Wild Mushrooms in Nepal: Some Potential Candidates as Antioxidant and ACE-Inhibition Sources

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Twenty-nine mushrooms collected in the mountainous areas of Nepal were analyzed for antioxidant activity by different methods, including Folin-Ciocalteu, ORAC, ABTS, and DPPH assays. Intracellular $\text{H}_2\text{O}_2$-scavenging activity was also performed on HaCaT cells. The results showed that phenolic compounds are the main antioxidant of the mushrooms. Among studied samples, Inonotus andersonii, and Phellinus gilvus exhibited very high antioxidant activity with the phenolic contents up to 310.8 and 258.7 mg GAE/g extracts, respectively. The $\text{H}_2\text{O}_2$-scavenging assay on cells also revealed the potential of these mushrooms in the prevention of oxidative stress. In term of ACE-inhibition, results showed that Phlebia tremellosa would be a novel and promising candidate for antihypertensive studies. This mushroom exhibited even higher in vitro ACE-inhibition activity than Ganoderma lingzhi, with the IC$_{50}$ values of the two mushrooms being 32 $\mu$g/mL and 2 $\mu$g/mL, respectively. This is the first time biological activities of mushrooms collected in Nepal were reported. Information from this study should be a valuable reference for future studies on antioxidant and ACE-inhibitory activities of mushrooms.

1. Introduction

For millennia, mushrooms have been used as a part of the human diet and as medicinal sources. In term of nutrition, mushrooms are recognized as a healthy food as they are low in calories and fat but rich in proteins and dietary fiber [1, 2], while pharmacologically, the potential of medicinal mushrooms is considered enormous but mostly untapped [3]. With their wide variety of components, mushrooms—both edible and medicinal—have a broad spectrum of bioactivities [4, 5]. According to FAOSTAT data [6], the total world production of mushrooms including truffles has sharply increased from 2.0 million metric tons in 1990 to nearly 7.4 million metric tons in 2010 and the market of mushroom-derived dietary supplements is also quickly growing and is valued at more than US $15 billion today [7]. This tendency may reflect an increase in the recognition of the value of mushrooms as a healthy food and an important source of medicinal compounds.

Oxidative stress is a chronic imbalance between antioxidant ability of biological systems and production of reactive
oxygen species (ROS) that is involved in many diseases including skin aging and hypertension [8–10]. UV exposure is initial step of ROS generation, causes many alterations and mutations in skin [11–13]. The skin itself has antioxidant defense system used to deactivate ROS, but when this system is overwhelmed there is a need of antioxidant supplement through food or treatment therapies [12, 14]. Abundance of studies have reported about beneficial effect of antioxidant on skin protection against ROS and were thoroughly reviewed in recent reports [12, 15–17]. Experimental and clinical studies have also indicated that hypertension occurs after a 2 Evidence-Based Complementary and Alternative Medicine

...studies have reported about beneficialeffect of antioxidants on defensesystemusedtodeactivateROS, butwhenthissystem

...osmotic water and ethanol (Wako Pure Chemical Industries, Japan), using an orbital shaker for obtaining 58 extracts. Water extracts were lyophilized, while ethanol extracts were rotary evaporated to dryness when preparing samples for assays. The resultant extracts were kept in glass-capped vials sealed with parafilm and stored in a cool place until assayed.

2.3. Phenolic Content Determination. Total phenolic content was determined by a method described by Singleton and Gillespie [36, 37] with some minor modifications. This assay is based on the electrons transferred in alkaline medium from phenolic compounds to blue-colored phosphomolyb-dic/phosphotungstic acid complexes which have maximum absorbance at 765 nm. Details of the procedures are as follow: 50 μL of sample solution was mixed well with 100 μL of 10% Folin–Ciocalteu solution (a mixture of Na2WO4, Na2MoO4,
Li$_2$SO$_4$, HCl, and H$_3$PO$_4$ with an appropriate ratio) in a 1.5 mL plastic tube. The mixture was equilibrated for several minutes and then 400 µL of 7.5% Na$_2$CO$_3$ was added to the tube and the reaction mixture was incubated at room temperature for 60–90 minutes. After the incubation period, reaction tubes were centrifuged at 6000 rpm for 2 minutes whenever necessary, 200 µL of supernatant of samples (or blank) was transferred to an optically clear 96-well microplate, and the absorbance was measured at 765 nm using Molecular Devices FlexStation 3 Microplate Reader. Data were managed by SoftMax Pro 5.4.1 software. Gallic acid was used as the standard and was measured in the same conditions as the samples.

