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Phytochemical Analysis and *In-vitro* Antioxidant Activities of Some Selected Higher Fungi from Oyo State, South West of Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the completion of this work. The study's design, statistical analysis, protocol and initial writing of the manuscript were all handled by author FCO. The study's design, analysis and discussions were supervised and handled by authors SGJ and ATS. The discussions, editing, and literature searches were overseen by authors VOA and SAL. The final manuscript was read and approved by all authors.

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ABSTRACT

Higher-fungi (Hf) of the polypore mushrooms are considered to have unique secondary metabolites, making them reservoirs of therapeutically significant bioactive compounds. Phytochemical and antioxidant properties of the Hf were accessed in this study.

Four Hf, which were found in several wild locations in Oyo state, Nigeria, were collected. At the University of Ibadan Botany Department Laboratory, the species of the four Hf were determined. In-vitro antioxidant activity were assessed using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP), and Hydrogen peroxide (H2O2) assays using methanol extracts of air-dried and powdered Hf. Results were presented as Mean SEM, graphs were created

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in Excel, one-way ANOVA was used for the analysis, and p ≤0.05 was regarded as significant.
These Hf were identified as *Lycoperdon rimlatum* (*Lr*) FFUI1, *Trametes versicolor* (*Tv*) FFUI2, *Ganoderma lucidum* (*Gl*) FFUI3, and *Daedelia quarcina* (Dq) FFUI4 and were recorded on the NCBI with accession numbers EU833664.1, JQ621899, JQ520179.1 and KP171209.1, respectively. All the Hf showed positive tests for the presence of saponin, tannin, alkaloid, terpenoid, carbohydrate,tannin and flavonoids. The Hf showed antioxidant activities, the highest DPPH inhibition was by *Tv* (94.48%), FRAP was by *Gl* (0.16 mg/g) and H_2O_2 inhibition was by *Lr* (70.90%). The antioxidant activities observed were due to the presence of useful phytochemicals making them therapeutically significant.

Keywords: Higher- fungi; phytochemicals; bioactive compounds; in-vitro antioxidant activities.

1. INTRODUCTION

Fungi constitute an undescribed, undocumented lineage of eukaryotes with huge ecological and economic effects [1]. Higher fungi have been considered one of the highly diversified biological resources in the world [2]. They are diverse, heterotrophic organisms with unique nutritional and ecological requirements [3]. These Mushrooms have been reported as foods with therapeutic value used in the management of hypercholesterolemia, hypertension, and cancer [4]. Natural products produced by microorganisms, plants, and animals that play important roles in defense are secondary metabolites [5].

This metabolite from fungi has been receiving great consideration globally since the 1940s when antibiotics were discovered [6]. Oxidation is essential for energy production in several living organisms for the fueling of physiological processes [7]. Although oxygen is essential for life, it can also worsen the harm done by oxidative events within the cell. Oxygen's oxidative property is vital in a range of biological activities. The production of ATP (adenosine triphosphate) by the mitochondria, which is used by the cell to produce energy, results in the synthesis of free radicals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are byproducts of the biological redox process, are both deadly and beneficial molecules in humans. Free radicals can be produced in humans all around the world by environmental factors like noise, radioactivity, smoke, and weedicides. Most endogenous and physiological reactive oxygen species (ROS) are by-products of the oxidative reaction process that occurs in the mitochondrial respiratory chain [9]. Reactive oxygen species (ROS) have a variety of consequences on cell physiology, including aiding in the death of invasive bacteria, wound healing, and regenerative processes [9]. Consuming antioxidants on a regular basis can help to reduce free radicals' potential for harm. Unquestionably, the delicate balancing act between these two opposing effects is crucial in life. At low to moderate concentrations, reactive species have positive impacts on immune response and cellular redox signaling; yet, at high concentrations, they can impair cell structures and function by inducing oxidative stress. The hydrogen-based scavenging, regulation of free radicals, and radical peroxide oxidation processes are all part of the mushroom's antioxidant action [10]. By strengthening the immune system, these antioxidants lower the risk of infection, cancer, and cardiac issues. External antioxidants from food supplements are required when endogenous antioxidants are insufficient to sufficiently protect the organisms from free radicals [11].

