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Determination of Fatty Acids Profile, Antioxidant, and Anticholinesterase Activities of *Ganoderma lucidum* collected from Swat, Pakistan

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Abstract: The current study was conducted on methanol extract of *Ganoderma lucidum* to evaluate its antioxidant, enzyme inhibition activities, and fatty acids profile. The dominant fatty acids were found to be linoleic acid (53.6 %), oleic acid (24.2 %), and palmitic acid (11.5 %) among the ten different fatty acids detected in the methanol extract using GC/MS techniques. Fatty acids groups like $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, and $C_{18:3}$ were also found from trace to middle amount (0.15 %, 0.15 %, 1.11 %, 0.26 %, 0.27 %, 2.85 %, and 5.9 %) respectively. Polyunsaturated fatty acids concentration of *G. lucidum* exposed the significance for lowering the blood cholesterol level. Methanol extract of *G. lucidum* showed significant antioxidant potential against *β*-carotene-linoleic acid assay (IC_{50}: 687.12 ± 2.8 µg/mL), metal chelating activity (IC_{50}: 870.00 ± 10.05 µg/mL), and CUPRAC reducing potential ($A_{0.5}$: 16.55 ± 4.53 µg/mL) respectively. The CUPRAC assay supports the lipid peroxidation inhibitory activity results. Against AChE enzyme, methanol extract of *G. lucidum* demonstrated IC_{50} value 19.11 ± 2.4 µg/mL, while exhibited weak butyrylcholinesterase (BChE) inhibitory activity (IC_{50} > 100 µg/mL). Thus, *Ganoderma lucidum* may defend people in contradiction of free radical damage and may be an important source of potential new acetylcholinesterase (AChE) inhibitory drugs.

Keywords: *Ganoderma lucidum*; fatty acids profile; antioxidant; anticholinesterase inhibitory activity; medicinal edible mushroom.

1. Introduction

Kingdom fungi are one of the valuable gifts of nature. Ancient nations like Egyptians and Aztecs (4600 years ago) had mushrooms on their menu and they believed mushrooms as the plant of immortality. King of Egypt (Pharaoh) was so fascinated by the delicious essence of mushrooms that

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they uttered mushrooms as the food for the royal family. The mushroom was practised as a ritual by various civilizations (China, Greece, Latin America, Mexico, and Russia) [1]. Mushroom (can be hypogynous or epigeous) is a "macro fungus" with the characteristic fruiting body and can be hand-picked" [2]. Mushrooms have visible variations in colour, taste, aroma, and shape. Mushrooms have different appearances and shapes; umbrella-shaped is the most common one [3]. The aroma of mushrooms plays a significant role amongst diverse mushroom species [4].

Medicinal fungi (mushrooms) are like workshops manufacturing valuable mycochemicals most effectively way through specific selectivity. Numerous mushroom bioactive components have been gained, and their medicinal properties have been established through intensive research [5]. Medicinal mushrooms have been practised for over 2000 years in China and the Far East [6,7]. However, due to the side effects of the alternative drug, the interest in natural product research, particularly on mushrooms, has been conducted to deliver lead compounds to cure long-lasting diseases like cancer, immunosuppression, anti-infective arthritis, metabolic disorders, tumours, and inflammatory processes [8].

As a source of therapeutic agents, the use of mushroom-based medicines will continue in the future; for example, high dietary fibre supplements from mushrooms and mycelia products are available with potential healing impacts on consumers' health [9-13]. Many edible species have therapeutic properties; hence it is important to differentiate between edible and medicinal mushrooms, harvested either wild or cultivated [14]. As part of our daily diet, mushrooms can easily provide recommended dietary fiber intake daily (25%) (Cheung, 2010). According to International Life Sciences Institute (ILSI Europe), edible mushrooms have been classified as an efficient food [15].