2.4. Free Radical Scavenging by the ORAC Assay. This assay measures the oxidative degradation of the fluorescence of fluorescein after being mixed with the free radical generator AAPH (2,2′-azobis(2-amidino-propane)dihydrochloride). Heating AAPH is said to produce the peroxyl radical, which damages fluorescein molecules resulting in the loss of fluorescence. Antioxidants suspected to be contained in extracts were pretreated with a small amount of acetone, buffer (pH 7.4) for use in the ORAC assays, but ethanol and ethanol extracts were dissolved in 75% of ethanol so that it gave an absorbance of 0.7 ± 0.02 units at 734 nm for a making working solution. One milliliter of working solution was mixed with 10 µL of mushroom extracts (maximum dissolved concentration) and shaken well for 10 seconds; after 4 minutes of incubation at 30°C, the absorbance of the reaction mixture was measured at 734 nm (UVmini-1240, Shimadzu, Kyoto, Japan) to give “$A_b$” values. Ethanol 99.5% was used as a blank (absorbance was “$A_{b0}$”) and the inhibition rates were calculated using (*).

2.6. Free Radical Scavenging by ABTS Radical. ABTS assay was mostly based on the methods described previously [42] in which ABTS+, the oxidant, was generated by persulfate oxidation 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). Specifically, to 5 mL of 7 mM ABTS ammion aqueous solution, 88 µL of 140 mM potassium peroxysulfate (K$_2$S$_2$O$_8$) was added and the resulting mixture was then allowed to stand at room temperature for 12–16 hour to yield a dark blue solution. The mixture was then adjusted by 99.5% ethanol so that it gave an absorbance of 0.7 ± 0.02 units at 734 nm for a making working solution. One milliliter of working solution was mixed with 10 µL of mushroom extracts (maximum dissolved concentration) and shaken well for 10 seconds; after 4 minutes of incubation at 30°C, the absorbance of the reaction mixture was measured at 734 nm (UVmini-1240, Shimadzu, Kyoto, Japan) to give “$A_b$” values. Ethanol 99.5% was used as a blank (absorbance was “$A_{b0}$”) and the inhibition rates were calculated using (*).

2.7. Detection of Intracellular UVB-Induced H$_2$O$_2$. Intracellular H$_2$O$_2$ was assessed using immortal human keratinocyte line (HaCaT) as cell model. HaCaT cells (Cell Line Service, Eppelheim, Germany) were cultured in DMEM supplemented with L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin antibiotic solution. After one more day incubation, cells were transferred to 96-well cells/well and incubated for 24 hours. Nucleus was stained by Hoechst 33342 (Dojindo, Kumamoto, Japan) and the amount of intracellular H$_2$O$_2$ was quantified based on the amount of difluorofluorescein (DFF) released from the reaction of H$_2$O$_2$ and BES-H$_2$O$_2$-Ac (Wako Chemical, Osaka, Japan). Determination procedures were as follows: 3 mL of 6 × 10$^{-5}$ M DPPH$^*$ solution (prepared daily) was mixed with 100 µL of methanolic solutions of mushroom extracts (maximum dissolved concentration); after 20 min incubation for at 37°C, absorbance decrease of the mixture was monitored at 515 nm ($A_j$). Blank samples with 100 µL of methanol in the above DPPH$^*$ solution were prepared and measured daily at same wavelength ($A_b$). The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula.

$$\text{Inhibition rate} \ (\%) = \left( \frac{A_{b0} - A_j}{A_{b0}} \right) \times 100. \quad (*)$$

2.5. Free Radical Scavenging by DPPH Radical. The radical scavenging activity of mushroom extracts against the DPPH$^*$ radical (2,2-diphenyl-2-picrylhydrazyl hydrate; Sigma-Aldrich, Steinheim, Germany) was determined by the method of Brand Williams modified by Dudonné et al. [40, 41]. DPPH radicals have an absorption maximum at 515 nm; upon reduction by the antioxidant, the solution color fades and the reaction progress is easily monitored by a spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan). Determination procedures were as follow: 3 mL of 6 × 10$^{-5}$ M DPPH$^*$ solution (prepared daily) was mixed with 100 µL of methanolic solutions of mushroom extracts (maximum dissolved concentration); after 20 min incubation for at 37°C, absorbance decrease of the mixture was monitored at 515 nm ($A_j$). Blank samples with 100 µL of methanol in the above DPPH$^*$ solution were prepared and measured daily at same wavelength ($A_b$). The experiment was carried out in triplicate. Radical scavenging activity was calculated using the following formula.

$$\text{Inhibition rate} \ (\%) = \left( \frac{A_{b0} - A_j}{A_{b0}} \right) \times 100. \quad (*)$$
then applied to Spotfire Decision Site Client 8.2 software for visualizing the results. Cells unexposed to UVB irradiation were used as controls; cells exposed to UVB and cultured in the presence or absence of resveratrol (10 ppm of final concentration) were used as positive or negative control, respectively.

