and in vivo models); antimicrobial and antiviral	[73–80]
Geranylgeraniol	5.26	-	0.89	Diterpenoid alcohol	Anti-inflammatory (NF-κB inhibition; ↓ IL-1β, TNF-α, IL-6, COX-2); pain relief; bone and muscle support (muscle regeneration and prevents bisphosphonate-related bone damage); antimicrobial activity; hormonal balance; glucose homeostasis	[81–84]
Hinokione	2.9	5.5	0.9	Abietane diterpene	Anticancer; anti-inflammatory; hypoglycemic & β-Cell regenerative properties (promotes β-cell differentiation and improved glycemia in zebrafish); antibacterial; antioxidant	[85–87]
Nerolidol acetate	-	1.70	-	Sesquiterpene ester	Anticancer; anti-inflammatory; neuroprotective; antimicrobial; antifungal; antioxidant	[88–90]
Retinoic acid	-	-	0.50	Retinoid	Acne and photoaging (promotes cell differentiation and skin repair); anti-cancer (induces differentiation of malignant promyelocytes in acute promyeloid leukemia); neuroprotective	[91–93]

4. Discussion

This study demonstrated the solvent-dependent extraction of bioactive compounds from *G. lucidum* collected from high-altitude regions of Nepal, supporting our initial hypothesis that unique environmental factors at these altitudes influence the mushroom's secondary metabolite profile. The observed variability in extraction yield across different solvents, with acetone yielding the highest, followed by ethanol, methanol, and water, demonstrates the role of solvent polarity in determining extraction efficiency. This observation aligns with established principles of phytochemistry, where solvent polarity dictates the solubilization and subsequent extraction of specific compound classes [33,94–101].

GEE displayed the highest TPC and TFC, correlating strongly with its superior antioxidant and cytotoxic performance (Figure 4-10). GEE's TPC exceeded that of 62 wild mushrooms previously reported in Nepal and outperformed other *Ganoderma species* and various commercial mushrooms [16,17,36]. GEE also contained approximately 4.7 times more flavonoids than GME, with flavonoid levels surpassing those reported in *G. applanatum* and *G. resinaceum* [36,102]. This suggests that flavonoids likely constitute a significant portion of the total phenolic content in the tested extracts.

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Collectively, these findings support the efficacy of ethanol in extracting phenolic and flavonoid-rich fractions with potential nutraceutical value.

Carotenoid extraction with methanol proved to be most effective among the tested solvents due to its polar nature, disrupting the cellular matrix to release hydrophobic carotenoids, consistent with previous reports of carotenoid content in *G. lucidum* [94]. Nevertheless, overall carotenoid yields were relatively low compared to the phenolic and flavonoid content (Figure 4, 5). The values obtained for GME were higher than those previously reported in Turkish mushrooms [103] and some Indian strains of *G. lucidum*, but still lower than those observed in wild Portuguese mushrooms [47]. This reinforces the role of solvent polarity in selective compound recovery.

Multiple in vitro antioxidant assays, including DPPH, superoxide, hydroxyl, nitric oxide radical scavenging, and reducing power assays, confirmed the strong antioxidant potential of the extracts. All extracts scavenged over 80% of DPPH radicals at 80–100 µg/mL, with GEE and GME showing the strongest activity, likely due to their higher phenolic content. Superoxide scavenging was highest in GEE (72.24 ± 0.61%), suggesting strong potential to neutralize ROS via electron donation [39]. Hydroxyl radical IC_{50} values for GWE, GEE, and GME were lower than that of ascorbic acid, indicating a potent ability to counter lipid peroxidation and DNA damage [40–42]. Nitric oxide scavenging by GEE (81.51%) indicates its role in mitigating nitrosative stress and possible prevention of nitrosamine-mediated carcinogenesis. Reducing power, another key antioxidant indicator, was highest in GEE and significantly exceeded values for *Boletus edulis* and *Pleurotus ostreatus* [45–48]. This capacity is linked to the hydrogen-donating ability of flavonoids and phenolics [49,50].

The cytotoxicity of the extracts was assessed against HeLa cells using the MTT assay. All extracts demonstrated dose-dependent inhibition of cell viability. At 1000 µg/mL, GEE exhibited strongest cytotoxic effect (82.53 ± 1.46%), followed by GWE (Figure 10). These results are consistent with earlier reports on the anticancer effects of *G. lucidum*, suggesting that ethanol and water extracts contain compounds that may induce apoptosis, modulate immune responses, and arresting cell cycle progression [48,94,104,105]. The anti-proliferative effects of *G. lucidum* extracts are well documented in the literature and have been reported in a variety of cancer cell lines, including HeLa (cervical cancer), A549 (lung cancer), LS174 (colon cancer), and MCF-7 (breast cancer) cells [105,106]. As described in recent studies, including the work by Prabhu et al. (2023), these cytotoxic effects are largely attributed to the presence of bioactive compounds such as pentadecanoic acid, 14-methyl ester; hexanoic acid; (Z,Z)-9,12-octadecadienoic acid methyl ester; ergosta-4,6,8(14),22-tetraen-3-one (ergosta-tetraenone); 7,22-ergostadienone; and various *Ganoderma*-derived polysaccharides [105]. Notably, our GC-MS profiling confirmed the presence of these compounds in the solvent extracts of *G. lucidum*, providing mechanistic support for the observed cytotoxicity in HeLa cells and reinforcing their potential therapeutic relevance in cancer treatment.

