

FREE RADICAL SCAVENGING AND ANTIMICROBIAL PROPERTIES OF EXTRACTS OF WILD MUSHROOMS

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ABSTRACT

Antioxidant and antimicrobial potentials of extracts obtained from four wild mushrooms, *Termitomyces clypeatus* (TCE), *Termitomyces robustus* (TRE), *Lentinus subnudus* (LSE) and *Lenzites* species (LZE) collected in Nigeria were investigated. LSE and LZE displayed good scavenging activity against 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) and ferrous ion radicals at concentration of 2 mg/mL. However, TRE and TCE exhibited better superoxide anion scavenging effect at 2 mg/mL. All extracts (TCE, TRE, LSE and LZE) had comparable scavenging effect on hydroxyl radicals as butylated Hydroxytoluene (BHT) used as control. Moreover, extracts from the wild mushrooms were able to inhibit the growth of all indicator organisms at concentrations between 12.5 mg/mL to 100 mg/mL. LSE and LZE, however, showed better antimicrobial effect on the indicator organisms. The results suggest that extracts obtained from the four wild mushrooms may serve as sources of new bioactive compounds with effective antioxidant and antimicrobial activity.

Key words: wild mushrooms, antimicrobial activity, free radical scavenging

INTRODUCTION

In the last three decades, the search for natural bioactive compounds that can serve as antioxidant and antimicrobial agents had increased tremendously. The reasons for these are increasing understanding of the harmful nature of reactive oxygen species (ROS) produced during oxidation processes, harmful nature of synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and the increasing resistance posed by microorganisms to synthetic antibiotics. Extracts from mushroom have received attention based on their safety and records of health promotion.

Mushrooms produce a wide range of secondary metabolites with high therapeutic value (9). Health promoting properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects, have been reported for some species of mushrooms (5,19,20). Both fruiting body and the mycelium contain compounds with wide-ranging antioxidant and antimicrobial activities (5,11).

The antioxidative and free radical scavenging properties of the phenolic content of mushroom methanol extracts have been reported, suggesting possible protective roles of these compounds, due to their ability to capture metals, inhibit lipoxygenase and scavenge free radicals (19). Recently, Ferreira *et al.* (11) reported the antioxidative properties of two mushrooms, *Lactarius deliciosus* (L.) Gray and *Tricholoma portentosum* (Fr.) Que2 l obtained from northeast Portugal. Moreover, the activity of the exudates from mushroom mycelia against protozoa such as the parasite that causes malaria, *Plasmodium falciparum* (14,17) and other microorganisms (16) had been reported. Chinese Shiitake mushroom (*Lentinus edodes*) has also been reported to possess both anti-tumour and antimicrobial properties (15). In an earlier study, Suay *et al.* (25) reported that extracts of more than 75% polypores mushroom species surveyed showed antimicrobial activity and 45% of 204 mushroom species inhibited wide variety of microorganisms. Hence, mushrooms may be a source of new antimicrobial capable of inhibiting microorganisms that are resistant to common antibiotics.

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Wasser (26) reported that the number of mushrooms on earth is estimated at 140,000 yet only 1400 (10%) are known. In essence, pharmacological potentials of about 90% of mushrooms on earth are yet to be exploited. A large number of the unknown species of mushrooms whose health promoting properties are unknown reside in Africa and probably in Nigeria. This is because there are little or no data about them. Most available data on these mushrooms are on their nutritional compositions (1,2,22). Most of these mushrooms are still obtained in the wild. Diverse species of mushrooms are found in Nigeria. Those that are common belongs to the following species; *Termitomyces*, *Plerotus*, *Lentinus*, *Lenzites*, *Trametes*, *Ganoderma* etc. The present study was aimed at assessing antimicrobial and free radical scavenging properties of four wild mushrooms, *Termitomyces clypeatus*, *Termitomyces robustus*, *Lentinus subnudus* and *Lenzites* species, obtained from Ado Ekiti, Nigeria.