2.8. Angiotensin-Converting Enzyme Inhibitory Assay. Water extracts were dissolved in milli-Q water (Millipore, MA, USA) and those that were difficult to dissolve in water were pretreated with a small amount of ethanol before being dissolved in milli-Q water (final concentration of organic solvent in enzyme reactions was less than 1%). Both types of extract were subjected to ACE-inhibitory assay using Dojindo ACE Kit-WST test kit (Dojindo Laboratories, Kumamoto, Japan). Details of the method’s principle can be found elsewhere [43]. Briefly, the enzymatic reaction was initiated by the ACE and aminoacylase in the mixture containing 3HB-GGG (3-hydroxybutyrate glycyglycylglycine) and the ACE-inhibitor. The mixture was then incubated at 37°C for 60 min. During this incubation, the substrate, 3HB-GGG, was enzymatically cut into 3HB-G and G-G and then 3HB and G. The yield of 3HB was monitored indirectly through formazan concentration, which was measured at 450 nm after 10 minute reaction at 25°C.

Testing procedures were run according to the manufacturer’s instructions using a 96-well plate without modification, and the inhibition rate was calculated based on a comparison of the optical absorbance of samples-treated wells \(A_s\), control wells \(A_c\), and blank wells \(A_b\). Absorbance was measured at 450 nm using the microplate reader Biotek-ELX800 (BioTek, Vermont, USA). Inhibition rates were calculated using the following equation.

\[
\text{Inhibition rate (\%) = } \left( \frac{A_c - A_s}{A_c - A_b} \right) \times 100. \tag{1}
\]

Samples were suspected to inhibit the ACE activity, and therefore inhibit the formation of formazan. The more strongly inhibitory the activity of the samples, the less color and therefore inhibit the formation of formazan. The more strongly inhibitory the activity of the samples, the less color and therefore inhibit the formation of formazan.

2.9. Statistical Analysis. Each ORAC experiment was repeated four times, while the ACE-inhibitory assay and phenolic content determination were performed in triplicate. The results are expressed as mean ± SD. The correlation coefficient between phenolic content and antioxidant assays was determined by least-square linear regression analysis using Microsoft Excel 2007.

3. Results and Discussion

3.1. Mushroom Collection and Identification. As shown in Table 1, 29 mushroom samples, collected from the mountainous area of Nepal were first identified by morphological observation. Some mushrooms for which ITSs were obtained were subjected to a BLAST search via INSDC.

Mushroom samples were collected at mass from 11.6 to 117.7 g in dried weight. Purified genome DNAs were successfully obtained from the mushroom samples. DNA fragments containing the ITS sequence were amplified in the 705 to 894 bp range. From BLAST search results, the mushroom listed in Table 1 were identified. Six samples (from N001 to N006) were included in the genus Ganoderma, and three samples (N016, N018, and N019) belonged to the genus Phellinus. Samples N009, N011, N014, N027, and N028 were identified as Trametes versicolor.

3.2. Antioxidant Activities

3.2.1. Free Radical Scavenging by the ORAC Assay. Many methods have been developed for measuring antioxidant capacity in vitro. The underlying chemistry, advantages, and disadvantages have also been well documented and reviewed [44, 45]. Among these methods, the oxygen radical absorbance capacity (ORAC) method, with some modifications that have been made over time, has been widely used to evaluate the antioxidant activity of many herbal extracts, food additives, and even biological samples [46]. The existence of the (USDA) US Department of Agriculture ORAC database and the recently launched web-based database for this index [47] show the scientific community’s estimation of the ORAC assay for measuring antioxidant capacity of herbal samples. In the initial checking for antioxidant activity of mushroom extracts in this study, we ran ORAC experiments in which fluorescein was used as fluorescent probe in a 96-well plate assay as described above.

The results of ORAC assays of samples are shown in Table 2. ORAC values (μmol TE/g extract) ranged from 342.8 to 21015.4 for ethanol extracts and from 83.2 to 1196.9 for water extracts. Among the samples Inonotus andersonii and Phellinus gilvus ethanol extracts showed extremely high activity. This is the first time such high ORAC values have been seen for mushroom extracts. Until now, such high ORAC values have only been reported for extracts of well-known antioxidant spices like cloves, pimento, and cinnamon [41, 48]. High antioxidant activities have also been reported for some mushrooms in Inonotus species such as I. hispidus and I. obliquus [49, 50], but we could not find any published report on the antioxidant capacity of the I. andersonii mushroom. It is worth noting here that the main antioxidative compounds isolated from above-mentioned Inonotus mushrooms are hispidin and hispidin moiety-contained compounds such as inonotusin A and B in I. hispidus [49], inonoblins, and phelligrinids in I. obliquus [51]. From these results, we think that I. andersonii may also contain such compounds and this mushroom should be a good candidate for future antioxidant researches.