God designed plants and other herbal products with specific therapeutic or curative properties, which are known as "phytochemicals," to benefit people [12]. Phytochemicals, as their name implies, are chemicals produced by plants from their primary or secondary metabolites [13]. These phytochemicals come in various kinds depending on their medicinal capabilities. Many plants and herbal items include a number of chemical components known as secondary metabolites that combine to produce therapeutic effects. Given that they have fewer or no side effects as compared to conventional synthetic medications and that many natural therapies have their own curative powers, the therapeutic properties of many plants and herbal items are becoming more and more well-known and preferred [14]. The phytochemicals that medicinal plants and herbs create for defense are quite abundant, and it is these components that give them their therapeutic properties [15].

Medicinal plants and herbs may contain a range of phytochemicals, including saponin, which can be used to lower blood cholesterol, nitrogen-rich alkaloids, which can be used as stimulants, tannins, which act as natural antibiotics, anthraquinones, which act as laxatives and dyes, cardiac glycosides for cardiac drugs, flavonoids, and antioxidant phenols, as well as other compounds [16]. The objective of this research is to assess the phytochemical and antioxidant properties of the selected higher fungi.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Mushroom Samples

Fresh fruiting parts of the selected macro- fungi were collected from the wild in Saki, Ibadan, Ogbomosho, and Iseyin in Oyo State, southwest of Nigeria between August and September 2016 and 2017. They were validated at the University of Ibadan's Botany Department Laboratory. They were identified using descriptions from Alexopoulos et al. [17]. The macro-fungi DNA was extracted with Hexadecyl trimethyl ammonium bromide (cTAB) [18]. The internal transcribed spacer (ITS) region was amplified using the primer pairs, pITS4-F (5'-TCCGTAGGTGAACCTGCCG-3') and pITS1-R (5'-TCCTCCGCTTATTGATATGC-3'). The PCR data was analyzed by electrophoresis on a 1 % agarose gel at the International Institute of Tropical Agriculture in Ibadan, Oyo state, Nigeria, prior to sequencing. Using the NCBI basic alignment search tools (BLAST), the generated sequences were analyzed to determine the closest sequence matches for taxonomy classification [19]. Their basidiocarps were gathered and kept in the macro-fungi collection owned by Jonathan Gbolagade at the University of Ibadan's Botany Department.

2.2 Extraction of Phytochemicals from Selected Higher Fungi

To obtain the fraction of the methanol extract, 1.5 kg of the air-dried powdered sample was placed separately into the soxhlet chamber extractor and extracted with 7.5 liters of 95 % methanol for 24 hours at 40o C. As reported by Evans, [20], the filtrate was evaporated using a rotating electrical evaporator in a vacuum. Crude methanol extract yield was measured and correctly labeled and maintained in hygienic glass containers at room temperature until it was ready for use. The crude methanol yield was calculated after the above extraction, and determined by deducting the final weight of the extract obtained from the initial weight multiply by 100.

$$\% \text{ yield} = W1 - W2 \times 100,$$

Where W1 initial weight, W2 =final weight..

2.3 Qualitative Phytochemical Analysis

Methanol extracts from medicinal mushrooms were subjected to numerous chemical tests to classify particular bioactive constituents using standard procedures [20].

2.4 Quantitative Determination of Phytochemical Constituents [20]

Under lower pressure, filtered raw mushroom extracts (200 ml) are concentrated and segmented with 70 percent (V/V) sequential extractions of n-hexane, chloroform, ethyl acetate, and ethanol. These four fractions were tested on secondary metabolites using qualitative phytochemical reactions. Triterpene/steroids, alkaloids, flavonoids, saponins, carbohydrates, tannins, and terpenoids are measured. As an empirical response to those measures, colour intensity was used.

