IPS Journal of Natural Products
IPS J. Nat. Prod., 1(1): 19-25 (2025)
DOI: https://doi.org/10.54117/ijnp.v1i1.35

In Vitro Antioxidant and Free Radical Scavenging Activities of *Pleurotus tuber-regium*

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Abstract

This study evaluated the *in vitro* antioxidant potential of the methanol extract of *Pleurotus tuber-regium* (PT) using a range of established spectrophotometric assays. These included ferric reducing antioxidant power (FRAP), nitric oxide scavenging activity, ABTS radical scavenging activity, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, metal ion chelating activity, and hydrogen peroxide scavenging activity. The results revealed that the extract exhibited concentration-dependent antioxidant activities across all assays. Although the scavenging effects of the extract were consistently lower than those of the standard antioxidant, gallic acid, it demonstrated notable efficacy, particularly in metal ion chelation, with maximum inhibition observed at 40 mg/ml. The extract also showed moderate reducing power and the ability to scavenge various free radicals. These findings suggest that *P. tuber-regium* possesses significant antioxidant properties and could serve as a promising source of natural antioxidants. Therefore, it holds potential for development into plant-based pharmaceutical or nutraceutical agents for managing oxidative stress-related disorders.

Keywords: *In vitro antioxidants, P. tuber-regium, free radicals, oxidative stress*

Article History
Received: 25 Apr 2025
Accepted: 20 May 2025
Published: 25 May 2025

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How to cite this paper: Ilechukwu, C. C., Nwaka, A. C., Ifemeje, J. C., Ezenwelu, C. O., Ilechukwu, J. N., Ekesiobi, A. O., Lukong, C. B., Okechukwu, A. U., & Iheukwumere, I. H. (2025). In Vitro Antioxidant and Free Radical Scavenging Activities of Pleurotus tuber-regium. *IPS Journal of Natural Products*, *1*(1), 19–25. https://doi.org/10.54117/ijnp.v1i1.35

Introduction

Free radicals are very reactive molecules or atoms that contain unpaired electrons, making them unstable and capable of damaging cellular components (Fletcher *et al.*, 2023). Free radicals can interact with and damage various biological macromolecules, including proteins, lipids, and DNA. They are produced endogenously through normal metabolic processes, such as electron transport chain, oxidative phosphorylation, and mitochondrial respiration, and exogenously from environmental factors like pollution, smoking, poor nutrition, drugs, certain chemicals, radiation exposure and from industrial effluents (Ilechukwu *et al.*, 2014, Ilechukwu *et al*., 2025a, Ilechukwu *et al*., 2025b, Ilechukwu *et al.*, 2025c). While free radicals play a role in normal physiological functions, excessive production can contribute to pathological conditions, including cancer (Harrison *et al.*, 2024). Although our bodies have antioxidant defense mechanisms capable of neutralizing free radicals, an imbalance between their production and neutralization leads to oxidative stress, a key factor in the development of neurodegenerative, cardiovascular and metabolic diseases (Ilechukwu *et al*., 2014). Oxidative stress is the imbalance

between the production of free radicals and the body's ability to neutralize these with its antioxidants defense mechanism. This imbalance leads to accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which cause damage to cellular components such as lipids, proteins, and DNA (Ilechukwu *et al*., 2014, Ilechukwu *et al*., 2025a, Ilechukwu *et al*., 2025b, Ilechukwu *et al*., 2025c). Antioxidants are molecules that prevent oxidative damage by neutralizing free radicals and maintaining cellular integrity (Jackson *et al.*, 2023). Antioxidant defense mechanisms are essential for protecting cells from oxidative stress and maintaining cellular homeostasis (Akinyemi *et al*., 2020). These defense systems neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS), preventing damage to lipids, proteins, and DNA (Allen *et al*., 2019).