The therapeutic properties of mushrooms are not new. Over 3000 mushrooms are used for medicinal purposes, and 2000 are edibles out of ten thousand known mushroom species [16]. Amongst *Ganoderma* is an imperative therapeutic genus [17-20]. Various species of *Ganoderma* were used as diets and as medications in China, Japan, Korea, and some parts of Africa. Currently, *Ganoderma* species are used for the treatment of various cancers and in the preparation of antioxidants and nutraceuticals [2,21]. Among the *Ganoderma* species especially, *Ganoderma lucidum* is of great importance and cultivated by many people due to its market value. *Ganoderma lucidum* is available in the form of tea, coffee, and as dietary supplements consumed by healthy and sick people. The major chemical constituents of *Ganoderma lucidum* are carbohydrates, polysaccharides, triterpenes, sterols, lectins, amino acids, proteins, vitamins, minerals, and fats, which have therapeutic effects on different diseases. The *Ganoderma lucidum* and *Ganoderma tsugae* methanolic extracts also demonstrated antioxidant abilities [4,22]. Extracts isolated from *G. lucidum* exhibited anti-hypertensive, anticancer, antibiotic, immunomodulatory activities, and blood lipid-lowering effects [23,24].

Search for safe antioxidants from natural products is extremely important because synthetic antioxidants (BHA, BHT, and TBHQ) have been described to be carcinogenic and can spoil the liver [25,26]. Synthetic antioxidants have been employed in various food industries to avoid oxidative spoilage. Likewise, for the cure of Alzheimer's disease (AD), some medicines have reported harmful side effects on the liver [26,27]. Therefore, to prevent these adverse effects produced by synthetic antioxidants, natural origins are important in the pharmaceutical and food industries to develop safe bioactive components.

Polyunsaturated fatty acids play an important role in human basal metabolism. The dry weight of mushrooms contains almost 6–8% of beneficial fatty acid components, mainly polyunsaturated fatty acids [28]. Deficiency or inefficient metabolism of fatty acids results in cardiovascular disease [29].

Since the therapeutic practices of *Ganoderma lucidum* are perfectly recognized, the variances in different origins, such as geographic, environmental, and climate conditions, change the chemical proportion of mushrooms [30]. It was aimed to study the fatty acids profile, antioxidant potential, and anticholinesterase enzyme inhibition potential of this mushroom collected from Swat valley of Khyber Pakhtunkhwa-Pakistan. This area is famous for a variety of edible and medicinal mushrooms. A typical example is *Ganoderma lucidum*, which is a wild mushroom and is a very famous traditional medicinal mushroom that is used in many folk medicines by the native people [4,22-24].

2. Materials and Methods

2.1. Collection and Extraction of Mushroom Materials

1.5 kg of *Ganoderma lucidum* Karst were collected from the premises of Swat valley and brought to the Department of Agricultural Chemistry and Biochemistry, The University of Agriculture Peshawar. *Ganoderma lucidum* was identified and authenticated by a nation eminent mycologist at the department of Plant Pathology under catalogue number 9741, The University of Agriculture Peshawar. The mushroom sample was cleaned, dried, and ground and the powdered sample was soaked in 2.0 L methanol for one week. The methanol solution was filtered and concentrated on a rotatory evaporator (Heidolph Laborota 4000) at 40 °C. The crude methanol extract (79.28 g) was refrigerated at 4 °C for further analysis.

2.2. Preparation of Methyl Esters of Fatty Acids (FAMEs)

In a flask (25 mL), 25 mg of methanol extract was thawed in 0.5 N NaOH (1.5 mL). Flask was covered and warmed in a boiling water bath for five minutes, removed, allowed to cool, and 2 mL BF_3-MeOH was added. The mixture was covered and heated again in a boiling water bath for three minutes, then left until it cooled down, 5 mL of saturated NaCl solution has added to the mixture and shacked. Finally extracted twice with n-hexane [1,31-32].

2.3. Fatty Acids Methyl Esters Analysis by GC–MS

For the GC-MS analyses, an Ion trap mass spectrometer (MS) and a non-polar capillary column (DB-1 MS fused silica, length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm) were used. An e.i system (i.e. of 70 eV) was used. He was used as a sample carrier (1.3 mL/min.) with a pressure of 15 psi. The transfer line temperature of the injector was 220 °C, and the MS temperature was 290 °C, respectively. The oven temperature was 100 °C for an initial 5 min, then rose at the rate of 3 °C/min, and the ending temperature was kept at 238 °C for 9 min. 0.5 µL (1/25, w/v, in hexane) sample was infused manually in the split ratio mode (50:1). MS scanning was performed at 70 eV ionization energy, with the mass range from *m/z* 50 - 650 amu. 0.5 s was scan time with inters scan delays of 0.1 s. NIST and Wiley 2005 GC-MS libraries were used to interpret chromatograms. To compare GC-chromatograms of FAMEs mixture, Supelco™ (Catalog no: 47885-U) was used.