When IC_{50} values were compared across assays, ethanol emerged as the most effective solvent for extracting multifunctional bioactives (Figure 11). GEE had the lowest IC_{50} values in superoxide (328.95 µg/mL), nitric oxide (57.67 µg/mL), and reducing power (78.04 µg/mL) assays. Although GWE had stronger hydroxyl radical inhibition, GEE consistently performed across multiple assays and demonstrated superior cytotoxicity (IC_{50}: 520.19 µg/mL). These findings highlight ethanol's extraction of polar antioxidant and anticancer agents with broad-spectrum activity.

GC-MS analysis confirmed solvent-specific extraction efficiency, identifying steroids, terpenoids, diterpenoids, triterpenoids, polyphenols, and fatty acids. Polyunsaturated fatty acids were most abundant in ethanol and methanol. One of the major bioactive constituents gaining a lot of attention recently is found in all three extracts was Hinokione, an abietane-type diterpene known for its significant anticancer and anti-inflammatory activities [85]. Hinokione, an abietane-type diterpene with established anticancer and anti-inflammatory properties, was identified in all extracts. Hinokione has been shown to exhibit cytotoxicity against MV-3 and MIAPaCa-2 human cancer cell lines with IC_{50} values of 34.1 and 17.9 µM, respectively, and has demonstrated β-cell regeneration and hypoglycemic effects in zebrafish [85,107,108]. Ferruginol, another abietane diterpenoid with neuroprotective and anticancer activity, was exclusively present in GEE. It has shown

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antiproliferative activity in melanoma (Sk-MEL28) and various cancer cell lines, including prostate, lung, gastric, and breast cancers, as well as efficacy in CL1-5 xenograft mouse models [73,75,77,78]. Methanol extract contained nerolidol acetate, a sesquiterpene with antioxidant, antibacterial, anti-biofilm, antifungal, and anticancer properties [88–90]. Geranylgeraniol, an anti-inflammatory isoprenoid, was also detected in methanol and ethanol extracts, likely contributing to their antioxidant activity [81–84]. GAE was rich in ergosterol and retinoic acid, with ergosterol comprising more than two-thirds of the total extracted compounds. As a vitamin D precursor, ergosterol has potential for addressing vitamin D deficiency-associated diseases, including cancers, rheumatoid arthritis, and multiple sclerosis [109]. Estrogenic derivatives such as 7,22-ergostadienone and 9(11)-dehydroergosteryl benzoate, known for their therapeutic applications, were found across all extracts (Table 3).

Collectively, the GC-MS dataset underscores the profound impact of solvent choice on the chemical profile of mushroom extracts and the types of bioactive molecules recovered. These solvent-dependent metabolic signatures not only explain the variation in antioxidant and cytotoxic activities observed across assays but also provide mechanistic insight into the functional contributions of specific compound classes. The selective enrichment of fatty acids, sterols, and terpenoids by distinct solvents offers a strategic basis for tailoring extraction protocols to maximize therapeutic yield. Building upon these findings, future investigations should focused on isolating and functionally characterizing the specific bioactive compounds responsible for the observed activities through selective extraction and purification of bioactive candidate compounds. Testing these isolated compounds will provide a clearer understanding of their therapeutic potential.

5. Conclusions

Our study aimed to investigate the therapeutic potential of *G. lucidum* from Nepal's high-altitude regions, and our findings strongly confirms it as a key source of bioactive compounds. Through this work, we have shown that the choice of extraction solvent is critical, significantly impacting not only the yield but also the specific bioactive compounds obtained, and consequently, their biological activities. While acetone yielded the most crude extract, ethanol and methanol extract showed higher phenolic and flavonoid content, correlating with high antioxidant activity across a spectrum of in vitro assays. The ethanol and water extracts also demonstrated a powerful ability to inhibit HeLa cell growth. GC-MS analysis identified diverse array of beneficial compounds, including fatty acids, sterols like ergosterol, and various terpenoids (diterpenoids, triterpenoids). The specific distribution of these compounds varied depending on the extraction solvents, and they collectively contribute to the observed health benefits. Future research should focus on optimizing extraction methods and characterizing these individual compounds to maximize specific bioactivities, which will be critical for bridging the gap between traditional use and modern applications.

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