Mushrooms for example *P. tuber-regium* just like plants produce secondary metabolites or phytochemicals which possess antioxidant properties. Numerous studies done in the past thirty years have shown that these phytochemicals play an important role in preventing chronic diseases like cancer, diabetes, coronary heart disease, viral and bacterial diseases (Egbuna *et al*., 2025a; Egbuna *et al*., 2025b; Iheukwumere *et al.*, 2025a; Iheukwumere *et al.*, 2025b). Plants, herbs, spices and mushrooms contain phyto-nutrient or phytochemicals which possess antioxidant activity, antiviral activity and antibacterial activity (Okpe *et al.*, 2012; Anameze *et al*., 2023; Ekesiobi *et al*., 2025a; Ekesiobi *et al.*, 2025b; Ekesiobi *et al*., 2025c; Iheukwumere *et al.*, 2025c; Iheukwumere *et al.*, 2025d, Iheukwumere *et al.*, 2025e). Plants, mushrooms, herbs and spices have been demonstrated to possess significant antioxidant properties in various studies suggesting its potential protective ability against oxidative stress and therefore play an important role in the chemo-prevention of diseases that have their etiology, progression and pathophysiology in reactive oxygen species (Ilechukwu *et al*., 2014; Ilechukwu and Okafor; Okolo *et al.*, 2017; 2020; Ilechukwu, 2022; Ifemeje *et al.*, 2025; Ilechukwu *et al*., 2025a; Ilechukwu *et al*., 2025b; Ilechukwu *et al*., 2025c).

Pleurotus tuber-regium, the king tuber mushroom, is an edible gilled fungus native to the tropics including Africa, Asia and Australasia (Oso, 1997). It has been shown to be a distinct species incapable of cross breeding and phylogenetically removed from other species of *Pleurotus* (Vilgalys *et al*., 1996). Medicinal mushrooms are known to contain several phytoactive constituents which are mainly secondary metabolites like flavonoids, tannins, alkaloids, phenols etc (Okolo *et al.*, 2017). *Pleurotus tuber-regium* is a saprotroph found on dead wood, including Daniellia trees in Africa (Okhuoya, 1990). As fungus consumes the wood, it produces a sclerotium, or storage tuber, either within the decaying wood or in the underlying soil. These sclerotia are round, dark brown with white interiors, and up to 30cm wide. The fruiting bodies then emerge from the sclerotium and the fruiting bodies are edible (Oso, 1997). In addition to being saprotrophic, *P. tuber-regium* is also nematophagous, catching nematodes by paralyzing them with a toxin (Hibbett, 1994*). Pleurotus tuber-regium* has a history in Africa as food and as a medicinal mushroom (Isikhuemhen, 2004).

Materials and Methods

Materials

Chemicals and reagents

All assay kits used was purchased from Randox Laboratories Ltd Ardmore, UK Chemical and reagents used was purchased from Sigma Chemical Company St. Louis U.S.A and was of analytical grade.

Macrofungi (mushroom) collection and identification

Pleurotus tuber-regium was purchased at Eke Awka market Awka and was identified by a taxonomist Prof. G. C. Ukpaka in the herbarium unit of Biological Science department Chukwuemeka Odumegwu Ojukwu University Uli.

Extraction of phytochemicals

1g of sample was weighed and transferred in a test tube and 15ml ethanol and 10ml of 50%m/v potassium hydroxide was added. The test tube was allowed to react in a water bath at 60^0c for 60mins. After the reaction time, the reaction product contained in the test tube was transferred to a separating funnel. The tube was washed successfully with 20ml of ethanol, 10ml of cold water, 10ml of hot water and 3ml of hexane, which was all transferred to the funnel. This extracts were combined and washed three times with 10ml of 10%v/v ethanol aqueous solution. The solution is dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1000ul of pyridine of which 200ul was transferred to a vial for analysis.

Determination of in-vitro antioxidant activity of *Pleurotus tuber-regium*

The in vitro antioxidant capacity of the samples was determined using the following methods (DPPH) radical scavenging activity assay, ferric reducing antioxidant potential assay (FRAP) and hydroxyl radical scavenging assay (HRSA) and nitric oxide scavenging assay.

DPPH Spectrophotometric Assay

The 1,1 Diphenyl 2-picryl Hydrazyl (DPPH) Radical scavenging activity assay was performed according to the method described by Mensor *et al*. (2001)

Reagents

1. DPPH – 2,2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

The leaf samples (20µl) were added to 0.5ml of 0.1mM methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the sample, served as the positive control while butylated hydroxytoluene (BHT) served as reference. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer (Genesys 10-S, USA). The radical scavenging activity was calculated as follows:

$$\text{Scavenging activity \%} = \frac{\text{100-A518 (sample) - A518 (blank)} \times 100}{\text{A518 (blank)}}$$

Ferric Reducing Antioxidant Property Assay (FRAP)

Ferric ions reducing power was measured according to the method of Pulido *et al*. (2000).