3.2.2. Phenolic Content, ABTS, and DPPH Radical Scavenging Assays. Phenolic compounds are considered one of the major groups of nonessential dietary components which have been suggested to be beneficial for human health and their physiological importance is said to relate to their abilities to chelate metals, inhibit lipoxygenase, and scavenge free radicals [52]. The Folin-Ciocalteu method is often used to estimate the phenolic content of plant extract samples although the
Table 1: Information related to mushrooms used in present study.

| Number | Scientific name      | Locus*       | Habitat                | INSDC Acc. number |
|--------|----------------------|--------------|------------------------|-------------------|
| N001   | *Ganoderma* *carnosum* | Mt. Phulchoki/2765m | Decayed wood          | AB763348          |
| N002   | *Ganoderma* *lingzhi*   | Mt. Phulchoki/2765m | Decayed wood          | AB811848          |
| N003   | *Ganoderma* *australe*  | Mustang/3150m   | Decayed wood          | AB811849          |
| N004   | *Ganoderma* *australe*  | Mt. Phulchoki/2765m | Decayed wood          | AB811850          |
| N005   | *Ganoderma* *australe*  | Mt. Phulchoki/2765m | Decayed wood          | Not determined    |
| N006   | *Ganoderma* *australe*  | Mt. Phulchoki/2765m | Decayed wood          | AB811852          |
| N007   | *Postia* *stiptica*     | Dawachok/1500m   | Decayed wood          | AB811853          |
| N008   | *Phlebia* *tremellosa* | Mt. Phulchoki/2765m | Soil                  | AB811854          |
| N009   | *Trametes* *versicolor* | Mt. Phulchoki/2765m | Decayed wood          | AB811855          |
| N010   | *Inonotus* *andersonii* | Mt. Phulchoki/2765m | Soil                  | AB811856          |
| N011   | *Trametes* *versicolor* | Mt. Phulchoki/2765m | Decayed wood          | AB811857          |
| N012   | *Inonotus* sp. 1        | Mt. Phulchoki/2765m | Living tree           | Not determined    |
| N013   | *Heterobasidion* *linzhiense* | Surya Binayak/1400 | Living tree           | AB811859          |
| N014   | *Trametes* *versicolor* | Mt. Phulchoki/2765m | Living tree           | AB811860          |
| N015   | *Heterobasidion* *linzhiense* | Mt. Phulchoki/2765m | Living tree           | AB811861          |
| N016   | *Phellinus* *gilvus*    | Mt. Phulchoki/2765m | Decayed wood          | AB811862          |
| N017   | *Inonotus* sp. 2        | Mt. Phulchoki/2765m | Decayed wood          | Not determined    |
| N018   | *Phellinus* *conchatus* | Nagarkot/2500m   | Decayed wood          | AB811863          |
| N019   | *Phellinus* *conchatus* | Nagarkot/2500m   | Decayed wood          | AB811864          |
| N020   | *Inocybe* sp.           | Mt. Phulchoki/2765m | Soil                  | Not determined    |
| N021   | *Collybia* *peronata*   | Nagarkot/2500m   | Fallen leaves         | Not determined    |
| N022   | *Inonotus* sp. 3        | Mt. Phulchoki/2765m | Decayed wood          | AB811865          |
| N023   | *Lactarius* *hatsudake* | Mustang/3150m   | Soil                  | Not determined    |
| N024   | *Lentinus* *betulina*   | Mt. Phulchoki/2765m | Soil                  | AB811866          |
| N025   | *Panellus* sp.          | Mt. Phulchoki/2765m | Decayed wood          | Not determined    |
| N026   | *Rigidoporus* sp.       | Surya Binayak/1400 | Decayed wood          | Not determined    |
| N027   | *Trametes* *versicolor* | Mt. Phulchoki/2765m | Decayed branch**      | AB811867          |
| N028   | *Trametes* *versicolor* | Mt. Phulchoki/2765m | Decayed branch**      | AB811868          |
| N029   | *Tricholoma* *caligatum* | Mt. Phulchoki/2765m | Soil                  | Not determined    |

* Number in "m" is average height of samples-collection area; ** Decay branch of living tree.