2.5 *In-vitro* Antioxidant Assays

2.5.1 DPPH radical scavenging activity method

Following the protocol established by Shimada, et al. [21], the scavenging ability of mushrooms was assessed with some modifications. First, 0.5 ml of the aliquot sample extract was put in the test tubes with radical 2.9 ml of 200 µmol DPPH at various ethanol concentrations. The mixture was shaken vigorously and allowed to stand for 30 minutes at room temperature in the dark. Using a UV spectrophotometer, the reaction blend was measured at 515 nm. The solvent extraction was used as a blank, without an extract. The base norm used as ascorbic acid. The scavenging effect was determined based on the following formulation:

$$\text{Scavenging effect (\%)} = 1 - [(\text{Absorbance sample} / \text{Absorbance control}) \times 100]$$

2.5.2 Ferric Reducing Antioxidant Power (FRAP) assay

The process described in Benzie & Strain [22], Huang, et al. [23] was applied. The freshly

formed FRAP reagent was regulated and incubated in a water bath for 10 minutes at 30°C. At 0 min., absorbance was then recorded (t0). The test tube was directly exposed to 100–500 l of mushroom sample extract and 100 l of distilled water for 30 minutes at about 30°C. The absorbance was then measured at a wavelength of roughly 700 nm (t30). The reference substance was ferrous sulphate. The sample extract's antioxidant capacity was assessed using a conventional ferrous sulfate curve, and the FRAP value was determined as being equal to M Fe2 + per gram of extract using the formula:

FRAP value = Absorbance (sample + FRAP reagent) – Absorbance (FRAP reagent)

2.5.3 H_2O_2 radical scavenging assay

The Ruch et al. [24] method was used to examine the extract's capacity to scavenge hydrogen peroxide. In the phosphate buffer, a solution of hydrogen peroxide (2mol/l) was made (pH 7.4). Extracts were added to the hydrogen peroxide solution at a rate of 1 to 10 g per ml (0.6 ml). The hydrogen peroxide absorbance at 230 nm was estimated using a blank solution devoid of hydrogen peroxide and comparing the results with ascorbic acid and the reference substance after 10 minutes.

H_2O_2 activity (%) = (Abs control − Abs sample) / Abs (control) x 100

2.6 Statistical Analysis

Results were presented as Mean ± SEM, graphs plotted using Microsoft Excel, analysed using one-way ANOVA and $P < 0.05$ was significant.

3. RESULTS AND DISCUSSION

After the sequenced data were edited using bio edit and blasted in the NCBI blast data based. The identity of our fungi were revealed. as *Lycoperdon rimlatum* FFUI1, *Trametes versicolor* FFUI2, *Ganoderma lucidum* FFUI3, and *Daedelia quarcina* FFUI4 and were recorded on the NCBI with accession numbers EU833664.1, JQ621899, JQ520179.1 and KP171209.1, respectively as shown in Table 1.

The percentage yield of metabolites from the Higher Fungi is represented in Table 2. The highest percentage yield was observed with the organism *Lycoperdon rimulatum* at 46.98%, while the lowest was observed with *Trametes versicolor* at 26.26%.

Table 1. Identity of the fungi strains

Strain	ID(NCBI) Submision	Accession number	Identified name	Blast search similarity
STB112	F1	EU833664.1	*Lycoperdon rimlatum*	99%
EMB5	F2	JQ621899	*Tramates versicolor*	99%
IUM4100	F3	JQ520179.1	*Ganoderma lucidum*	99%
Dai12697	F4	KP171209.1	Daedelia quarcina	99%

The qualitative phytochemical analysis of the Higher fungi samples is represented in Table 3. In the analysis, it was observed that all the organisms showed positive tests for the presence of saponin, tannin, alkaloid, terpenoid, carbohydrate and tannin. The steroid tested positive with only *Tramates versicolor* while anthocyanin and phlobatannin tested positive with only *Ganoderma lucidum*. Flavonoids tested positive with all the Higher fungi except *Daedelea quercina.*

Table 2. Percentage yield of metabolites

Organisms	% Yield
Trametes versicolar	26.26
Daedelia quercina	38.68
Ganoderma lucidum	36.19
Lycoperdon rimulatum	46.98