Principle

The principle of the assay is the quantification of ferric degradation product, by its condensation with the extract

Procedure

An aliquot, 0.25 ml of the extracts was mixed with 0.25 ml of 200mM Sodium phosphate buffer pH 6.6 and 0.25 ml of 1% Potassium ferrocyanide. The mixture was incubated at 50oC for 20 min, thereafter 0.25 ml of 10% trichloroacetic acid was added and centrifuged at 2000 rpm for 10 min, 1 ml of the supernatant was mixed with 1 ml of distilled water and 0.2 ml of ferric chloride and the absorbance was measured at 700 nm.

Measurement of Hydroxyl Radical Scavenging Activity

The extent of hydroxyl radical scavenging from Fenton reaction was quantified using 2'-deoxyribose oxidative degradation as described by Kunchandy and Rao (1990).

Principle

The principle of the assay is the quantification of 2'-deoxyribose degradation product, malondialdehyde, by its condensation with thiobarbituric acid.

Reagents

1. Deoxyribose (2.8mM)
2. Ferric chloride (0.1mM)
3. EDTA (0.1mM)
4. H_2O_2 (1mM)
5. Ascorbate (0.1mM)
6. KH_2PO_4-KOH buffer (20mM, pH 7.4)
7. Thiobarbituric acid (1%)

Procedure

The reaction mixture contained 0.1ml of deoxyribose, 0.1ml of FeCl3, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH2PO4-KOH buffer and 20µl of sample samples in a final volume of 1.0ml. The mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1.0 ml of TBA was added and heated at 95°C for 20 minutes to develop the colour. After cooling, the TBARS formation was measured spectrophotometrically (Genesys 10-S, USA) at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the -absorbance of the control with that of the samples. The per cent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative per cent TBARS was calculated for the sample treated groups.

Chelation Power on Ferrous (Fe^{2+}) Ions

The chelating effect on ferrous ions of the prepared extracts was estimated by the method of Dinis with slight modifications. Briefly, 100 µL of each test sample (1 mg/mL) was taken and raised to 3 mL with methanol. 740 µL of methanol was added to 20 µL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 µL of 5 mM ferrozine into the mixture, which was then left at room temperature for 10 min and then the absorbance of the mixture was determined at 562 nm.

Measurement of Nitric Oxide Scavenging Activity

The extent of inhibition of nitric oxide radical generation *in vitro* was followed as per the method reported by Green *et al*. (1982). Sodium nitroprusside in aqueous solution, at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that are estimated spectrophotometrically at 546nm. The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of leaf samples (50mg) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the samples. The absorbance was read at 546nm against the reagent blank, in a spectrophotometer (Genesys 10-S, USA).

ABTS Radical Scavenging Activity Measurement

Principle

The ABTS assay is a common method used to evaluate the antioxidant activity of plant extracts. It measures the ability of antioxidants in the extract to scavenge the ABTS radical cation, which is a blue-green chromophore. The decolorization of this chromophore, measured spectrophotometrically, is an indicator the antioxidant capacity of the extract. The reaction is based on the ability of antioxidants to donate a hydrogen atom or an electron to neutralize the ABTS•+ radical, leading to decolorization.

Procedure

The diluted ABTS•+ solution was mixed with the plant extract solution and the mixture is incubated for a set time (e.g., 6-30 minutes). After incubation, the absorbance of the solution is measured spectrophotometrically, typically at 734 nm. The percentage inhibition of the ABTS•+ radical is calculated using a formula that compares the absorbance of the reaction mixture with a control (ABTS•+ solution without extract). A higher percentage of inhibition indicates a greater antioxidant activity

Statistical Analysis

The data obtained was expressed as mean of three determinations ± SD. Statistical analysis was carried out using the statistical package for social sciences (SPSS). The data was analyzed using oneway analysis of variance (ANOVA).

