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Total Phenol, Antioxidant and Cytotoxic Properties of Wild Macrofungi Collected from Akure Southwest Nigeria

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Abstract

Three wild macrofungi, *Lenzites betulina* (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers) collected in Akure southwest of Nigeria were assessed for their total phenol, antioxidant and cytotoxic activities. The antioxidant and cytotoxic properties of the hexane, ethyl acetate and ethanol extracts from the fruit bodies of these macrofungi were assessed using DPPH scavenging capacity, inhibition of the formation of thiobarbituric acid reactive species (TBARS), and the protein-binding dye sulforhodamine B (SRB) microculture assay to measure cell growth using six human cancer cell lines. Total phenol content ranged from 8.22µgGAE/mg to 60.54 µgGAE/mg. The DPPH and inhibition of the formation of TBARS ranged between 7.97% to 91.18% at 1000µg/mL. Ethanolic extracts (LET, TET and CET) with higher phenol content exhibited better antioxidant property. The inhibition of human cancer cell lines varies from one extract to the other. However, ethanolic extract of *Trametes vesicolor* (TET) demonstrated the best cytotoxic activity with 100% inhibition of HCT-5, MCF-7 and SKLU-1 human cancer cell lines. The study suggests that these three wild macrofungi could be source of effective antioxidant and anticancer agents.

Keywords: Wild Macrofungi; Total Phenol; Antioxidant; Anticancer.

1. Introduction

Macrofungi have long been used as valuable food source and as traditional medicines around the world, especially in the orient (Wasser, 2002). Macrofungi are known to produce large and diverse variety of secondary metabolites (Liu, 2007). These secondary metabolites have health promoting properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects (Anderson, 1992; Mizuno, 1999; Mau *et al*., 2004). Some common bioactive compounds isolated from these macrofungi include glycolipids, compounds derived from shikimic acid, aromatic phenols, fatty acid derivatives, polyacetylamine, polyketides, nucleosides, sesterterpenes, and many other substances of different origins (Lorenzen and Anke, 1998; Wasser and Weis, 1999; Mizuno, 1999; Liu, 2007).

The number of these macrofungi on earth has been estimated to be 140,000 out of which only 14,000 (10%) are identified (Hawksworth, 2001). The pharmacological potential of about 90% of these macrofungi is yet to be explored. A large number of the unknown species of mushrooms which may possess health promoting properties are not well studied especially in Africa (Oyetayo, 2011). There are no data on these macrofungi and their medicinal potentials.

The search for safe and effective pharmacological substances had increased of recent. Bioactive compounds obtained from macrofungi maybe the answer to these novel pharmacological agents. The antioxidative and free radical scavenging properties of mushroom have been reported (Mau *et al*., 2002; 2004; Ferreira *et al*., 2007). Chinese Shiitake mushroom (*Lentinus edodes*) has also been reported to possess both anti-tumour and antimicrobial properties (Jong and Birmingham, 1993). Natural products have been the source of most of the active ingredients of medicines (Harvey, 2008). Natural substances provide a large reservoir for screening of anti-HIV-1 agents with novel structure (Liu, 2007). The present study seeks to assess the total phenol, antioxidant and cytotoxic effects of hexane, ethyl acetate and ethanol extracts obtained from three wild non-edible macrofungi, *Lenzites betulina* (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers), collected from Akure, South West region of Nigeria.

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2. Materials and Methods

2.1. Collection of Macrofungi

Lenzites betulina (Fries), *Trametes vesicolor* (Lloyd) and *Coriolopsis polyzona* (Pers) were collected in the wild between June and September, 2011 in forest around the campus of Federal University of Technology, Akure, Nigeria (Lat. 07° 14^{I}N Long. 05° 11^{I}E). The morphological and ecological characteristics of these macrofungi were recorded in their natural habitats. Dried samples of the macrofungi were numbered and kept in polythene bags. The collected macrofungi were identified based on their macroscopic and microscopic characteristics and the related literature (Watling, 1973; Moser, 1983). Voucher specimens of dried macrofungi were deposited in the herbarium of Department of Microbiology, Federal University of Technology, Akure, Nigeria.

