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# Enzyme Inhibitory and Antioxidant Activity of Combination of Two Edible Mushrooms of *Ganoderma lucidum* and *Pleurotus ostreatus*

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ABSTRACT

Edible mushrooms such as *Ganoderma lucidum* and *Pleurotus ostreatus* are of great significance in traditional medicine. This study evaluated the *in vitro* antioxidant and α-amylase inhibition potentials of the hydro-methanol extracts of the fruiting bodies of *Ganoderma lucidum* (GL), *Pleurotus ostreatus* (PO) and their combination (GL+PO). The extracts were obtained by hydro-methanol (1:40) extraction. The extracts showed a dose-dependent antioxidant and α-amylase inhibitory activities. The combined extracts (GL+PO) exhibited higher antioxidant and α-amylase inhibitory activities than that shown by the individual extract. The combination of GL and PO exhibited maximum (98.65%) antioxidant activity at the highest concentration. GL extract showed the highest amount of total flavonoid (6.58 mg quercetin Eq/g) and total phenolic content (13.67 mg catechol Eq/g) and strong DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging (24.58 µg/mL), superoxide scavenging (652.39 µg/mL), and hydroxyl radical scavenging (555.43 µg/mL) activities while PO exhibited 26.36 µg/mL, 775.65 µg/mL, and 769.23 µg/mL, correspondingly. The combination of GL and PO showed the highest free radical scavenging capabilities (15.01 µg/mL, 495.59 µg/mL, and 312.85 µg/mL for DPPH radical scavenging, superoxide scavenging and hydroxyl radical scavenging activities, respectively). Methanol extracts of mushroom, GL, PO, and GL+PO showed a dose-dependent enzyme inhibitory potential against α-amylase activity with $IC_{50}$ of 386.04 µg/mL for GL, 391.74 µg/mL for PO, and 363.07 µg/mL for GL+PO, while that of acarbose was 269.15 µg/mL. The results indicate that the combination of *Ganoderma lucidum* and *Pleurotus ostreatus* extracts has significantly higher antioxidant and alfa-amylase inhibitory activities compared to the single individual extract.

***Keywords:*** Free radical, Edible mushrooms, *α*-amylase, Cancer, Oyster.

## Introduction

Edible mushrooms have many beneficial components which make them healthy food for human and their use as medicine for more than 5000 years.[1-3] About 80 species of Ganoderma are available growing on rotten trees and the common species is *Ganoderma lucidum*. It has been used as medicine for more than 2000 years in the treatment of cancer, diabetes and other pathological conditions.[4] The use of *Ganoderma lucidum* for the treatment of many diseases are based on laboratory and preclinical studies. It has been found to be beneficial to cancer patients.[5] On the other hand, Pleurotus comprises about 40 species and are commonly called "oyster mushroom" They are propagated widely in humid areas. *Pleurotus ostreatus* is considered as a special mushroom due to its high nutritional and medicinal properties.[6]

Free radicals have been implicated as a significant contributory factor in the development of degenerative diseases such as cancer, organ toxicity (hepatotoxicity) and so on. Phytoconstituents, mainly polyphenol and flavonoids have been shown to possess free radicals scavenging activity or as reducer of singlet oxygen and thus delay or inhibit oxidative stress.[7] *Ganoderma lucidum* and *Pleurotus ostreatus* are rich in polyphenols, flavonoids and vitamins which are responsible for their antioxidant potential.[8-9] Jayakumar *et al.* (2009) reported the concentration-dependent antioxidant activity of *G. lucidum* in mouse heart homogenate.[10] On the other hand, *Pleurotus ostreatus* has been shown to be a potential antioxidant agent *in vitro* and *in vivo* by exhibiting free radical scavenging activity by quenching the singlet oxygen and by augmenting the antioxidant role of enzyme and non-enzyme antioxidants.[9] Besides, the antioxidant activity of *Ganoderma lucidum* and *Pleurotus ostreatus*, in this present study have been documented with the screening of antioxidant and anti-α-amylase activity of the combinations of *G. lucidum* and *P ostreatus* extracts.