Results

The antioxidant activities of *Pleurotus tuber-regium* extract were evaluated through various in vitro assays. The nitric oxide scavenging activity showed a slight concentration-dependent increase but remained significantly lower than that of gallic acid, which exhibited a strong dose-dependent response (Figure 1). Similarly, the ABTS radical scavenging activity of the extract increased gradually with concentration, though gallic acid consistently demonstrated superior scavenging potential at all levels tested (Figure 2). The hydroxyl radical scavenging activity also rose with increasing concentrations of the extract but remained lower than the activity observed for gallic acid (Figure 3). A comparable trend was seen in the DPPH assay, where the extract exhibited a concentration-dependent increase in radical scavenging but still fell short of the standard (Figure 4). In terms of ferric reducing antioxidant power (FRAP), the extract showed moderate antioxidant potential, with maximum activity at 40 mg/ml, though not reaching the level of gallic acid (Figure 5). Interestingly, the extract demonstrated strong metal ion chelating activity, with the highest inhibition recorded at 40 mg/ml (Table 1), suggesting its potential in mitigating oxidative stress through metal chelation.

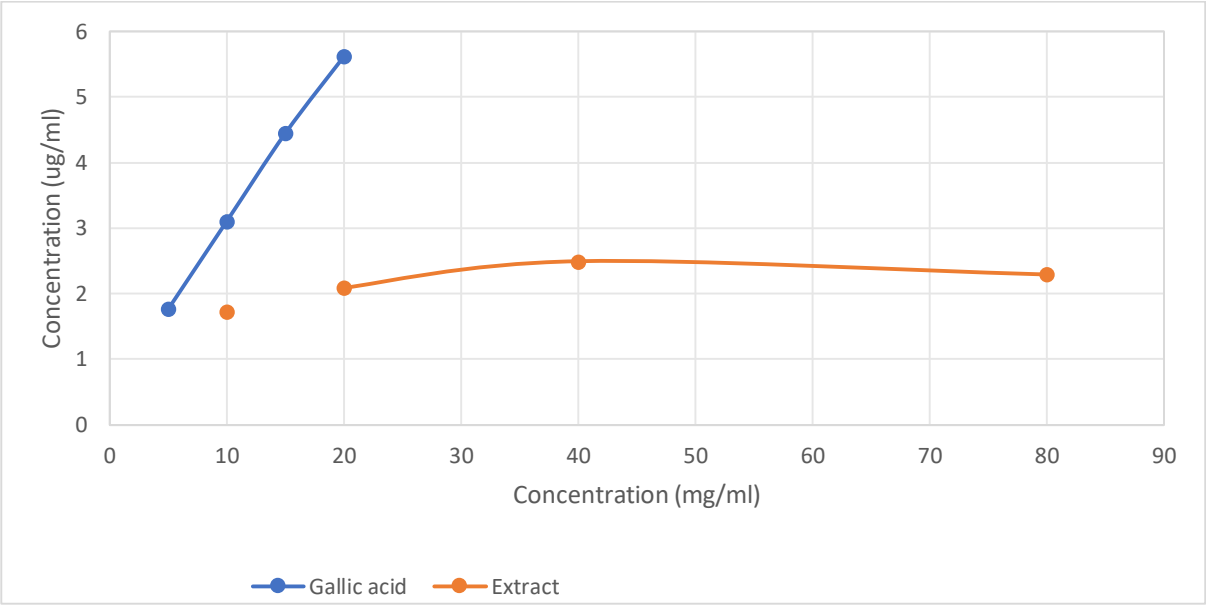

Figure 1: Nitric oxide scavenging activity of *P. tuber-regium*

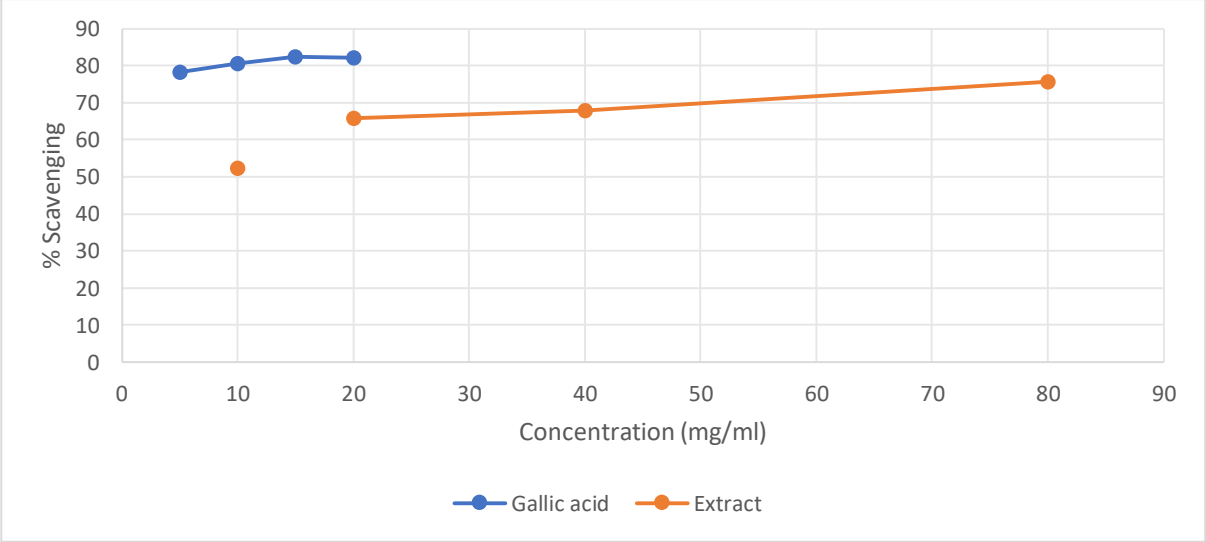

Figure 2: The ABTS radical scavenging activity of *P. tuber-regium*

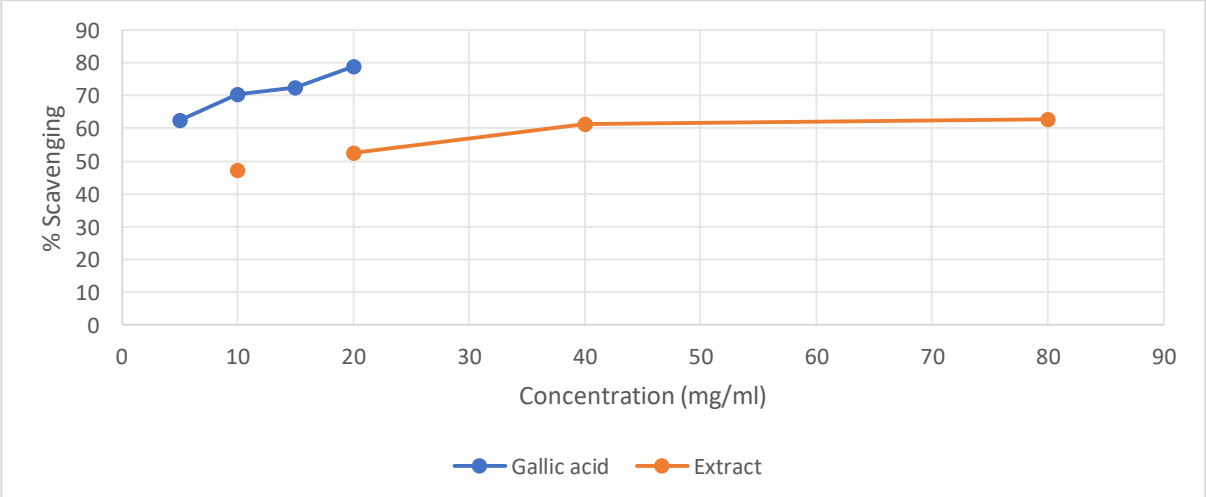

Figure 3: The in-vitro Hydroxyl radical scavenging activity of *P. tuber-regium*

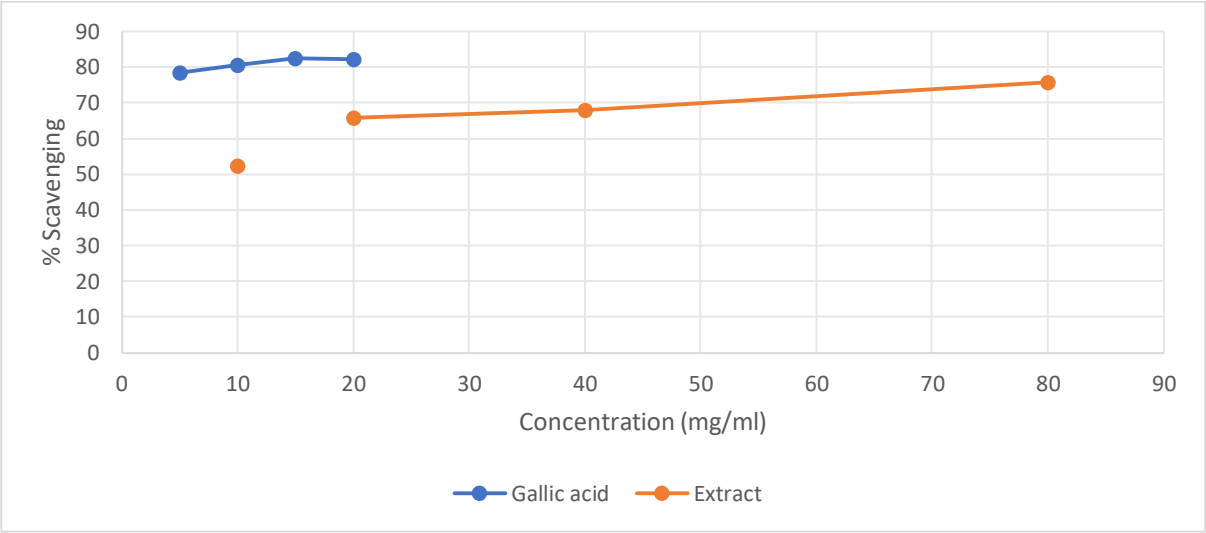

Figure 4: The result of the 2,2-diphenyl-2-picryl-hydroxyl hydrate (DPPH) in vitro scavenging activity of *P. tuber-regium*

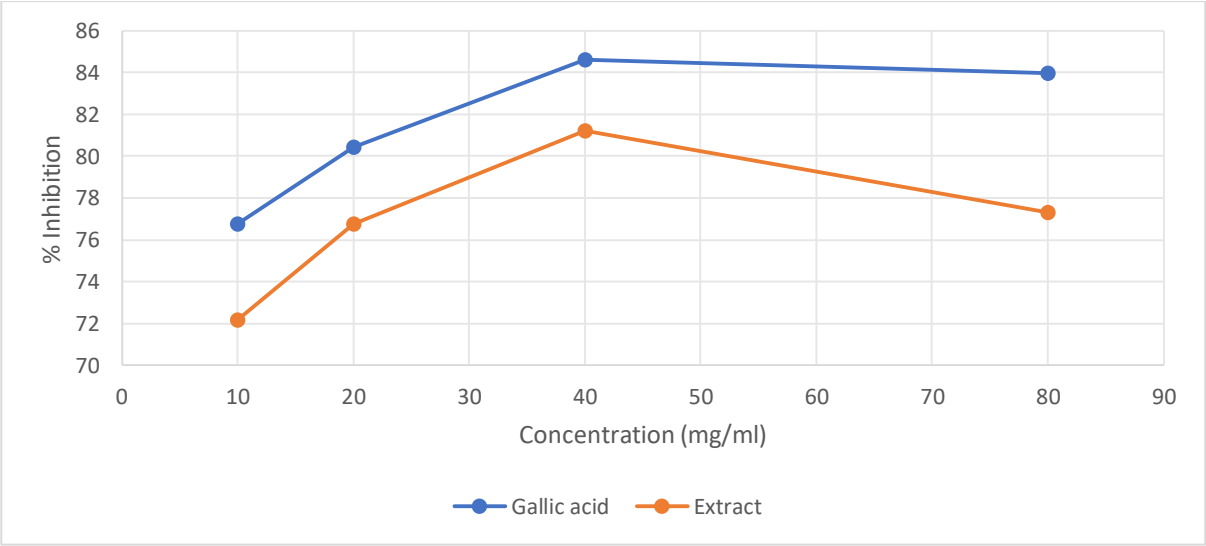

Figure 5: The in-vitro ferric reducing antioxidant property (FRAP) of *P. tuber-regium* extract