2.4. Biological Activities

2.4.1. β-carotene-Linoleic Acid Assay

The entire antioxidant activity was analyzed by applying the test of *β*-carotene-linoleic as described by [26,33-35] with slight modifications. 0.5 mg of *β*-Carotene, 25.0 µL of linoleic acid, mixed with Tween 40 emulsifier 200 mg in 1 mL of $CHCl_3$. The $CHCl_3$ was vaporized beneath a low vacuum, 50 mL of distilling H_2O saturated with O_2, which was added by strong shaking. 160 µL of this mixture was shifted into 40 µL of the samples at various concentrations. The initial absorbance at zero time and final absorbance after two hours of incubation (at 50 °C) was calculated at 470 nm employing a 96-well microplate reader. The IC_{50} activity was measured from the % antioxidant activity and extract concentration graph. Standards (BHT, quercetin, and *α*-tocopherol) were used for comparison. For bleaching rate (R) calculation, the following equation was used.

$R = \frac{ln\frac{a}{b}}{t}$; where ln represents the natural log, a is the initial absorbance (zero-time), and b is the final absorbance (120 min). Antioxidant activity as inhibition percent was calculated by the following equation:

% inhibition = [$R_{control}$ - R_{sample} / $R_{control}$] x 100.

2.4.2. Ferrous Ions Chelating Assay

The methanol extract of *G. lucidum* on ferrous ions assay was calculated by applying the method of [26,35] with slight modifications. First, 80 µL of extracts solution was run in C_2H_5OH in various concentrations, then 40 micro-litre (0.20 milli-mole) Ferrous chloride was added, and the reaction was started by adding 85.0 micro-litres (0.50 milli-mole) ferene.

This mix was gently shaken vigorously and incubated at 20-30 ºC for 10 to 12 minutes. The absorbance was determined at 593 nm after the mixture attained stability. The activity of metal-chelating was estimated by the succeeding equation.

$$\text{Ferrous ions chelating activity (\%)} = [A_{control} - A_{sample} / A_{control}] \times 100$$

50% metal chelating activity (IC_{50}) was measured from the graph of Fe^{2+} (Ferrous ions) chelating effects percentage against extract concentration. Quercetin and EDTA antioxidant standards were used for the comparison of the activity results.

2.4.3. Cupric Reducing Antioxidant Capacity (CUPRAC) Test

According to the method of [33,38], the cupric reducing antioxidant capacity of the *G. lucidum* methanol extract was analyzed with a slight modification in the spectrophotometric method. 40 µL of extract at various concentrations, 50 µL 10 mM Cupper, 50 µL 7.50 mM neo-cuproine, and 60 µL NH_4 Acetate buffer (1 M, pH 7.0) solutions were added to every well in a 96 well plate to formulate the final volume 200 µL. The absorbance of the sample solution was determined at 450 nm compared to a component blank after one hour by applying a microplate a 96-well reader. The results were given as $A_{0.50}$ (µg/mL), which corresponds to the sample concentration giving 0.50 absorbance. The BHT and *α*-tocopherol antioxidant standards were used for comparison of the activity.

2.4.4. Anticholinesterase Inhibitory Activities

The inhibitory activities of acetylcholinesterase and butyrylcholinesterase were calculated by slight modification in the spectrophotometric method [34,37]. Acetylcholinesterase (AChE) from electric eel and Butyrylcholinesterase (BChE) from horse serum was used, while as substrates of the reaction, butyryl thiocholine chloride and acetylthiocholine iodide were employed. The 5, 50-Dithio-bis 2-nitrobenzoic acid (DTNB) was used for the cholinesterase activity determination. Briefly, 150 µL of 100 mM Na_2PO_4 buffer (pH 8.0), 10 µL of sample solutions were dissolved in ethanol at different concentrations, and 20 µL Acetylcholinesterase (5.3210-3 U) or Butyrylcholinesterase (6.85 10-3 U) solution was mixed and incubated for 15 to 18 minutes at 25 °C. Then frequently about 10 µL of DTNB (0.5 mM) was added.