Anti-*α*-amylase activity of mushrooms are implicated in the prevention of secondary health complications of diabetes (nephropathy, retinopathy, hypertension and neuropathy).[11] It has been shown that the antioxidant activity of medicinal mushrooms are dependent on their total phenolic content and *Ganoderma lucidum* have been found to exhibit high free radical scavenging activity which also showed a positive correlation to their phenolic content.[12]

The aim of this study was to screening and compare the antioxidant and α-amylase inhibitory activities of the hydro-methanol extracts of *Ganoderma lucidum*, *Pleurotus ostreatus*, and their combination.

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## Materials and Methods

*Mushrooms collection and Preparation of extracts*
Edible mushrooms, *Ganoderma lucidum* and *Pleurotus ostreatus* were collected from the Mushroom Development Institute, Department of Agriculture Extension, Ministry of Agriculture, Bangladesh. Mushrooms used in this study were identified and authenticated by Scientific Research Officer from Mushroom Development Institute, Bangladesh. About 250 g powder of each mushroom was macerated in 80% methanol and kept at 25ºC for 7 days. Extracts were collected, concentrated, dried and stored in the refrigerator for future use.

*Phytochemicals screening*
The qualitative and quantitative determination of total phenol, flavonoids content and phytochemical screening were carried out according to standard method.[13-15]

*Determination of total phenol content*
The total phenol content of the mushrooms was determined spectrophotometrically, using catechol as standard.[16] Briefly, 1.0 mL of the diluted extract was transferred into three separate test tubes containing 5.0 mL of diluted Folin-Ciocalteu's reagent (1:10). Subsequently, 4.0 mL $Na_2CO_3$ solution (7.5%) was added into all test tubes and were allowed to stand for 1 h at 25ºC. Thereafter, the absorbance was measured at 765 nm against a blank. Total phenol content was expressed as mg catechol equivalent / g extract.

*Determination of total flavonoid content*
The aluminum chloride method[17] was used to determine the total flavonoid content of the mushroom extracts. Briefly, 50 µL of crude extract (1 mg/mL) was made up to 1 mL with methanol and was mixed with 4 mL distilled water. After 5 min incubation, 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% $AlCl_3$ was added and was allowed to stand for 5 min. Subsequently, 2 mL of 1M NaOH was added and the volume of the reaction mixture was made up to 10 mL with distilled water. After 15 min of incubation, the absorbance of the resulting solution was measured at 510 nm. Quercetin was used as a standard for generating the calibration curve. The total flavonoid content was calculated from the equation of the curve and results were expressed as mg Quercetin equivalent per g dry weight of extract.

*In vitro antioxidant activity screening*
Appropriate amount of each sample was dissolved in 95% methanol to make a concentration of 1 mg/mL and different concentrations were prepared by serial dilution technique.

*DPPH radical scavenging*
Triplicate reaction mixture which comprised 50 µL of each mushroom extract and 5.0 mL of DPPH solution (0.04% w/v in ethanol) followed by 80% ethanol as a blank preparation.[18] All test tubes were shielded by Aluminum foil and after 30 minutes of incubation, absorbance of the reaction mixture was measured at 517 nm. DPPH scavenging effect was determined using the following equation;

DPPH scavenging effect (%) = (Ao-As)/Ao x 100

Where Ao was the absorbance of control, As was the absorbance of selected sample.

BHT was used as the positive control and for comparison with each sample's activity. The effective concentrations that exhibit 50% of free radical scavenging activity were determined using standard curve ($r^2$=0.9994).