2.2. Preparation of Macrofungi Extracts

The method described by Yu *et al*. (2006) was adopted with slight modification. Briefly, dried samples of *Lenzites betulina*, *Trametes vesicolor* and *Coriolopsis polyzona* were ground into fine powder with an electric mill. The bioactive components were sequentially extracted from non-polar to polar solvents using hexane, ethylacetate and ethanol. The extraction by the solvent was performed in Erlenmeyer flask at room temperature for 48 h. The extracts obtained were dried to constant weight in a laboratory hood overnight (12 hours). The extracts were designated LHE (hexane extract of *L. betulina*), LEA (ethyl acetate extract of *L. betulina*), LET (ethanol extract of *L. betulina*), THE (hexane extract of *T. vesicolor*), TEA (ethyl acetate extract of *T. vesicolor*), TET (ethanol extract of *T. vesicolor*), CHE (hexane extract of *C. polyzona*), CEA (ethylacetate extract of *C. polyzona*) and CET (ethanol extract of *C. polyzona*).

2.3. Total Phenol Determination

The method of estimating total phenols as described by Singleton *et al*. (1999) was used. Fifty microlitre (50µL) of extract was added 250µL of undiluted Folin-Ciocalteau-reagent. After 1 min, 750µL of 20% (w/v) aqueous Na_2CO_3 were added and the volume was made up to 5.0mL by adding 3.95mL of water. The control contained all the reaction reagents except the extract. The preparation above was incubated for 2 h at 25^{o}C and the absorbance was measured at 760nm and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract) in triplicate. Each experiment was repeated three times and the results were expressed as average values.

2.4. Scavenging Effect of Extracts on DPPH Radicals

The method of Blois (1958) was used in determine the effect of extracts of *Lenzites betulina*, *Trametes vesicolor* and *Coriolopsis polyzona* on DPPH• radicals with some modifications. A solution of DPPH (0.5mmol/L) in ethanol and in 0.05 mol/L acetate buffer (pH 5.5) was prepared. Extract in solution (0.1mL of 2mg/mL) was mixed with 2mL of acetate buffer, 1.9mL of absolute ethanol and 1mL DPPH solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in dark for 30 min. The decrease in absorbance at 517nm was measured using Ultra Microplate Reader (Elx 808, BIO TEK Instruments Inc). 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.

2.5. Estimation of Lipid Peroxidation.

Thiobarbituric acid reactive species (TBARS) levels were measured using rat brain homogenates according to the method described by Ng *et al*. (2000) with some modifications. Adult male Wistar rats (200-250 g) were provided by the Instituto de Fisiologı´a Celular, UNAM, and their use was approved by the Animal Care and Use Committee. Rats were maintained at 25 °C on a 12/12 h light/dark cycle with free access to food and water and killed under mild ether anesthesia. Cerebral tissue was rapidly dissected from the whole brain and homogenized in phosphate-buffered saline (PBS; 0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 2.16 g of $NaHPO_4 \cdot 7\ H_2O$/L, pH 7.4) to produce a 1 in 10 homogenate (w/v) (Rosato *et al*., 2002). The homogenate was centrifuged for 10 min at 3400 rpm, and the resulting pellet was discarded. The protein content of the supernatant was measured according to the method of Lowry *et al*. (1951), and samples were adjusted to 2.5 mg of protein/mL with PBS. The supernatant (400 μL, 1 mg of protein) was preincubated with sample (50 μL) at 37 °C for 30 min, then peroxidation was initiated by the addition of 50 μL of freshly prepared FeSO4 solution (final concentration) 10 μM), and the sample was incubated at 37 °C for an additional 1 h (Ng *et al*., 2000). The TBARS assay was determined as described by Ohkawa *et al*. (1979) with the modification, 0.5 mL of TBA reagent (1% thiobarbituric acid in 0.05 N NaOH and 30% trichloroacetic acid, 1:1) was used and the final solution was cooled in ice water bath for 10 min, centrifugated at 10000 rpm for 5 min, and then heated at 95 °C in a boiling water bath for 30 min. BHT and α-tocopherol were used as positive controls. Then, the absorbance was measured at 532 nm in the Ultra Microplated Reader (Elx 808, BIO TEK Instruments Inc). Results are expressed as nanomoles of TBARS per milligram of protein, with percent inhibition after 30 min calculated as the inhibition ratio (IR), where C = absorbance of the control and E = absorbance of the test sample.

$$\text{IR (\%)} = [(C - E)/C] \text{ X } 100$$

These values were plotted against the log of the concentrations of individual extracts. Each experiment was replicated three times and the results were expressed as average values.