MATERIALS AND METHODS

Fruit bodies of *Termitomyces clypeatus*, *Termitomyces robustus*, *Lentinus subnudus* and *Lenzites* species were collected during raining season between April and October 2006 in the forest along University of Ado Ekiti Road, Nigeria (N07°41' 06.2" and E 005°14' 45.7"). The fruit bodies were morphologically identified by Dr. T.-Z, Wei of Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences. Molecular identification was carried out by amplifying and sequencing the Internal Transcribed Spacer (ITS 4 and ITS 5) of nuclear ribosomal DNA (nrDNA) of the mushrooms. The sequenced DNA fragments were compared with data set obtained from NCBI GenBank. The fruit bodies were dried and ground into powder prior to extraction.

Preparation of macrofungi extracts

The powder of *T. clypeatus*, *Termitomyces robustus*, *Lentinus subnudus* and *Lenzites* species were respectively extracted by soaking in ethanol in Erlenmeyer flasks shaken on orbital shaker at 180rpm at room temperature for 24 hour. The process of extraction was repeated as stated above for each of the mushroom samples. The extracts obtained from the extracting medium were dried to constant weight in a SUN LAB proctector laboratory hood overnight to a constant weight. The extracts were designated *Termitomyces clypeatus* extract (TCE), *Termitomyces robustus* extract (TRE), *Lentinus subnudus* extract (LSE) and *Lenzites* species extract (LZE).

Chemicals

2-deoxy-o-ribose, 1,1-diphenyl-2-picrylhydrazyl (DPPH•), Folin-Ciocalteu-reagent, Gallic acid were products of Sigma-Aldrich (Steinheim, Germany); 2-thiobarbituric acid (TBA) from Acros Organics (Geel, Belgium); ethylenediaminetetraacetic acid (EDTA) from Amresco (Ohio, USA); sodium acetate trihydrate,

acetic acid, hydrogen peroxide (H₂O₂), trichloroacetic acid (TCA) and ascorbic acid from Beijing Chemical Reagents Company (Beijing, China); butylated hydroxytoluene (BHT) from China National Pharmaceutical Group Shanghai Chemical Reagents Company (Shanghai, China); detection Kit of superoxide anion radical scavenging activity from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All reagents were of analytical grade.

Radical scavenging activities of extracts

Multimechanistic radical scavenging assays as listed below were employed in assessing the antioxidant property of the wild mushroom extracts (TCE, TRE, LSE and LZE).

Scavenging effect of DPPH radicals

The method of Blois (6) was used in studying the effect of extract (TCE, TRE, LSE and LZE) on DPPH• radicals with some modifications. A solution of DPPH (0.5 mmol/L) in ethanol and 0.05 mol/L acetate buffer (pH 5.5) were prepared. Extracts in solution (0.1ml) at different concentrations was mixed with 2 ml of acetate buffer, 1.9 ml of absolute ethanol and 1ml DPPH solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark for 30 min. The decrease in absorbance at 517 nm was measured using a UNICO 2100 spectrophotometer. BHT was used as positive control and the sample solution without DPPH was used as blank. The radical scavenging activity was measured as a decrease in absorbance of DPPH and calculated as:

$$\text{Scavenging activity (\%)} = \frac{Ab - (As - Asb)}{Ab} \times 100$$

Where Ab, As and Asb are absorbances at 517nm of DPPH of the blank, extract or control and sample blank respectively.

Scavenging effect on hydroxyl radical

The determination of scavenging effect of extracts (TCE, TRE, LSE and LZE) on hydroxyl radicals was carried out as described by Halliwell *et al.* (12). The reaction mixture in a final volume of 1.0 ml, containing 0.4 ml of 20 mmol/mL sodium phosphate buffer (pH 7.4), 0.1 ml of 0.0625 mg/mL to 1 mg/mL extracts, 0.1 ml of 60 nmol/L deoxyribose, 0.1 ml of 10 mmol/L hydrogen peroxide, 0.1 ml of 1 mmol/L ferric chloride, 0.1 ml of 1.04 mmol/L EDTA and 0.1 mL of 2 mmol/L ascorbic acid was incubated at 37°C for 1h. Solutions of FeCl₂ and ascorbic acid were made up immediately before use in de-ionised water. The reaction was stopped by adding 1 ml of 17 mmol/L thiobarbituric acid (TBA) and 1 ml of 17 mmol/L trichloroacetic acid (TCA). The mixture was boiled for 15 min, cooled in ice and then the absorbance measured at 532 nm using a UNICO 2100 spectrophotometer. BHT was used as positive control while distilled water in place of extracts or BHT was used as

blank and the sample solution without adding deoxyribose as sample blank.

$$\text{Scavenging activity (\%)} = \frac{Ab - (As - Asb)}{Ab} \times 100$$

Where Ab is absorbance at 532 nm of the blank; As is absorbance of blank at 532 nm and Asb is absorbance of extract or BHT at 532 nm.