*Superoxide Radical Scavenging*
This was done according to the method previously described by Villaño *et al* (2007).[19] The reaction mixture comprised of equal volume of 936 µM NADH, 120 µM PMS, sample extracts, and 300 µM NBT and final volume made up to 1 mL with 100 mM buffer solution (pH 7.4). After 5 min of reaction incubation at 25ºC, absorbance of each solution was recorded at 560 nm. The superoxide radical scavenging was determined by the following formula;

Percent effect of superoxide radical scavenging = (1-As/Ao) x 100

where As was the absorbance of sample and Ao the absorbance of control.
Ascorbic acid was used as the as the positive control, for evaluating sample's radical scavenging activity. Trend equation of % scavenging effect of samples and standard was used to calculate the effective concentration ($EC_{50}$) provided that 50% radicals scavenging by samples. The $EC_{50}$ value was expressed as mg/mL.

*Hydroxyl radical scavenging*
The experiment was done according to the method of Klein *et al*.[20] The reaction mixture comprised 1 mL Fe-EDTA solution, prepared by 0.13% ferrous ammonium sulfate in 0.26% EDTA, 0.5 mL of 0.018% EDTA and 1 mL DMSO solution prepared by mixing 0.85% DMSO in phosphate buffer saline (pH 7.4) including appropriate volume of samples and standards. Termination of this reaction was done by applying 1 mL ice cold TCA and subsequently, 3 mL Nash reagent was added as well as incubated for 15 minutes. The absorbance of this reaction mixture was measured at 412 nm with spectrophotometer. Gallic acid was used as a standard and the experiment was repeated in the absence of EDTA. The difference between two readings at selected concentration was recorded and the hydroxyl radical scavenging activity was calculated by the following formula;

Hydroxyl radical scavenging (%) =1-(difference in absorbance of sample/ difference in absorbance of blank) x 100.

*Alpha-amylase inhibitory activity study*
The α-amylase inhibition was measured by the method described earlier.[21] In brief, triplicate volume of 250 µL of sample extracts was taken in test tube and followed by 250 µL of $Na_2PO_4$ buffer (0.02 M, pH 6.9) comprising alpha-amylase solution (0.5 mg/mL). After 10 minutes incubation at room temperature, 250 µL of 1% starch solution in $Na_2PO_4$ buffer (0.02 M, pH 6.9) was taken and was incubated for 10 minutes again at 25ºC. Finally, 500 µL of DNS solution was added to the reaction mixture to terminate reaction.
After incubation for 5 minutes in boiling water and cooling to 25ºC, the reaction solution was diluted with distilled water (5 mL) and the absorbance was measured at 540 nm. Using the same technique, control was prepared by replacing the extract with distilled water. Enzyme inhibition was calculated as percentage inhibition according to the formulae;

% inhibition = (Ac-As)/Ac x 100

Where Ac was the absorbance of control and As was the absorbance of sample

Inhibitory concentration ($IC_{50}$) comprising 50% alfa-amylase inhibition was calculated from trend line equation ($r^2$=0.997).

*Statistical Analysis*
GraphPad Prism 6 (Pad Software, Inc., USA) was used to analyze data, and results are expressed as mean ± SD.

## Results and Discussion

The results revealed the presence of alkaloids, flavonoids, polyphenols, saponin, steroids, coumarins, terpenoids, vitamin C, cardiac glycosides, and anthocyanin in the selected mushrooms extracts (Table 1). The extract of *Ganoderma lucidum* had a significantly ($p < 0.05$) higher total phenol and flavonoid content (13.673±0.238 mg Catechol Eq/g and 6.580 ± 0.211 mg Quercetin Eq/g, respectively) compared to *Pleurotus ostreatus* mushroom extract (Table 2). Figure 1 shows the dose-dependent curve of DPPH radical scavenging effects of methanol extracts of *G. lucidum* and *P. ostreatus* and their combination (GL + PO), compared with Butylated hydroxytoluene (BHT). It was observed that the antioxidant activities of GL, PO, GL+PO, and BHT were recorded as 48.31 - 96.71%, 48.12 - 96.23%, 53.23 - 98.65%, and 53.12 - 95.74%, respectively at a concentration of 31.25 - 500 µg/mL. The extracts showed a dose-dependent radical scavenging activity and the combination of GL and PO exhibited maximum antioxidant activity compared to BHT ($p < 0.05$). The results of DPPH radical scavenging effect of GL (and BHT), PO (BHT), and their combination, GL+PO (and BHT) are shown in figure 1A, 1B, and 1C, respectively, this clearly