Reagent used for determining phenolic content does not react exclusively with phenolics and has even been proven to be affected by a variety of compounds such as thiol derivatives, vitamin derivatives, amino acids, and metal complexes [53]. Thus, the reagent often overestimates the phenolic contents in samples, but because it is a cheap, simple, convenient and, in some aspects, useful method for determining total phenolic content, Folin-Ciocalteu is still widely used to estimate the total antioxidant capacity of samples. Many studies on spices, vegetables, fruits, and plants extracts have shown a good relationship between phenolic content and antioxidant activity [41, 53–55]. It is also generally accepted that the main antioxidants in mushrooms are phenolics, mainly phenolic acids [56]. To confirm the relationship of phenolic compounds in mushrooms and their antioxidant activities we selected 10 mushroom samples which had the highest ORAC values and carried out experiments for determining phenolic content and other radical scavenging activities. The correlation coefficients between phenolic content and antioxidant activity resulting from different assays were also calculated and the results were shown in Table 3.

Experimental results showed good relationships between phenolic content and antioxidant activities in which the correlation coefficient $R$ of phenolics and ORAC, ABTS, and DPPH activities were 0.923, 0.936, and 0.986, respectively. The close correlation between phenolic content and ABTS inhibition is not surprising since the methods used to determine phenolics and ABTS inhibition rates are both based on the electron transfer ability of the sample's components. However, while the ORAC assay is based on hydrogen atom transfer reactions, we still could see a good correlation between phenolic content and ORAC values. This may come from the fact that phenolic compounds are not only a rich electron source, but the phenolic hydroxy group can also act as a hydrogen donor supplying hydrogen atom to wipe out peroxy radicals by forming stabilized phenoxyl radicals in the ORAC assay. In this context, phenolic compounds can be both electron and hydrogen atom donors, and therefore can be in good correlation with both the ORAC and ABTS assays. The very high correlation found between DPPH assays and total phenolic content with $R$ equal to 0.986 indicates a close relationship between phenolic compound concentration in
## Table 2: ORAC values ($\mu$mol TE/g extract) of mushrooms extracts.

| Number | Scientific name          | EtOH        | ORAC values      |
|--------|--------------------------|-------------|------------------|
| N001   | *Ganoderma carnosum*     | 1938.6 ± 64.5 | 764.2 ± 29.8    |
| N002   | *Ganoderma lingzhi*      | 2136.2 ± 100.8 | **1046.1 ± 18.5** |
| N003   | *Ganoderma australe*     | 1406.9 ± 71.4 | 663.0 ± 19.4     |
| N004   | *Ganoderma australe*     | **1602.9 ± 88.5** | 497.1 ± 69.7 |
| N005   | *Ganoderma australe*     | 1781.4 ± 123.3 | 813.5 ± 23.6     |
| N006   | *Ganoderma australe*     | 2578.5 ± 99.0 | **1196.9 ± 48.7** |
| N007   | *Postia stipita.*        | 449.5 ± 27.7  | 660.5 ± 15.5     |
| N008   | *Phlebia tremellosa*     | 960.1 ± 38.1  | 629.2 ± 14.6     |
| N009   | *Trametes versicolor*    | 615.6 ± 34.0  | 650.0 ± 6.4      |
| N010   | *Inonotus andersonii*    | **21015.4 ± 121.3** | 83.2 ± 31.9     |
| N011   | *Trametes versicolor*    | 2168.0 ± 33.1  | 111.3 ± 36.7     |
| N012   | *Inonotus sp.* 1         | 1848.0 ± 77.6  | 761.1 ± 15.4     |
| N013   | *Heterobasidion linzhiense* | 616.1 ± 83.1  | 655.9 ± 26.8     |
| N014   | *Trametes versicolor*    | 410.6 ± 49.8  | 745.3 ± 3.7      |
| N015   | *Heterobasidion linzhiense* | 691.0 ± 22.1  | 419.7 ± 19.8     |
| N016   | *Phellinus gilvus*       | 9564.0 ± 281.5 | 280.7 ± 22.4     |
| N017   | *Inonotus sp.* 2         | **1746.6 ± 100.9** | 358.2 ± 43.0    |
| N018   | *Phellinus conchatus*    | 3856.4 ± 296.6 | 558.1 ± 18.9     |
| N019   | *Phellinus conchatus*    | **4431.5 ± 211.6** | 570.3 ± 22.4 |
| N020   | *Inocybe sp.*            | 472.7 ± 38.8  | 447.6 ± 35.8     |
| N021   | *Collybia peronata*      | 802.4 ± 45.9  | 461.3 ± 15.9     |
| N022   | *Inonotus sp.* 3         | 342.8 ± 7.9   | 936.5 ± 69.5     |
| N023   | *Lactarius hatsudake*    | 425.5 ± 27.4  | 941.9 ± 54.9     |
| N024   | *Lenzites betulina*      | 548.9 ± 14.3  | 741.6 ± 13.8     |
| N025   | *Panellus sp.*           | 462.8 ± 12.0  | **1070.1 ± 42.1** |
| N026   | *Rigidoporus sp.*        | 1025.9 ± 48.7 | 534.1 ± 28.9     |
| N027   | *Trametes versicolor*    | 435.3 ± 14.2  | **984.4 ± 14.1** |
| N028   | *Trametes versicolor*    | 522.2 ± 18.8  | 499.9 ± 26.5     |
| N029   | *Tricholoma caligatum*   | 620.5 ± 18.8  | 247.1 ± 10.8     |