The reaction was then started by the addition of 10 µL of butyryl thiocholine chloride (0.2 mM) or by the addition of 10 µL of acetylthiocholine iodide (0.71 mM). The hydrolysis of these substrates was measured spectrophotometrically by the development of yellow 5-thio-2- nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, unconstrained by the enzymatic hydrolysis of acetylthiocholine iodide or butyryl thiocholine chloride, correspondingly, at 412 nm utilizing a 96-well microplate reader. The percent inhibition of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) enzymes were calculated by comparison of reaction rates of samples comparative (E) to blank sample (S) (ethanol in phosphate buffer pH 8) applying the given formula. Galantamine compound was used as a reference.

$$(E - S)\backslash E \times 100$$

2.5. *Statistical Analysis*

The data were recorded as mean ± S.E.M., and *t*-test was used to calculate S.E.M., p values < 0.05 were regarded as significant.

3. Results and Discussion

The current study was conducted on methanol extract of *Ganoderma lucidum* to evaluate the fatty acids profile, antioxidant, and enzyme inhibition activities. The results are discussed in this section.

3.1. Fatty Acid Composition

The fatty acids profile of Ganoderma lucidum is given in table 1. Totally ten fatty acids were detected. Linoleic acid and oleic acid were found in the highest concentration (53.6 % and 24.2 %), followed by palmitic acid (11.5 %), respectively. Fatty acids groups like $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, $C_{18:0}$, and $C_{18:3}$ were also found from trace to middle amount (0.15 %, 0.15 %, 1.11 %, 0.26 %, 0.27 %, 2.85 % and 5.9 %) respectively. The degree of unsaturation was 85.1 %.

The presence of unsaturated fatty acids upturned the nutritional value of *Ganoderma lucidum*. For people with high blood cholesterol, mushrooms with a high concentration of polyunsaturated fatty acids (PUFAs) are suggested. Polyunsaturated fatty acids concentration of *Ganoderma lucidum* (Table 1) exposed the significance for lowering the blood cholesterol level.

3.2. Bioassays

3.2.1. Antioxidant Activity

Several methods are more helpful and even essential for assessing the antioxidant potential of the extract. Therefore, three radical scavenging assays were used in this study.

Table 2 shows the antioxidant activity results of the methanol extract of *G. lucidum*. The results were expressed as half-maximum effective concentration (IC_{50}). In *β*-carotene-linoleic acid, methanol extract of *Ganoderma lucidum* showed (IC_{50}: 687.12 ± 2.8 µg/mL) lipid peroxidation inhibition activity. However, the extract was not so active as compared with the standards *α*-tocopherol, BHT, and quercetin (2.10 ± 0.08, 1.34 ± 0.09, and 1.80 ± 0.10 µg/mL), respectively. This method is also important to expose the level of inhibition of lipid peroxidation [36].

The methanol extract of *G. lucidum* indicated the metal chelating activity with an IC_{50} value of 870.0 ± 10.05 µg/mL (Table 2). At the same conditions, EDTA demonstrated 6.50 ± 0.07 µg/mL IC_{50} value. The metal chelating activity supports the lipid peroxidation inhibitory activity results. Through the Fenton reaction, transition ions (ferrous and cupric) reactive free radicals and hence accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to free radicals [39]. To retard the radicalic degradation, chelating agents are important.

Table 2 shows the cupric reducing antioxidant capacity. In this method, the latter is formed by the reduction of copper (II) in the presence of neocuproine, resulting in the formation of a stable complex between neocuproine and copper (I), and its absorbance was measured at 450 nm spectrophotometrically. The reducing power of the methanol extract is given in Table 2. The MeOH extract of *Ganoderma lucidum* revealed a reducing potential of 16.55 ± 4.53 µg/mL $A_{0.5}$ value. At the same conditions, the positive standards *α*-tocopherol and BHT indicated 0.012 ± 0.01 and 0.0038 ± 0.00 µg/mL $A_{0.5}$ values, respectively. The CUPRAC assay supports the lipid peroxidation inhibitory activity results. Consequently, it can be said from the conclusions that *Ganoderma lucidum* showed moderate antioxidant potential.

3.2.2. Anticholinesterase Inhibitory Activity

AChE and BChE are related to AD (Alzheimer's disease). There are some hypotheses about the formation of Alzheimer's disease, but the exact reason is unclear. The lack of acetylcholine in the brain is the only known hypothesis [40]. Therefore, acetylcholinesterase inhibitory drugs have been developed and used for the patients. *In vitro,* Ellman's method is frequently used to test any compound's inhibitory activity against the acetylcholinesterase enzyme. Scientists know that nature is full of various compounds that should be screened for acetylcholinesterase inhibitory activity using a simple and easy method.