The quantitative phytochemical analysis of the Higher fungi samples is represented in Table 4. In the quantitative analysis of carbohydrate content, the highest amount of carbohydrate was observed with *Lycoperdon rimulatum* at 2.38±0.03, while the lowest was observed with *Tramates versicolor* at 0.19±0.01. For the flavonoid content, *Ganoderma lucidum* had the highest amount with 0.2±0.01 while the lowest amount was observed with *Tramates versicolar* with 0.11±0.01. In the quantitative analysis of Terpenoid content, *Lycoperdon rimulatum* has the highest amount of terpenoid with 0.60±0.03, while *Tramates versicular* has the lowest with 0.20±0.0. For saponin content, *Ganoderma lucidum* has the highest amount with 0.18±0.03 while the lowest amount was observed with *Lycoperdon rimulatum* with 0.02±0.01. For alkaloid, the highest amount was observed with *Tramates versicolor* while the lowest amount was observed with *Daedelea quercina* with 0.02±0.01. For the quantitative presence of tannin, the highest amount was observed with *Ganoderma lucidum* with 7.73±0.0 while the lowest was observed with *Tramates vericolar* with 2.6±0.02.

The link between phytochemistry and pharmacology is critical to consider when designing studies on the medicinal potential of plants and herbal medicines. In general, the results showed that phytochemicals were present in all samples, but at varying quantities. Alkaloids, tannins, saponins, and phenols are considered anti-nutrients since they are poisonous when consumed in large doses. However, it has been shown that several of the phytochemicals found in mushrooms have therapeutic properties. The presence of bioactive phytochemical constituents found in all of the Higher fungi studied has been suggested as the reason for their traditional uses in the treatment of inflammation, pains, hemostatic, diuretic, nutrition, antibiotics, and antitumor agents, which is supported by the findings of Edeoga and Erita [25], who discovered alkaloids' significant pharmacological modulation. The presence of alkaloids in the samples shows that they have analgesics and bactericides medicinal value, confirming Stary's (1998) findings. Furthermore, presence of phenols in the Higher fungi samples makes them antiseptics and antifungal (Gill, 1992). The presence of flavonoids in the Higher fungi suggests that they have antioxidant healing properties, which backs up Okwu, [26] findings that flavonoids can prevent cancer and oxidative cell damage. External antioxidants from food supplements are required when endogenous antioxidants are insufficient to protect organisms from free radicals. This exogenous antioxidants can be obtained naturally from flavonoids, according to Litescu et al. [27]. The presence of tannin in the sample, on the other hand, is linked to wound healing, supporting Okwu, [26] results that the concentration of tannin present in mushrooms can draw tissues together to aid wound healing.

Table 3. Qualitative phytochemical analysis of methanolic extracts of the selected Higher Fungi

Test	*T.v*	*D.q*	*G.l*	*L.r*
Saponin	+	+	+	+
Tannin	++	+++	+++	++
Steroid	+	-ve	--ve	-ve
Alkaloid	+	+	+	+
Flavonoid	+	-ve	+	+
Coumarin	-ve	-ve	-ve	-ve
Emodin	-ve	-ve	-ve	-ve
Terpenoid	+	+	+	+
Carbohydrates	+	+	+	+
Anthraquinone	-ve	-ve	-ve	-ve
Anthrocyanin	-ve	-ve	+	-ve
Phlobatannin	-ve	-ve	+	-ve
Cardiac glycoside	-ve	-ve	-ve	-ve

Samples: KEY; + - Positive -ve - Negative
T.v - Tramates versicolar, D.q - Daedelea quercina, G.l - Ganoderma lucidum, L.r - Lycoperdon rimulatum.

Table 4. Quantitative phytochemical analysis of the higher fungi samples

Organism/Test	Carbohydrate	Flavonoid	Terpenoid	Saponine	Alkaloid	Tannin
T.v	0.19±0.01[a]	0.11±0.01[a]	0.20±0.0[a]	0.17±0.02[a]	0.13±0.03[a]	2.6±0.02[a]
D.q	0.65±0.03[b]	-	0.30±0.02[b]	0.15±0.03[a]	0.02±0.01[b]	7.71±0.0[b]
G.l	0.65±0.02[b]	0.2±0.01[b]	0.4±0.03[c]	0.18±0.03[a]	0.11±0.02[a]	7.73±0.0[b]
L.r	2.38±0.03[c]	0.12±0.02[a]	0.60±0.03[d]	0.02±0.02[b]	0.13±0.05[a]	4.73±0.07[c]

Values are expressed as Mean± SEM. (n = 3). Mean with the same letter in each column are not significantly different at 0.05 probability level.