The bold values show the samples with high ORAC value.

## Table 3: Phenolic content, ORAC values, ABTS, and DPPH radical scavenging results of top 10 extracts.

| Number | Scientific name          | Total phenolic (mg GAE/g) | Ethanol extract |
|--------|--------------------------|---------------------------|-----------------|
| N010   | *Inonotus andersonii*    | 310.8 ± 2.7               | 21015.4 ± 121.3 | 36.4 ± 1.0 (63.5) | 72.9 ± 2.1 (102.0) |
| N016   | *Phellinus gilvus*       | 258.7 ± 4.3               | 9564.0 ± 281.5  | 38.1 ± 0.4 (56.0) | 55.2 ± 3.2 (109.0) |
| N017   | *Inonotus sp.* 2         | 97.1 ± 0.9                | 1746.6 ± 100.9  | 24.1 ± 0.3 (62.5) | 22.4 ± 1.6 (106.0) |
| N006   | *Ganoderma australe*     | 88.3 ± 2.4                | 2578.5 ± 281.5  | 21.5 ± 0.7 (63.5) | 19.2 ± 0.7 (108.0) |
| N005   | *Ganoderma australe*     | 82.7 ± 8.2                | 1781.4 ± 121.3  | 16.1 ± 2.3 (53.5) | 14.7 ± 0.9 (100.0) |
| N019   | *Phellinus conchatus*    | 74.9 ± 1.3                | 4431.5 ± 211.6  | 19.3 ± 0.2 (58.5) | 14.5 ± 1.5 (103.0) |
| N018   | *Phellinus conchatus*    | 68.2 ± 0.2                | 3856.4 ± 296.6  | 12.6 ± 0.3 (47.5) | 17.0 ± 0.2 (115.0) |
| N001   | *Ganoderma carnosum*     | 70.2 ± 3.0                | 1938.6 ± 281.5  | 16.0 ± 2.4 (61.0) | 11.3 ± 1.3 (103.0) |
| N002   | *Ganoderma lingzhi*      | 66.8 ± 3.0                | 2168.0 ± 33.1   | 15.1 ± 0.4 (63.0) | 10.7 ± 0.6 (126.0) |
| N011   | *Trametes versicolor*    | 50.4 ± 0.5                | 2168.0 ± 33.1   | 10.3 ± 0.3 (74.0) | NA**               |

*Correlation coefficient* —  \( R = 0.923 \)  \( R = 0.936 \)  \( R = 0.986 \)

*Correlation coefficients in each column were between phenolic content and correspondent antioxidant activity; ** not available; numbers in bracket of ABTS and DPPH columns were final concentrations (µg/mL).
The bold values show high correlation coefficient.
mushroom extracts and their nitrogen-radical scavenging capacities.

Despite the fact that there have been many studies referring to the antioxidant activity of mushroom, almost no report has mentioned a correlation between mushroom genus (or family) and antioxidant activity. Our present results suggest such a relationship. For example, all studied Ganoderma (Ganodermataceae) samples had a medium antioxidant activity, while Phellinus and Inonotus samples (Hymenochaetaceae) showed quite high activity. Some previous discrete studies [51, 57–61] have also shown the high antioxidant capacity of many mushrooms in the Inonotus and Phellinus genera. This consistency across different studies results can be used to consolidate and direct future research on antioxidant activity. Assuming that this genus bioactivity relationship can be further established, we think that these genera could be good candidates for studies of mushroom’s antioxidants properties in the future.

3.2.4. ACE-Inhibitory Assay. Water and ethanol extracts of 29 mushrooms samples were used for screening the ACE-inhibitory effect using Dojindo ACE test kits. Each test was repeated three times and inhibition rates were calculated based on a comparison of blank and control samples. Results are shown in Table 5.