indicates the concentration-dependent DPPH radical scavenging activity of the selected edible mushrooms. The $EC_{50}$ value (Table 3) of GL was 24.58 µg/mL ($R^2$ = 0.9387) whereas that of PO was 26.36 µg/mL ($R^2$ = 0.9569) and GL+PO had $EC_{50}$ value of 15.014 µg/mL ($R^2$ = 0.8538) which was lower in comparison to BHT (16.11 µg/mL, $R^2$ = 0.8832). At 500-3000 µg/mL, the superoxide scavenging activity of GL, PO, and GL+PO were 44.12-96.03%, 42.04-82.07%, and 51.08-97.02% respectively and that of ascorbic acid was 50.20-88.03%. The superoxide scavenging activity of *G. lucidum* and *P. ostreatus*, combination and ascorbic acid is shown in Figure 2. The order of $EC_{50}$ values in superoxide scavenging activity were GL+PO > GL > PO (Table 3). The $EC_{50}$ of combination of GL and PO was found to be low. Besides, GL and PO ($EC_{50}$ 625.39 µg/mL and 775.69 µg/mL, respectively) acts as powerful superoxide anion scavenger. The potential of the mushroom extracts and gallic acid to inhibit hydroxyl radical was assessed at a concentration of 500-3000 µg/mL. Results are shown in Figure 3. The samples showed maximum activity at 3000 µg/mL, indicating that the hydroxyl radical scavenging activity were in a dose-dependent manner. The $EC_{50}$ values of hydroxyl radical scavenging by GL, PO, and GL+PO were 555.43 µg/mL, 769.23 µg/mL, and 312.85 µg/mL, respectively (Table 3). The α-amylase inhibitory potential of GL, PO, and GL+PO was investigated in this study. Acarbose (standard sample) was used without mushrooms extract and results obtained were compared with the test samples (500 - 2000 µg/mL). Figure 4 illustrates the enzyme inhibition (percent inhibition) of methanol extracts of *Ganoderma lucidum* and *Pleurotus ostreatus* with their combination (GL+PO) as well as acarbose. Results obtained revealed that the enzyme inhibition activity of the mushroom extracts was in a concentration-dependent and the combination (GL+PO) were comparatively more effective than GL and PO, respectively (Figure 4A and B). The GL extracts at 2000 µg/mL showed 88.5% of enzyme inhibition with an $IC_{50}$ value of 389.04 ± 1.45 µg/mL, while the $IC_{50}$ value of PO was 391.74 ± 2.11 µg/mL (Table 4). Although, in comparison to standard acarbose ($IC_{50}$ value was 269.15 ± 2.03) the $IC_{50}$ values of GL, PO, and GL+PO were significantly low ($p < 0.05$). In spite of this, the selected mushroom samples showed potential α-amylase inhibitory activity.

Antioxidants have the ability to defend the body against oxidative stress, which result as a consequence of an unevenness between free radical production and antioxidant defense.[22] Three *in vitro* antioxidant assays were performed for the mushrooms extract at different concentrations. These showed significant antioxidant activity compared with the positive controls (BHT, Vitamin C, and Gallic acids). The antioxidant properties of natural phytocompounds has been tested widely utilizing DPPH radicals scavenging. From this study, it could be concluded that the combination of GL and PO show stronger antioxidant activity than their single preparation (GL or PO). The antioxidant potency of mushrooms, especially for the combination of GL and PO was also evaluated by superoxide radical scavenging and hydroxyl radical scavenging tests. Superoxide radical is measured as one of the major reactive oxygen species in biological system, which contribute to the generation of oxidative stress.[23] The results of this study revealed that GL ($EC_{50}$ 652.39 µg/mL), PO ($EC_{50}$ 775.65 µg/mL), and their combination (GL+PO, $EC_{50}$ 495.59 µg/mL) had potential of foraging for superoxide radical which could be associated with their flavonoid content (Tables 2 and 3). Another potent reactive oxygen species is hydroxyl radical, produced from hydrogen peroxide in the biological system, which reacts with phospholipids in cell membrane resulting in cell damage.[24] Hydroxyl radical is recognized as an injurious molecules in metabolic processes and detrimental to biological system and contributes to cytotoxicity.[25] Low concentration levels of hydrogen peroxide ($H_2O_2$) are present in air, water, human body, plants, foods and microorganisms. In biological system, catalase rapidly decompose hydrogen peroxide into oxygen and water and the decomposition of hydrogen peroxide may generate hydroxyl radicals (OH), which can enhance lipid peroxidation and cause DNA damage.[26]