Scavenging effect on superoxide anion radicals

A commercial kit was used for this assay. Superoxide anion radicals were generated by xanthine/xanthine oxidase system and reacted with, 4-iodiphenyl-5-phenylterazolium chloride to form formazan, a coloured compound which was spectrophotometrically quantified at 550 nm. The production of formazan is inversely related to the superoxide anion radical scavenging activity of tested sample. The final results were expressed as the inhibition degree of formazan production. BHT was used as positive control and distilled water in place of extracts or BHT as blank. The % of inhibition of superoxide anion radicals was calculated using the formula below.

$$\text{Scavenging activity (\%)} = \frac{Ab - (As - Asb)}{Ab} \times 100$$

Where Ab is absorbance at 550 nm of the blank; As is absorbance of blank at 550 nm and Asb is absorbance of extract or BHT at 550 nm.

Ferrous ion chelating assay

The chelation of ferrous ion by the extracts was ascertained by the methods of Decker and Welch (8). One millilitre of extracts with concentration ranging between 0.25 to 4 mg/mL was mixed with 3.7 mL of deionised water and then the mixture was reacted with ferrous chloride (2 mmol/L, 0.1 mL) and ferrozine (5 mmol/L, 0.2 mL) for 20 min. The absorbance at 562 nm was determined spectrophotometrically. EDTA was used as positive control. Chelating activity on ferrous ion was calculated using the equation below:

$$\text{Chelating effect (\%)} = \frac{Ab - As}{Ab} \times 100$$

Where Ab is the absorbance of the blank without extract or EDTA and As is the absorbance in the presence of extract or EDTA.

Antimicrobial assay

Indicator organisms used for antimicrobial assay

A total of 8 microbial strains made up of 6 bacterial strains and 2 yeast strains were used. The microbial strains were *Bacillus*

subtilis ATCC 6633, *Bacillus cereus* CMCC1.1846, *Alcaligenes faecalis* CMCC1.1837, *Staphylococcus aureus* ATCC 6538, *Shigella dysenteriae* CMCC 51252, *Salmonella typhimurium* CMCC 1.1174, *Candida albican* ATCC10231 and *Cryptococcus neoformans* CMCC 1038. Microbial strains were obtained from China general microbial culture collection centre (CGMCC).

Antimicrobial activity

Antimicrobial activity of extracts was determined by the agar well diffusion method (23). Bacteria used as indicator organism were cultivated on nutrient agar medium at $36 \pm 1^\circ\text{C}$ for 24 hour while the yeast were cultivated on Yeast malt extract agar at $26 \pm 1^\circ\text{C}$ for 48 to 72 hour. Aliquot of culture (100 μl) was evenly spread on the surface of the solidified agar. Wells of 7 mm were bored in the agar with sterile cork borers. The extract (100 μl) dissolved in dimethylsulfoxide (DMSO) to concentration of 12.5 to 100 mg/mL and filtered through 0.22 μm membrane filter was introduced into the wells. The plates were incubated at $36 \pm 1^\circ\text{C}$ for 24 hour for bacteria while the fungi were incubated at $26 \pm 1^\circ\text{C}$ for 48 to 72 hour. Tetracycline and ampicillin were used as standard antibacterial while nystatin was used as antifungal standard under the conditions specified for bacteria and fungi respectively. The diameter of the inhibition zones were measured in milliliters. Inhibition zones were measured in triplicates (three plates per indicator organism).

Minimum inhibitory concentration

Dilutions of extracts (TCE, TRE, LSE and LZE) ranging from 12.5 mg/mL to 100 mg/mL were prepared. The agar diffusion method described above was used to screen the antimicrobial effect of the different concentrations of extracts. Agar well in which DMSO was added served as negative control. The tests were performed in triplicates.