High blood pressure is one of the major independent risk factors for cardiovascular diseases and is considered a worldwide health problem. Angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) plays a crucial role in blood-pressure regulation by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Therefore, the inhibition of ACE activity is a major target in the prevention of hypertension [68]. Until now, ACE-inhibitors have been mainly sourced from food protein, especially milk protein. Many milk protein-derived peptides have demonstrated inhibitory effects on ACE in vitro [69–71] and on antihypertension in vivo [72–74].

Recently, mushrooms have also been considered as good candidate sources of hypotensive agents. Several peptides and proteins extracted from mushrooms have been shown to have an ACE-inhibitory effect. Many mushroom extracts have been screened for this activity [26, 75–77], and most of the time, the dominant ACE-inhibition extracts have been aqueous. Consistently with previous results, our study also showed a predominance of water extracts for ACE-inhibition. While there were 15 water extracts which showed rather high efficiency against UVB-induced H$_2$O$_2$ generation in HaCaT cells, only 8 significant results were obtained.

**Table 4: Effect of mushrooms extract on UVB-induced intracellular H$_2$O$_2$ generation in HaCaT cells.**

| Number | Scientific name          | DFF$^*$ | Number | Scientific name          | DFF$^*$ |
|--------|--------------------------|---------|--------|--------------------------|---------|
| N016   | *Phellinus gilvus*       | 7.3 ± 6.1 | N019   | *Phellinus conchatus*     | 31.7 ± 3.1 |
| N001   | *Ganoderma carnosum*     | 19.0 ± 9.6 | N018   | *Phellinus conchatus*     | 32.7 ± 4.0 |
| N010   | *Inonotus andersonii*    | 26.7 ± 4.0 | N002   | *Ganoderma lingzhi*      | 35.3 ± 7.4 |
| N005   | *Ganoderma australe*     | 26.7 ± 7.6 | N003   | *Trametes versicolor*     | 35.7 ± 4.9 |
| N017   | *Inonotus sp. 2*         | 31.3 ± 10.7 | N006   | *Ganoderma australe*      | 53.3 ± 5.8 |

| Control | 26.3 ± 7.0 | 53.1 ± 4.0 | Negative control | 36.0 ± 9.8 | Resveratrol | 53.3 ± 4.0 |

$^*$ DFF: Difluorofluorescein fluorescent intensity; proportionally related to H$_2$O$_2$ concentration.

The bold values show low difluorofluorescein fluorescent intensity compared to control and negative control.
inhibitory effect with inhibition rates higher than 50% at 100 μg/mL, only one ethanol extract showed more than 50% inhibition at this concentration. Besides traditionally well-known mushrooms such as *Ganoderma lingzhi* and *Trametes versicolor*, other nonmedicinal mushrooms like *Phlebia tremellosa* and *Heterobasidion linzhiense* sp. samples also showed high inhibition activity. According to Lindequist et al. [4], the responsible bioactive compounds in mushrooms belong to several chemical groups; usually they are polysaccharides, triterpenoids, and proteins. As mentioned above, several ACE-inhibitory peptides and proteins have been identified from mushroom water extracts. From ethanol or methanol extracts only some ganoderic acids [23] and nicotianamine [76] with ACE-inhibitory capacity have been identified. Recent studies indicated that phenolic compounds can also play a role in the inhibition of ACE [77, 78]. In this study, the average inhibition against ACE of *I. andersonii* and *P. gilvus* ethanol extracts (the two highest-phenolic-content samples) could be explained by the action of phenolic compounds in the mushroom extracts.

Among the studied samples, it seems that *Phlebia tremellosa* contained potent compounds having high ACE-inhibitory capacity. To confirm the potential of mushrooms for ACE-inhibitory activity, we performed the IC50 value determination for water extracts of this mushroom and compared with *Ganoderma lingzhi*’s capacity. The IC50 of *Phlebia tremellosa* was 16 times higher than that of *Ganoderma lingzhi* sample, with values of 32 μg/mL and 2 μg/mL, respectively. These results confirmed the potential of this mushroom for ACE-inhibition, and it should be pursued in future studies.

Besides the fact that the ACE-inhibitory capacity of most mushrooms (except for *Ganoderma lingzhi*) in this study has never been reported, our results also indicated a clear relationship between mushroom genus and certain activities, as mentioned above. Five of six *Ganoderma* and four of five *Trametes* mushroom samples showed high inhibition rates at the studied concentration. This correlation may result from the similarity of the chemical structures of metabolites provided by fungal species belonging to the same genus [77]. Further investigation and more samples are needed.