**Table 1:** Phytochemical Screening of *Ganoderma lucidum* and *Pleurotus ostreatus*

| **Phytochemical** | *Ganoderma lucidum* | *Pleurotus ostreatus* |
|---|---|---|
| Alkaloids | + | + |
| Flavonoids | + | + |
| Polyphenols | + | + |
| Saponin | + | - |
| Steroids | + | + |
| Coumarins | + | - |
| Terpenoids | + | + |
| Vitamin C | + | + |
| Cardiac Glycosides | + | + |
| Anthocyanin | + | - |

"+" present and "-"absence

**Table 2:** Total polyphenol content (TPC) and total flavonoids content (TFC) of *Ganoderma lucidum* and *Pleurotus ostreatus* (mg/g dry samples).

| **Sample** | **TPC (mg Catechol Eq/g)** | **TFC (mg Quercetin Eq/g)** |
|---|---|---|
| *Ganoderma lucidum* | 13.673 ± 0.238** | 6.580 ± 0.211* |
| *Pleurotus ostreatus* | 5.101 ± 0.534 | 3.261 ± 0.132 |

Legends: TPC; Total Phenol Content, TFC; Total Flavonoid Content, *p < 0.05, **p < 0.01, Values are mean ± SD. The concentration of total phenol content in mushrooms was derived from a standard curve of Catechol ($r^2$ = 0.9987).

**Table 3:** $EC_{50}$ values (µg/mL) of radical scavenging activity.

| **Sample** | **DPPH radical** | **Superoxide radical** | **Hydroxyl radical** |
|---|---|---|---|
| GL | $24.58 \pm 1.54^b$ | $652.39 \pm 2.01^b$ | $555.43 \pm 1.28^c$ |
| PO | $26.36 \pm 2.09^b$ | $775.65 \pm 1.22^c$ | $769.23 \pm 2.00^c$ |
| GL+PO | $15.01 \pm 1.23^a$ | $495.59 \pm 3.06^c$ | $312.85 \pm 2.98^b$ |
| BHT | $16.11 \pm 1.53^a$ | - | - |
| Vitamin C | - | $562.10 \pm 2.36^a$ | - |
| Gallic Acid | - | - | $491.15 \pm 3.00^a$ |

Each value is presented as mean ± SD (n=3). Values followed by different letters (a-c) are significantly different ($p<0.05$). The same letter indicates the non-significance of mean difference ($p>0.05$). – Not determined.

**Table 4:** The $IC_{50}$ (µg/mL) values of enzyme inhibition.

| **Sample** | **$IC_{50}$ (µg/mL)** |
|---|---|
| Acarbose | $269.1534 \pm 2.03^b$ |
| *Ganoderma Lucidum* | $389.0451 \pm 1.45^b$ |
| *Pleurotus ostreatus* | $391.7418 \pm 2.11^b$ |
| *Ganoderma lucidum + Pleurotus ostreatus* | $363.0783 \pm 1.28^a$ |

Each value is presented as mean ± SD (n=3). Values followed by different letters are significantly different ($p < 0.05$).