Key; T.v - Trametes versicolor, D.q - Daedelea quercina, G.l - Ganoderma lucidum, L.r- Lycoperdon rimulatum

The percentage inhibition of radical of methanolic extract of the selected higher fungi and Vitamin C, using DPPH assay are represented in Fig. 1. At 100 µg/ml, the highest percentage inhibition was observed with Vitamin C, a standard antioxidant by 94.63% followed by *Trametes versicolor* by 94.48 % while the least percentage inhibition was observed with *Lycoperdon rimulatum* by 89.44 %,meanwhile at 500 µg/ml, the standard sample, vitamin c had the highest percentage inhibition by 87.1% followed by *Trametes versicolor* by 85 % while the least percentage inhibition was observed with *Lycoperdon rimulatum* by 48.3 %.Overall, it was observed that percentage inhibition reduced with an increase in the concentration of the extracts. The DPPH test assesses the reactivity of substances using a stable free radical called DPPH, which produces a potent visible-range absorption band at 517 nm. The absorbance decreases and the color of the DPPH solution changes from deep violet to light yellow when the odd electron pairs off in the presence of a free radical scavenger. The extent of the absorbance reduction reflects the extract's antioxidant strength [28].

The ferric reducing power activity of methanolic extract of the selected higher fungi and Vitamin C assay are represented in Fig. 2. At 100 µg/ml, the highest ferric reducing power activity was observed with *Ganoderma lucidum* by 0.14 while the least activity was observed with *Lycoperdon rimulatum* by 0.01. .At 500 µg/ml, the standard sample, vitamin c had the highest ferric reducing power activity by 0.37 followed by *Ganoderma lucidum,* by 0.36, while the least was observed with *Lycoperdon rimulatum* by 0.16.Overall, it was observed that ferric reducing power activity increased with an increase in the concentration of the extracts and the highest reducing power was observed with Vitamin C at 500 (µg/ml) with 0.37 while the lowest reducing power was observed with *Lycoperdon rimulatum* with 0.01.at 100 (µg/ml). Since a compound's reducing power is correlated with its capacity for electron transfer, it may be a useful predictor of its potential antioxidant action [29]. The extract's ability to reduce happened in a dose-dependent way. This can be ascribed to the extract's polyphenols' ability to donate electrons.

The percentage inhibition of methanolic extract of the selected higher fungi and Vitamin C.using, Hydrogen peroxide (H_2O_2) radical assay are represented in Fig. 3. At 20 µg/ml, the highest percentage inhibition was observed with Vitamin C, a standard antioxidant by 83.5% followed by *Lycoperdon rimulatum,Trametes versicolor and Daedelia quarcina* by 70.9 %, 65.3 %, and 65.3 % respectively while the least percentage inhibition was observed with *Ganoderma lucidum* by 55.28 %. At 100 µg/ml, the standard sample, vitamin C had the highest percentage inhibition by 54.1 % followed by *Daedelia quarcina* by 35.2 %, while the least percentage inhibition was observed with *Trametes Versicolor* by 24.1 %. In the overall, it was observed that percentage inhibition reduced with an increase in the concentration of the extracts, and *Lycoperdon rimulatum* gave the highest inhibition at 20(µg/ml) with 70.9 % while the least was observed at 100(µg/ml) with *Trametes Versicolor* at 24.1%. A precursor to the formation of hydroxyl radicals in cellular components is hydrogen peroxide. One of the quick initiators of the lipid peroxidation process, hydroxyl radicals take hydrogen atoms from polyunsaturated fatty acids to cause peroxidic reactions of membrane lipids [30].

The capacity of a chemical to transport electrons influences its reducing power. Reducing power is frequently used to assess the anti-inflammatory properties of polyphenols, such correlated with reductones' existence, which exerts antioxidant activity by severing the cycle of free radicals by giving atom of hydrogen [31] The FRAP value acts as a Fe (II) TPTZ extract's reduction power as measured.