### Table 5: ACE-inhibition rate of mushroom extracts at concentration of 100 μg/mL.

| Number | Scientific name | EtOH ACE inhibition (%) | H2O ACE inhibition (%) |
|--------|-----------------|-------------------------|-------------------------|
| N001   | *Ganoderma carnosum* | 22.99 ± 6.45 | 71.30 ± 2.22 |
| N002   | *Ganoderma lingzhi* | 21.42 ± 3.77 | 76.98 ± 1.22 |
| N003   | *Ganoderma australe* | 22.11 ± 5.03 | 38.61 ± 4.65 |
| N004   | *Ganoderma australe* | 15.37 ± 7.67 | 50.72 ± 6.43 |
| N005   | *Ganoderma australe* | 20.99 ± 3.44 | 65.26 ± 6.76 |
| N006   | *Ganoderma australe* | 33.10 ± 8.53 | 61.59 ± 2.98 |
| N007   | *Phlebia tremellosa* | 18.23 ± 1.16 | 26.81 ± 6.71 |
| N008   | *Phlebia tremellosa* | 6.14 ± 1.19 | 92.57 ± 1.25 |
| N009   | *Trametes versicolor* | 19.72 ± 2.11 | 38.18 ± 0.41 |
| N010   | *Inonotus andersonii* | 52.76 ± 1.80 | 39.38 ± 7.52 |
| N011   | *Inonotus andersonii* | 17.03 ± 0.82 | 58.92 ± 7.82 |
| N012   | *Inonotus sp. 1* | 18.25 ± 2.47 | 18.22 ± 1.40 |
| N013   | *Heterobasidion linzhiense* | 1.47 ± 3.33 | 54.97 ± 2.67 |
| N014   | *Trametes versicolor* | nd | 69.09 ± 1.41 |
| N015   | *Heterobasidion linzhiense* | nd | 73.38 ± 3.08 |
| N016   | *Phellinus gilvus* | 40.96 ± 2.60 | 13.80 ± 4.16 |
| N017   | *Inonotus sp. 2* | 14.54 ± 4.45 | nd |
| N018   | *Phellinus conchatus* | 18.21 ± 0.86 | nd |
| N019   | *Phellinus conchatus* | 19.56 ± 4.59 | 48.39 ± 4.00 |
| N020   | *Inocybe sp.* | nd | 56.05 ± 7.40 |
| N021   | *Collybia peronata* | 9.72 ± 1.23 | 38.99 ± 7.34 |
| N022   | *Inonotus sp. 3* | nd | 15.71 ± 1.71 |
| N023   | *Lactarius hutsudake* | nd | nd |
| N024   | *Lenzites betula* | 16.31 ± 1.03 | 84.87 ± 2.04 |
| N025   | *Panellus sp.* | 23.92 ± 1.49 | 35.36 ± 3.32 |
| N026   | *Rigidoporus sp.* | 1.95 ± 2.32 | 10.17 ± 9.07 |
| N027   | *Trametes versicolor* | 4.22 ± 5.10 | 40.96 ± 2.60 |
| N028   | *Trametes versicolor* | 0.17 ± 3.17 | 13.80 ± 4.16 |
| N029   | *Tricholoma caligatum* | nd | 55.40 ± 3.89 |

*IC50 = 32 μg/mL; **IC50 = 2 μg/mL; ***nd: not detected.*

The bold values show high ACE inhibition.
to confirm the speculation, but assuming such correlations can be established, this should be valuable information for directing future researches.

4. Conclusion

Twenty-nine mushroom samples of 21 species in 14 genera collected in Nepal were checked for antioxidant and angiotensin-converting enzyme in vitro inhibition capacity. Beside *Phellinus gilvus* which was reported as a potent mushroom for isolating antioxidant compounds in some previous studies, this time we showed that *Inonotus andersonii* is also a promising candidate for antioxidant investigation with an antioxidant capacity equivalent to the well-known antioxidant spice, cloves. The H$_2$O$_2$-scavenging assay on HaCaT cells also revealed the potential of these mushrooms in the prevention of oxidative stress. From the fact that other samples of the *Phellinus* genus also showed high antioxidant activity, we deduced the potential of this genus as an important antioxidant source for future studies. ACE-inhibition assays indicated that *Phlebia tremellosa* is a novel and potent candidate for antihypertensive studies. This mushroom exhibited even higher in vitro ACE-inhibition activity than *Ganoderma lingzhi*, with the IC$_{50}$ values of the two mushrooms at 32 µg/mL and 2 µg/mL, respectively. With half of the mushrooms samples herein being reported for antioxidant properties for the first time and most of the mushrooms having never been reported for ACE-inhibitory activity, information from this study should be a valuable reference for future studies on antioxidant and ACE-inhibitory activities of mushrooms.

Conflict of Interests

The authors declare that there is no conflict of interests and no potential of conflict with any trademarks mentioned in this paper.

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