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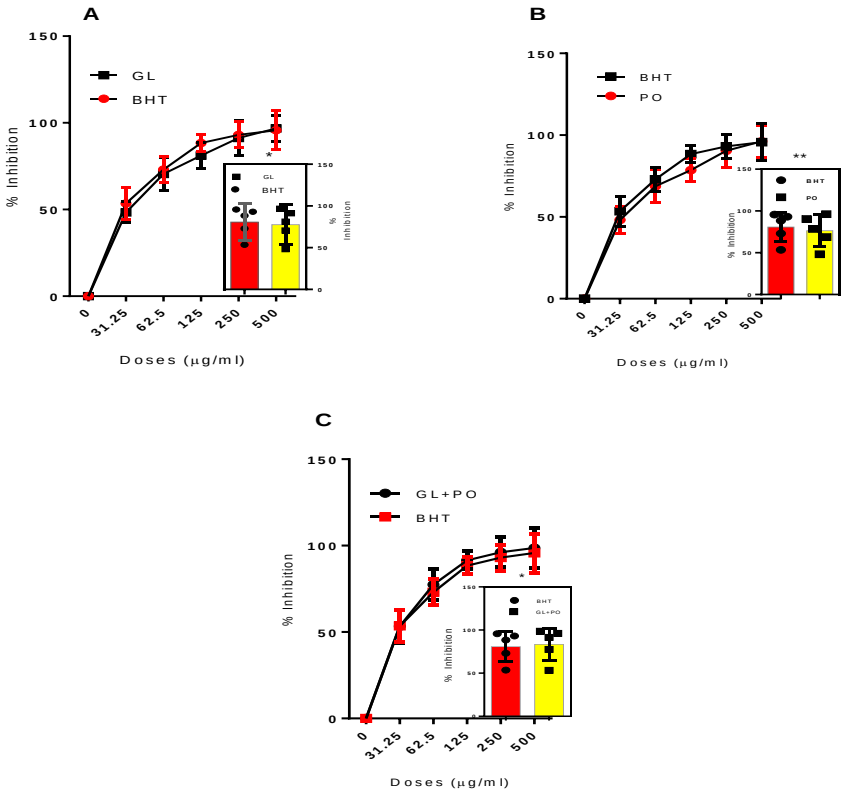


**Figure 1:** Antioxidant activities of methanol extracts of *Ganoderma lucidum* and *Pleurotus ostreatus* with their combination (GL+PO). It was measured based on the % of DPPH radical scavenging at assigned concentrations. GL- *Ganoderma lucidum*; PO- *Pleurotus ostreatus*; BHT- Butylated hydroxytoluene; *p<0.05. Values are expressed as mean ± SD (n=3); DPPH - 1,1-diphenyl-2-picrylhydrazyl