Table 1. Fatty acid composition (%) of methanol extract of *Ganoderma lucidum*.

No	Fatty acids	*Ganoderma lucidum* (%)
1	Myristic acid ($C_{14:0}$)	0.15 ± 0.001
2	Pentadecanoic acid ($C_{15:0}$)	0.15 ± 0.001
3	Palmitoleic acid ($C_{16:1}$)	1.11 ± 0.001
4	Palmitic acid ($C_{16:0}$)	11.5 ± 0.01
5	*cis*-10-Heptadecanoic acid ($C_{17:1}$)	0.27 ± 0.001
6	Heptadecanoic acid ($C_{17:0}$)	0.26 ± 0.001
7	Linolenic acid ($C_{18:3}$)	5.9 ± 0.02
8	Linoleic acid ($C_{18:2}$)	53.6 ± 0.09
9	Oleic acid ($C_{18:1}$)	24.2 ± 0.03
10	Stearic acid ($C_{18:0}$)	2.85 ± 0.001
	Total saturation %	14.9
	Total unsaturation %	85.1

Table 2 also shows the acetylcholinesterase and butyrylcholinesterase inhibitory activities of the methanol extract of *G. lucidum* compared with those of standard drug galantamine. Against the AChE enzyme, methanol extract of *Ganoderma lucidum* mushroom was found active, demonstrating a 19.11 ± 2.4 µg/mL IC_{50} value. On the other hand, methanol extract exhibited weak butyrylcholinesterase (BChE) inhibitory activity (IC_{50}>100 µg/mL). At the same conditions, galantamine demonstrated 0.005 ± 0.00 µg/mL IC_{50} value. *G. lucidum* may be an important source of potential new acetylcholinesterase (AChE) inhibitory drugs. The inhibitory activity results recommended that the mushroom should be further investigated for their constituents by anticholinesterase activity-guided fractionation.

Table 2. Antioxidant and anticholinesterase activity of methanol extract of *Ganoderma lucidum* by the· *β*-carotene linoleic acid, and metal chelating assays. [a]

Samples	***β*-carotene-linoleic acid assay IC_{50} (µg/mL)**	**Ferrous-Ferrin chelating assay IC_{50} (µg/mL)**	**CUPRAC assay $A_{0.50}$ (µg/mL)**	**AChE assay IC_{50} (µg/mL)**	**BChE assay IC_{50} (µg/mL)**
Ganoderma lucidum	687 ± 2.81	870 ± 10.05	16.55 ± 2.53	19.11 ± 2.4	>100
α-Tocopherol [b]	2.10 ± 0.08	*NT*	0.010 ± 0.01	*NT*	*NT*
BHT [b]	1.34 ± 0.09	*NT*	0.0038 ± 0.01	*NT*	*NT*
Quercetin [b]	1.80 ± 0.10	250 ± 0.07	*NT*	*NT*	*NT*
EDTA	*NT*	6.50 ± 0.07	*NT*	*NT*	*NT*
Galantamine [b]	*NT*	*NT*	*NT*	5.04 ± 0.0002	51.55 ± 1.01

NT= not tested.

[a] IC_{50} and $A_{0.50}$ values represent the means ± SEM of three parallel measurements (p<0.05).

[b] Reference compounds.

4. Conclusion

In this study, the antioxidant, anticholinesterase assays of *Ganoderma lucidum* collected from the Swat region of Khyber Pakhtunkhwa-Pakistan were studied. The fatty acid composition of this species is also studied.

In conclusion, the results confirmed the significance of the *Ganoderma lucidum* species and the native use of *Ganoderma lucidum* in different parts of Swat-Khyber Pakhtunkhwa-Pakistan is based on their therapeutic potential with a delightful taste. Thus, *Ganoderma lucidum* may be used against lipid peroxidation and free radical damage. *Furthermore,*

Ganoderma lucidum may be an important source of potential new acetylcholinesterase (AChE) inhibitory drugs. The inhibitory activity results recommended that the plants should be further investigated for their constituents by anticholinesterase activity-guided fractionation.

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Conflict of Interest

The authors proclaimed no conflict of interest.

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