Statistical analysis

All experiments were carried out in triplicates. Data obtained were analyzed by one way analysis of variance and means were compared by Duncan's multiple range tests (SPSS 11.5 version). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Generally, the most used genomic region for molecular characterization of fungi is the ITS region (internal transcribed spacer). It presents several characteristics making it a pertinent tool to identify and analyze phylogenetic molecules of fungi at species level differentiating between a large interspecific variability and a weak intraspecific variability (4). Hence, ITS region of rDNA of the fungi was used for their identification. Table 1 shows the molecular identity of macrofungi that were earlier identified using phenotypic data. The percentage identity of macrofungi obtained from BLAST analysis varies between

Table 1. Genomic identification based on rDNA ITS sequences of wild macrofungi collected from Ado Ekiti, Nigeria

*Code	Phenotypic identity	Closest relative from NCBI data base	% Identity with sequence from NCBI GenBank
LS	<i>Lentinus subnidus</i>	<i>Lentinus tigrinus</i>	80
TC	<i>Termitomyces clypeatus</i>	<i>T. striatus</i>	85
TR	<i>Termitomyces robustus</i>	<i>T. eurhizus</i>	96
LZ	<i>Lenzites species</i>	<i>Lenzites betulina</i>	97

* LS: *Lentinus subnidus*; TC: *Termitomyces clypeatus*; TR: *Termitomyces robustus*; LZ: *Lenzites species*.

80% and 97%. It has been reported that percentage similarity of 70% and above shows that strains identified are closely related (10).

Appreciable yield of 21.73 to 44.60mg/g was obtained as ethanolic extracts from TRE, TCE, LSE and LZE (Table 2). Ethanolic extracts of TRE and TCE were higher and significantly different ($P < 0.05$) from what were obtained in LSE and TZE.

Scavenging effect of extracts on DPPH radicals was concentration dependent (Fig. 1). LSE exhibited a significantly different ($P < 0.05$) DPPH scavenging effect at 2mg/mL than other extracts. However, BHT (positive control) has a significantly different ($P < 0.05$) DPPH scavenging effect at all concentrations than the extracts. Scavenging effect of BHT at all concentrations was not concentration dependent. In a previous report, the highest scavenging activities on DPPH radical obtained for edible mushroom such as *Lentinus edodes* (Berk.) Singer was 55.4% and *Volvariella volvacea* (Bull.) Singer 37.9%, at the concentrations of 1–9 mg/mL (7). The results of DPPH scavenging effect of LSE and LZE were higher than what was reported by Cheung *et al.* (7). DPPH assay is a widely used method to evaluate antioxidant activities in a relatively short time compared to other methods (21). It has the advantage of being unaffected by certain side reactions which is common in laboratory-generated free radicals such as the hydroxyl and superoxide anion (3).

In ferrous ion reduction, the general ability of the extracts to donate electrons is tested (13). Extracts exhibited a concentration dependent scavenging effect on ferrous ion (Fig. 2). However,

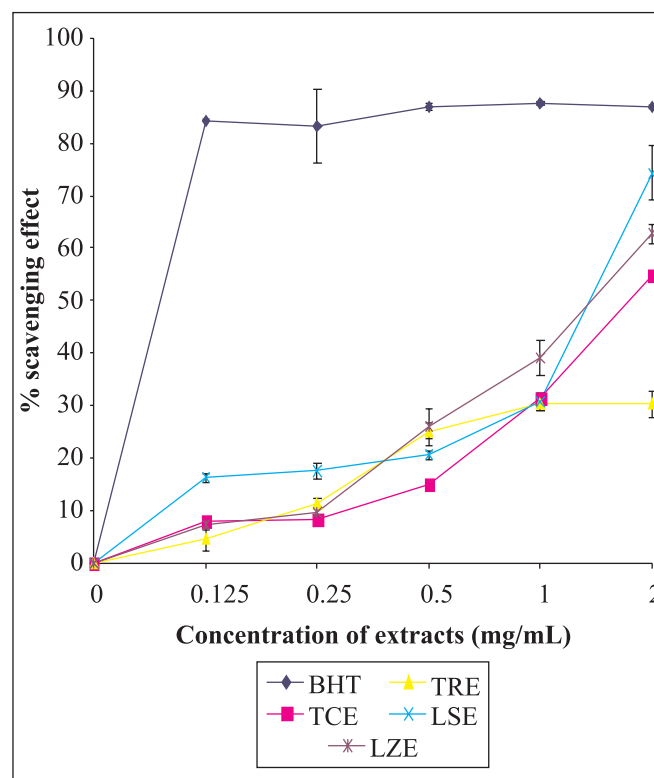
scavenging effect of EDTA (control) on ferrous ion was not concentration dependent. The ferrous ion scavenging effect of LSE at 4mg/mL concentration was 96.55%. This was higher and significantly different ($P < 0.05$) from ferrous ion chelating effects exhibited by the other extracts. TRE exhibited the lowest ferrous ion chelating effect of 23.08% at 4mg/mL concentration. In a previous study, Mau *et al.* (17) reported the ability of methanolic extracts from commercial medicinal mushrooms including *G. lucidiun*, *G. lucidium* antler and *G. tsugae* to reduce ferrous ion.