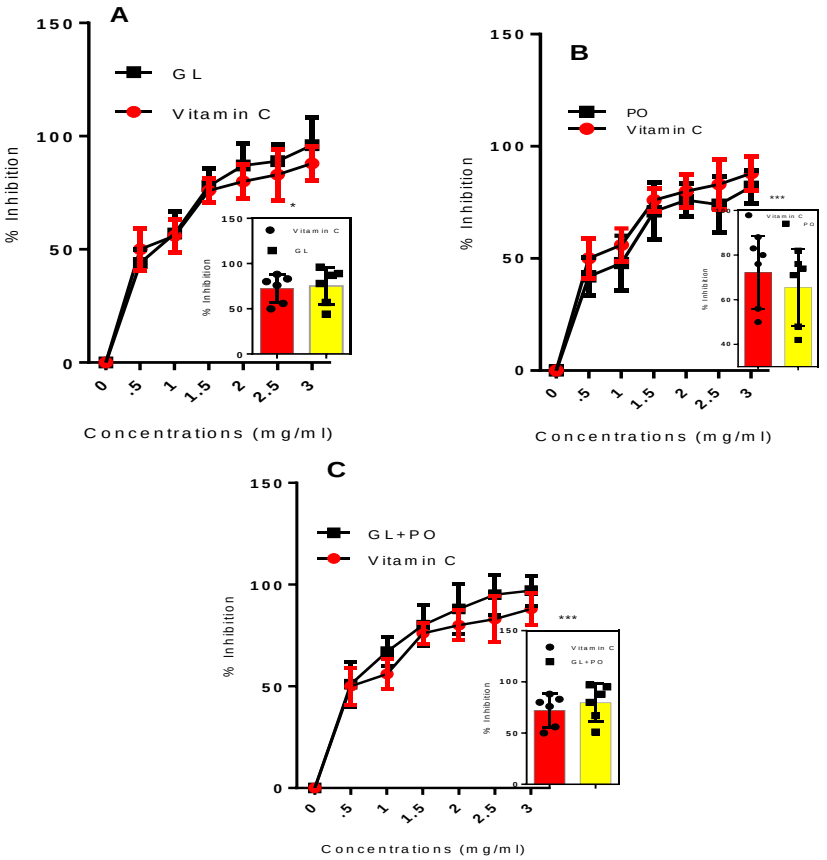


**Figure 2:** Superoxide scavenging activity of the extracts of GL, PO, and combination of GL and PO as well as Ascorbic acid. GL- *Ganoderma lucidum*; PO- *Pleurotus ostreatus*; *p < 0.05. Values are expressed as mean ± SD (n=3).

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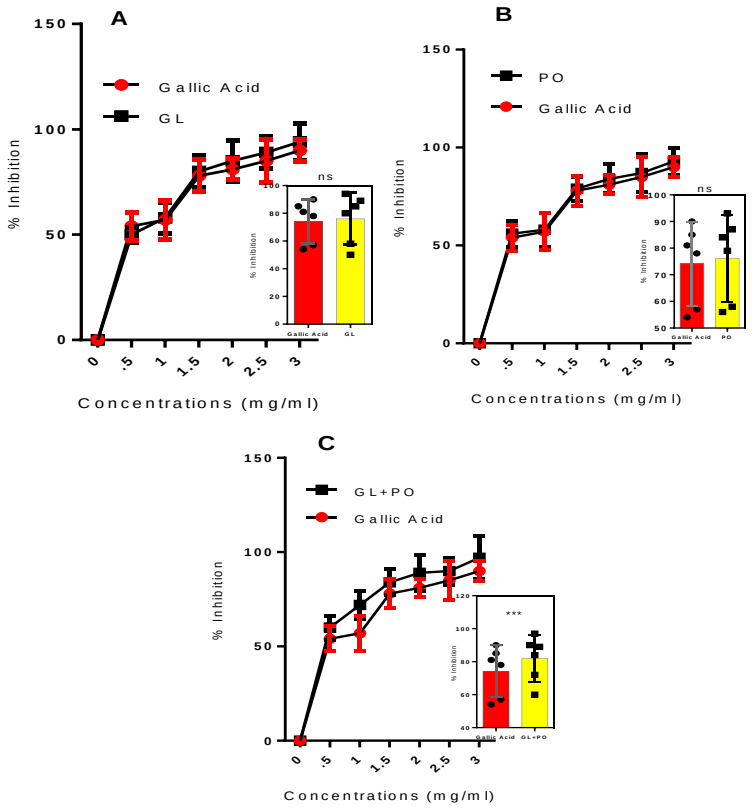


**Figure 3:** Hydroxyl radical scavenging activity by the extracts of *Ganoderma lucidum* and *Pleurotus ostreatus*, compared with Gallic acid. GL- *Ganoderma lucidum*; PO- *Pleurotus ostreatus*; *p < 0.05. Values are expressed as mean ± SD (n=3).