Table 1: The result of the ion chelating activity of *P. tuber-regium*

Concentration of the extract in mg/ml	% Inhibition
0 mg/ml	0.001
10 mg/ml	78.730
20 mg/ml	82.271
40 mg/ml	85.465
80 mg/ml	81.911

Discussion

Free radicals are very reactive molecules or atoms that contain unpaired electrons, making them unstable and capable of damaging cellular components (Fletcher *et al.*, 2023). Free radicals can interact with and damage various biological macromolecules, including proteins, lipids, and DNA. They are produced endogenously through normal metabolic processes, such as electron transport chain, oxidative phosphorylation, and mitochondrial respiration, and exogenously from environmental factors like pollution, smoking, poor nutrition, drugs, certain chemicals, radiation exposure and from industrial effluents (Ilechukwu *et al.*, 2014, Ilechukwu *et al*., 2025a, Ilechukwu *et al*., 2025b, Ilechukwu *et al.*, 2025c). While free radicals play a role in normal physiological functions, excessive production can contribute to pathological conditions, including cancer (Harrison *et al.*, 2024). Although our bodies have antioxidant defense mechanisms capable of neutralizing free radicals, an imbalance between their production and neutralization leads to oxidative stress, a key factor in the development of neurodegenerative, cardiovascular and metabolic diseases

(Ilechukwu *et al*., 2025a; Ilechukwu *et al*., 2025b; Ilechukwu *et al*., 2025c).

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteracts the effects of free radicals and other oxidants produced during normal metabolism and the ones acquired from the environment. The body can be protected from these free radicals by simply taking an ample amount of dietary antioxidants. From available evidence, foods containing antioxidants and antioxidant nutrients can be employed in disease prevention. Antioxidants are particularly important in improving the quality and quantity of life by preventing or delaying the onset of diseases that have their etiology in oxidative stress. Over the years, various methods have been used to investigate the antioxidant ability of substances both in vitro and in vivo.

In this study, the in vitro antioxidant properties of *P. tuber-regium* (King tuber mushroom) was evaluated using ferric reducing antioxidant property (FRAP), Nitric oxide scavenging activity, ABTS radical scavenging activity, DPPH (2,2-diphenyl-2-picryl-hydroxyl hydrate) scavenging activity, metal ion chelating property and hydrogen peroxide scavenging activity. The In-vitro nitric oxide scavenging activity of *P. tuber-regium. P. tuber-regium* shows lower nitric oxide scavenging activity when compared with the standard antioxidant garlic acid. The ABTS radical scavenging activity of *P. tuber-regium. P. tuber-regium* extract was able to scavenge for ABTS radical in a dose dependent manner. When compared with the standard antioxidant (garlic acid), *P. tuber-regium* shows similar scavenging activity but was less effective than garlic acid. The Hydroxyl radical scavenging activity of *P. tuber-regium. P. tuber-regium* extract was able to scavenge for hydroxyl radical in a dose dependent manner. When compared with the standard antioxidant (garlic acid) *P. tuber-regium* shows similar scavenging activity but was less effective than garlic acid. The 2,2-diphenyl-2-picryl-hydroxyl hydrate (DPPH) scavenging activity of *P. tuber-regium. P. tuber-regium* extract was able to scavenge for DPPH radical in a dose dependent manner. When compared with the standard antioxidant (garlic acid) *P. tuber-regium* shows similar scavenging activity but was less effective than garlic acid.
In all, the *in vitro* antioxidant scavenging assay of the extract showed that PT has the ability to scavenge for free radical, reduce power of some ions and chelate metal ions in a dose dependent manner. These results gotten is in close conformity with the works of (Bamigboye, 2016, Vishwakarma *et al.*, 2017, Bamigboye, 2019; Odjimogho *et al.*, 2024) which reported similar results.

Conclusion

The methanol extract of *P. tuber-regium* has antioxidant properties and may be very useful in the treatment of disorders caused by free radicals. Thus, this mushroom is worth considering as an important source of natural antioxidants which fewer or no side effects. It might as well be a good plant–based pharmaceutical or nutraceutical product or lead compound in drug development for the treatment of several diseases caused by free radicals.

Conflict of interests: Authors declare no conflict of interests.

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