Hydroxyl ion scavenging effect of extracts was not concentration dependent (Fig. 3). There was no significant

Table 2. Total ethanolic yield (mg/g) from wild mushrooms

Mushrooms	Total yield*
TRE	44.60 ^b ± 1.65
TCE	40.03 ^b ± 3.07
LSE	26.60 ^a ± 1.43
LZE	21.73 ^a ± 1.43

Values are mean ± standard deviation of three replicates. *Values along column with different superscript are significantly different ($P < 0.05$).

**Figure 1.** Scavenging activity of extracts on DPPH radicals. Each value is mean ± standard deviation (n=3).

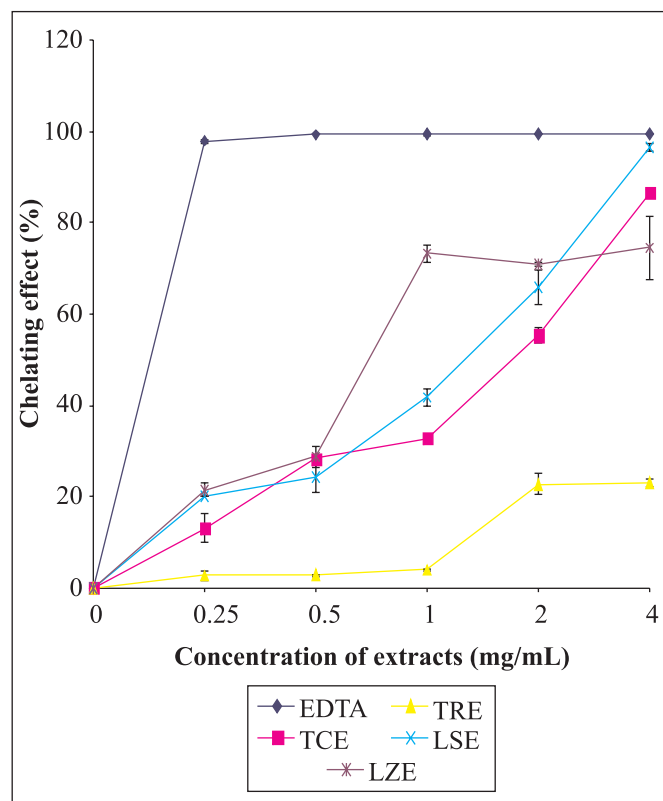


Figure 2. Ferrous ion chelating effect of extracts. Each value is mean \pm standard deviation (n=3).