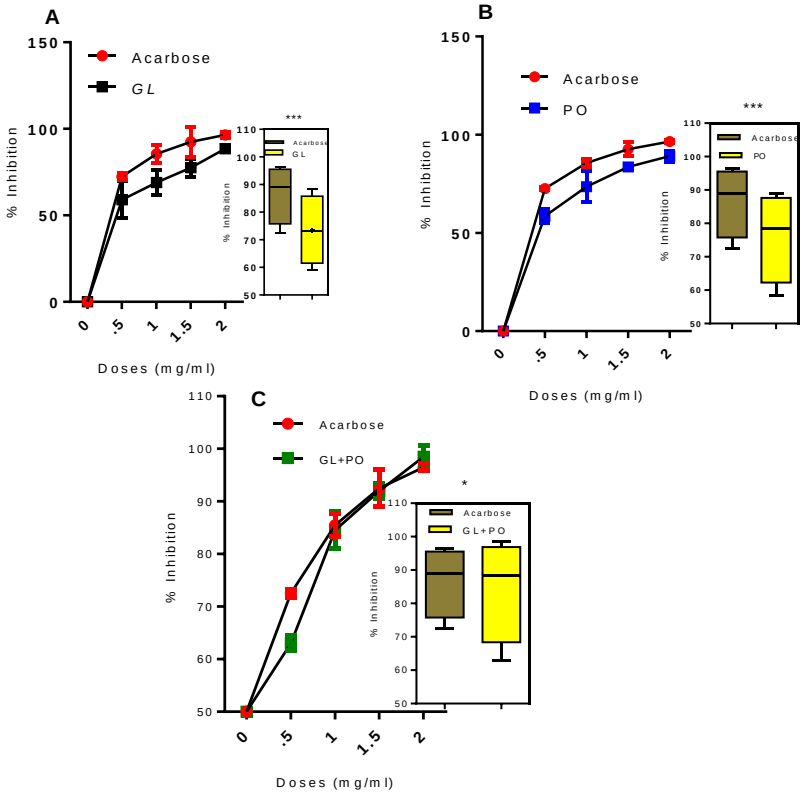


**Figure 4:** percentage α-amylase inhibition vs different concentrations of (A) *Ganoderma lucidum* [GL], (B) *Pleurotus ostreatus* [PO], and (C) combination of GL and PO. Yellow box plots represent the enzyme inhibition capabilities of mushrooms compared to acarbose.

Methanol extracts of the mushrooms efficiently scavenged hydroxyl free radical and the measured average $EC_{50}$ value of GL+PO was 312.85 µg/mL (555.43 µg/mL for GL and 769.23 µg/mL for PO) and 491.15 µg/mL for Gallic acid. It has been reported that phenolic compounds are associated with free radical scavenging and that they play a vital role in preventing lipid peroxidation.[23] Here, it can be concluded that the presence of polyphenols and flavonoids may be responsible for the antioxidant activity of the mushrooms extract. The α-amylase inhibitory activity of the mushrooms was explored in the present study. Enzyme inhibition by methanol extract of *Ganoderma lucidum* and *Pleurotus ostreatus*, and their combination (GL+PO) was scrutinized at different concentrations ranging from 500 to 2000 µg/mL. Among the three mushroom extracts, combination of *Ganoderma lucidum* and *Pleurotus ostreatus* was appreciably more effective than that of the extract of *Ganoderma lucidum* and *Pleurotus ostreatus* alone in inhibiting α-amylase. At high concentration, 2000 µg/mL, GL+PO showed 2.07 % more α-amylase inhibitory potential than that of acarbose as well as GL ($IC_{50}$ 389.04 ± 1.45 µg/mL) and PO ($IC_{50}$ 391.74 ± 2.11) which exhibited 11.36% and 8.41% less α-amylase inhibition in comparison to GL+PO ($IC_{50}$ 363.07 ± 1.28 µg/mL). Antiamylase potential of edible mushrooms tested in this study has shown their potential role in the control of blood glucose levels in the early treatment of diabetes mellitus. So, the prevention of carbohydrate absorption, which is assisted by the inhibition of enteric enzymes including α-amylase after food intake is the therapeutic approach for treating diabetes mellitus.

## Conclusion

The findings from this study has shown the antioxidant, and α-amylase inhibitory activities of *Ganoderma lucidum*, *Pleurotus ostreatus*, and their combination. The findings therefore necessitate further comprehensive studies into the fundamental mechanism responsible for their antioxidant and α-amylase inhibitory activities.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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