A protective framework that counteracts unpaired radicals fortifies living things. Oxidative enzymes

make up the safety structure. The body's anti-oxidative defense system has so far successfully adjusted free radicals in controlled environments. A defense mechanism that balances unpaired radicals strengthens living things, and this defense mechanism is made up of oxidative enzymes. Accordingly, antioxidant food supplements can help the body's defense mechanism in neutralizing or mitigating oxidative harm, supporting the findings of the American Dietetic Association [32] that a healthy diet can provide all of the antioxidants needed and Cadenas' [33] early findings that dietary antioxidant intake can improve protection against free radicals. All the higher-fungi tested positive for antioxidant properties *in vitro* using DPPH, FRAP, and Hydrogen peroxide assays, which is consistent with Mau et al. [34] findings, that natural bioactive products generated by microorganisms, macro-fungi, plants, and animals have antioxidant properties. The hydrogen peroxide scavenging activity of the Higher-fungi can be attributed to the proton donating potential of their phytochemical components. It also corroborated the findings of Chang et al. [35] that hydrogen scavenging is a key component of the antioxidant action of mushrooms, keeping free radicals and radical peroxide oxidation in check.

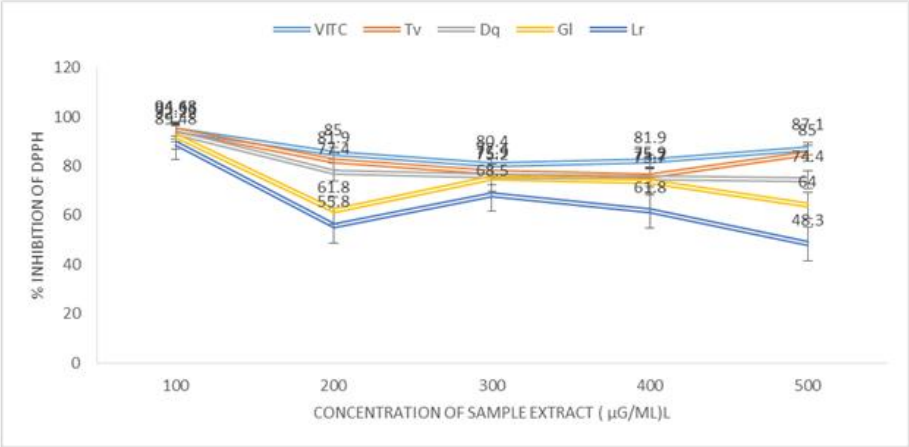

Fig. 1. Inhibition of radical by methanolic extract of the selected higher fungi and vitamin C, using DPPH assay

T.v - Trametes versicolor, D.q- Daedelea quercina, G.l - Ganoderma lucidum, L.r- Lycoperdon rimulatum

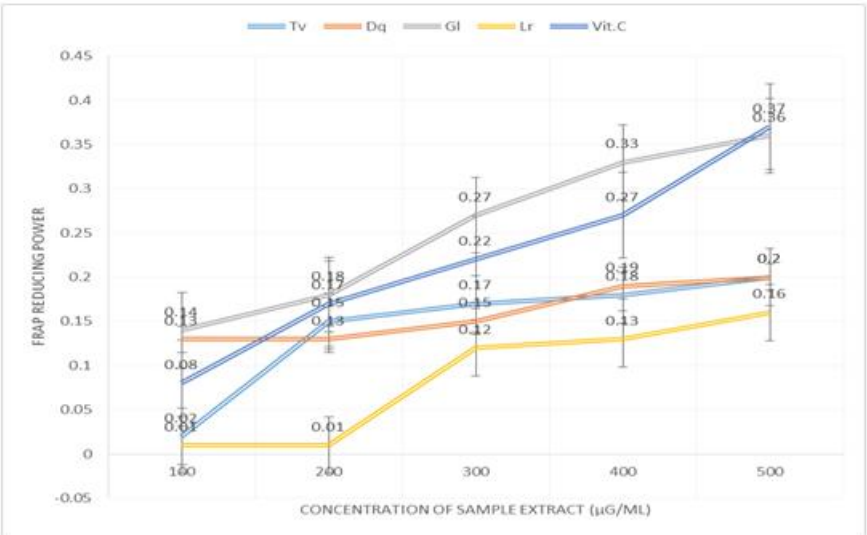

Fig. 2. Ferric reducing power activity of the selected higher fungi and Vitamin C