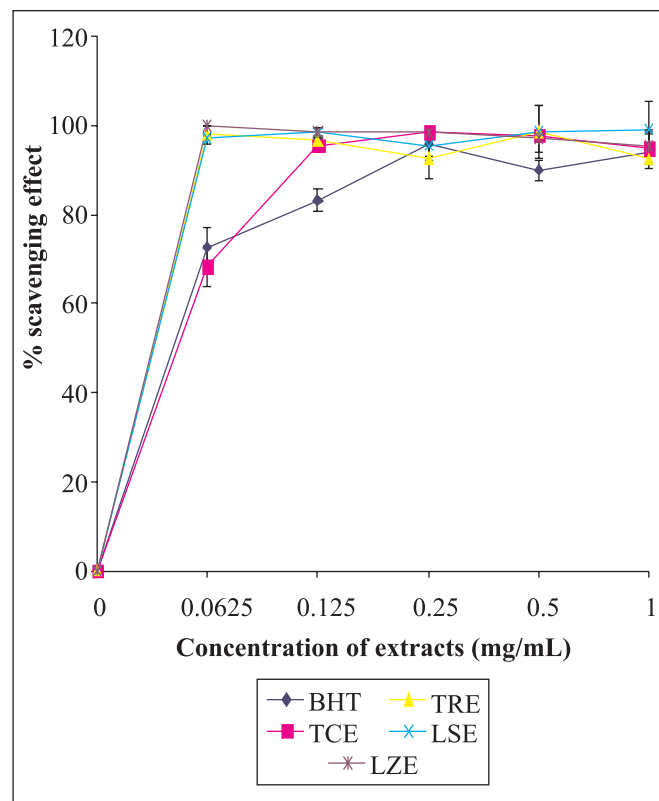


Figure 3. Scavenging effect of extracts on hydroxyl radicals. Each value is mean \pm standard deviation (n=3).

difference in the hydroxyl ion scavenging effect of extracts and BHT at concentrations of 0.0625 and 1mg/ml. TCE and LPE, however, exhibited a higher and significantly different hydroxyl ion scavenging effect at 0.5 mg/mL concentration. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (24). A very high hydroxyl ion scavenging ability exhibited suggests that extracts have potentials of being used as alternative to synthetic antioxidants in arresting oxidative activity of hydroxyl ion.

Superoxide anion scavenging activity of extracts and BHT were concentration dependent (Fig. 4). TRE and TCE exhibited a significant superoxide anion scavenging effect of 72.95% and 98.80% respectively at 2.0 mg/mL concentration. Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals hence, they are very harmful to cellular components of biological systems (27).

Table 3 shows the results of antimicrobial effect of extracts on selected microorganisms. Extracts showed good antimicrobial activity against the two yeasts, *C. albicans* and *C. neoformans*. There were no observable differences in the susceptibility of Gram positive and Gram negative bacteria to

the antimicrobial effect of extracts. Only *Alcaligenes faecalis*, a Gram positive bacteria, that exhibited more susceptibility to the antimicrobial effect of extracts. The MIC of extracts ranged between 12.5 mg/mL to 100 mg/mL (Table 4). *C. albicans*, *C. neoformans* and *Alcaligenes faecalis* were more susceptible to the antimicrobial effect of LSE and LZE at the lowest concentration of 12.5mg/mL. Mushrooms need antibacterial and antifungal compounds to survive in their natural environment (16). Hence, they may be rich sources of natural antibiotics. Suay *et al.* (24) had earlier reported that extracts of more than 75% polypores mushroom species surveyed showed antimicrobial activity and 45% of 204 mushroom species inhibited wide variety of microorganisms. In essence, unexploited mushrooms such as the ones found in Nigeria may be sources of new antimicrobials that can tackle the problem of drug resistance that is rampant in the country at present.

Results obtained from this study revealed that LSE and LZE displayed better activity in scavenging for DPPH and ferrous ion radicals while TRE and TCE exhibited better superoxide anion scavenging activity. Scavenging activity of all extracts on hydroxyl radical was not significantly different from what was observed for BHT. The extracts were also able to inhibit the

Table 3. Inhibition zone (mm) of crude extracts 50mg/ml from wild mushrooms against indicator organisms.

Indicator organisms	LZE	LSE	TCE	TRE	Tetracycline (30 µg/ml)	Nystatin (40 µg/ml)
<i>Candida albicans</i>	15	15	11.5	10	NT	10.3±0.5
<i>Cryptococcus neoformans</i>	22	17.5	13	13	NT	9.3±1.2
<i>Bacillus cereus</i>	-	11	-	9	25.6±1.2	NT
<i>Bacillus subtilis</i>	11	9	-	-	35.0±1.5	NT
<i>Staphylococcus aureus</i>	-	8	-	-	5.0±1.0	NT
<i>Shigella dysenteriae</i>	-	-	-	-	4.0±0.0	NT
<i>Salmonella typhimurium</i>	10	-	-	-	4.0±0.0	NT
<i>Alcaligenes faecalis</i>	14.5	15	13	13	23.7±3.7	NT

Values are means of three replicates. NT: Not tested; -: No inhibition.