T.v - Tramates versicolar, D.q - Daedelia quercina, G.l - Ganoderma lucidum , L.r - Lycoperdon rimulatum

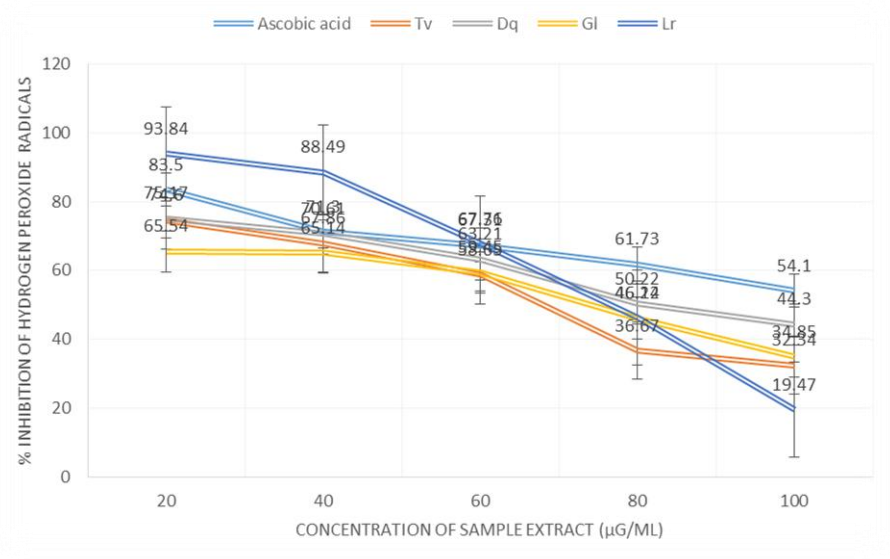

Fig.3. Percentage inhibition of methanolic extract of the selected Higher fungi and Vitamin C using Hydrogen peroxide (H_2O_2) radical assay

T.v - Tramates versicolar, D.q - Daedelia quercina, G.l - Ganoderma lucidum , L.r - Lycoperdon rimulatum

4. CONCLUSION

According to what has already been stated, the primary objective of researchers today is to discover natural antioxidants that will displace synthetic ones in the food, medicinal, and industrial applications [36]. Finding novel natural products from wild sources could benefit the food business by introducing better and safer antioxidants that offer strong defense against oxidative damage, which happens in both the body and our everyday foods. Therefore, new wild non- poisonous mushrooms could be introduced as natural supplies for this purpose. The researched Higher-fungi seems to be viable sources of bioactive substances that might have intriguing antioxidant effects in animal systems. Due to the Fungi's high phenolic content, it exhibits substantial antioxidant action. As a result, *Ganoderma lucidum, Tramates versicolor, Daedelia quercina,* and *Lycoperdon rumilatum* are recognized as superb source of bioactive substances that can be turned into medicines to treat oxidative stress.

The current research was able to identify indigenous mushrooms using molecular analysis, enriching and adding to our knowledge of mushroom biodiversity in Nigeria. The methanolic extracts of the studied Higher-fungi (*Tramates versicolar, Daedelia quercina, Ganoderma lucidum,* and *Lycoperdon rimulatum*) indicated the existence of flavonoids as well as antioxidant activity. Consuming these mushrooms can therefore serve as a source of exogenous antioxidants to supplement endogenous antioxidants in nutritionally supplemented diets, which can be extremely beneficial as protection against cancer, heart disease, boosting immunity, and anti-aging, supporting the findings of Omeonu et al. [38]. Preclinical and clinical studies are also needed to assess the effectiveness of the natural extracts of these mushrooms in the treatment or prevention of a variety of human diseases [39-47].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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