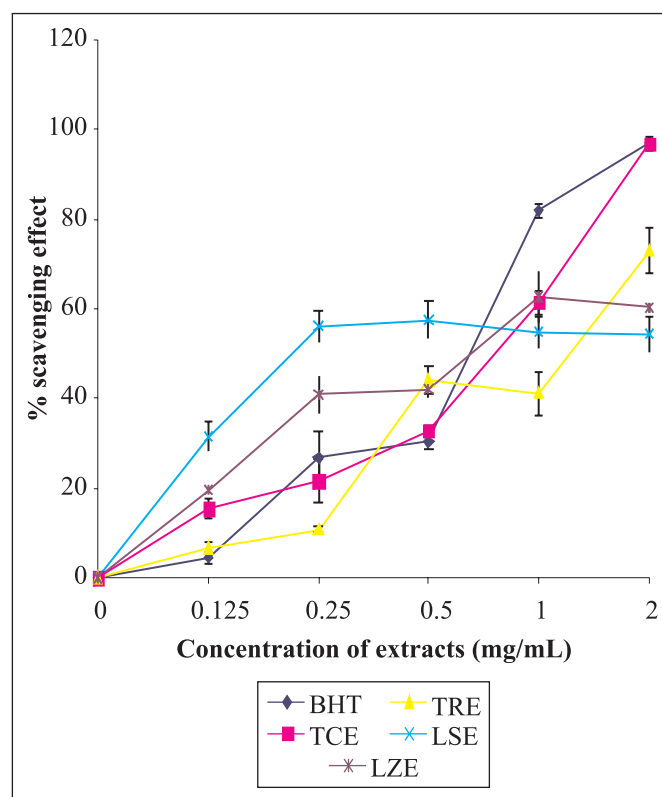


Figure 4. Scavenging effect of extracts on superoxide anion. Each value is mean ± standard deviation (n=3).

growth of all the indicator organisms at concentrations between 12.5 mg/mL to 100 mg/mL. LSE and LZE, however, displayed a better antimicrobial activity against the indicator organisms. Hence, extracts (LSE, TCE, TRE and LZE) have potential of being used as source of biopharmaceutical substances with antioxidant and antimicrobial properties. Further work aimed at isolation and identification of specific antioxidant and antimicrobial agents in extracts is the next focus of this research.

Table 4. Minimum inhibitory concentration (MIC) of extracts (mg/ml) from wild mushrooms against indicator organisms.

Indicator Organisms	LZE	LSE	TCE	TRE
<i>Candida albicans</i>	12.5	12.5	50	12.5
<i>Cryptococcus neoformans</i>	12.5	12.5	50	50
<i>Bacillus cereus</i>	100	50	100	50
<i>Bacillus subtilis</i>	50	50	100	100
<i>Staphylococcus aureus</i>	100	50	100	100
<i>Shigella dysenteriae</i>	100	50	100	50
<i>Salmonella typhimurium</i>	50	100	100	100
<i>Alcaligenes faecalis</i>	12.5	12.5	50	50

Values are means of three replicates.

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RESUMO

Atividade sequestradora de radicais livres e propriedades antimicrobianas de extratos de cogumelos selvagens

Foram investigadas as propriedades antioxidantes e antimicrobianas de extratos obtidos de quatro cogumelos selvagens da Nigéria: *Termitomyces clypeatus* (TCE), *Termitomyces robustus* (TRE), *Lentinus subnudus* (LSE) and *Lenzites species* (LZE). LSE e LZE, na concentração de 2 mg/ml,

apresentaram boa atividade sequestrante contra 2,2'-difetil-β-picrilhidrazil (DPPH) e radicais ferrosos. Entretanto, TER e TCE a 2 mg/ml apresentaram melhor efeito sequestrador de anions superóxido. Todos os extratos apresentaram efeito semelhante de seqüestro de radicais hidroxila como BHT usado como controle. Além disso, todos os extratos dos cogumelos selvagens, na concentração de 12,5 mg/ml até 100 mg/ml, foram capazes de inibir a multiplicação de todos os microrganismos indicadores testados, mas LSE e LZE apresentaram efeito antimicrobiano mais intenso. Os resultados sugerem que os extratos obtidos dos quatro cogumelos selvagens podem ser fontes de novos compostos bioativos com atividade antimicrobiana e antioxidante.

Palavras-chave: Atividade sequestradora de radicais livres, atividade antimicrobiana, cogumelos selvagens

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