2.6. Cytotoxicity Assay

The extracts were screened *in vitro* against human cancer cell lines: U251 (human glioblastoma), PC-3 (human prostatic adenocarcinoma), K562 (human chronic

myelogenous leukemia), HCT-15 (human colorectal adenocarcinoma), MCF-7 (human breast cancer cell line) and SKLU-1 (human lung adenocarcinoma). Human cancer cell lines were supplied by National Cancer Institute, USA. The protocol described by Monks *et al*. (1991) for assessing human tumor cytotoxicity was adopted. The cell lines were cultured in RPMI-1640 medium which was supplemented with 10% fetal bovine serum, 2mM L-glutamine, 10,000 units/ml penicillin G, Sodium, 10µg/mL streptomycin sulfate and 25µ/mL amphotericin B (Gibco) and 1% non-essential amino acids (Gibco). The cultures were maintained at 37^{o}C in a 5% CO_2 humidified atmosphere. The viability of the cell exceeded 95% as verified by trypan blue method.

The cells were removed from the tissue culture flasks by treatment with trypsin, and diluted with fresh media. One-hundred-microliters cell suspension aliquots, containing 5000 – 10,000 cell per well, were transferred into 96 well microtiter plates and incubated at 37^{o}C for 24h in a 5% CO_2 atmosphere. Stock solution of extracts initially dissolved in DMSO (20mM) were prepared and further diluted to a final concentration of 50µg/mL. One hundred microliter aliquots of diluted solution of extracts were added to each well. The cultures were exposed for 48h to the extracts at concentration of 50µg/mL. After the incubation period, cells were fixed to the plastic substratum by the addition of 50µL of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4^{o}C for 1h, washed with tap water and air-dried. The trichloroacetic-acid-fixed cells were stained by the addition of 0.4% sulforhodamine B (SRB). Free SRB solution was then removed by washing with 1% aqueous acetic acid. The plates were then air-dried, and the bound dye was solubilized by the addition of 100µL of 10mM-unbuffered Tris base. The plates were placed on a shaker for 5 min, prior to analysis. Optical density was determined in the Ultra Microplated Reader (Elx 808, BIO TEK Instruments Inc) while the absorbance was measured at 515nm. Each experiment was repeated three times and the results were expressed as average values.

3. Results and Discussion

Higher fungi are a major source of biological active natural substances among many diverse organisms which provide a rich variety of active metabolites (Liu, 2007). There are potentially many bioactivities and novel compounds still to be discovered in higher fungi since until now only a few numbers of higher fungi have been biologically and chemically investigated (Liu, 2007; Oyetayo, 2011). Nigeria is extraordinarily rich in higher fungi. However, there are few data on the medicinal uses of these fungi. The current study reports the total phenol, antioxidant and cytotoxic effects of extracts of three wild mushrooms, *Lenzites betulina* , *Trametes vesicolor* and *Coriolopsis polyzona* collected from Nigeria.

The total phenol content of the extracts ranged from 8.22µgGAE/mL to 60.54 µgGAE/mL (Figure 1). The highest phenolic content was recorded for CET (60. 54 µgGAE/mL). Generally, the phenolic content of ethanolic extracts was higher than extracts obtained with hexane and ethyl acetate. It has been reported that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g is ingested daily from a diet rich in fruits and vegetables (Tanaka *et al*., 1998).

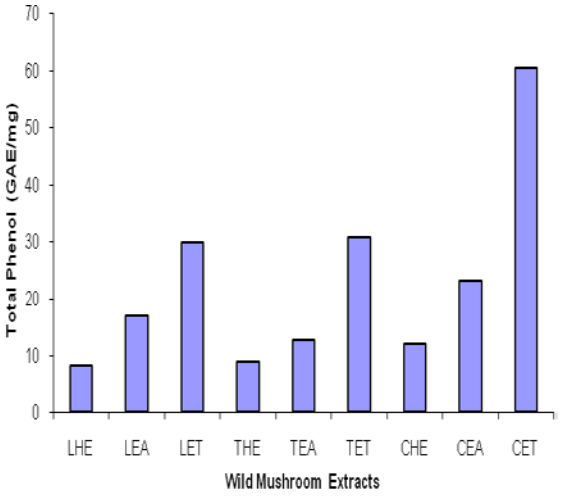

Figure 1. Total Phenol Content (GAE/mg) of Extracts of Wild Mushrooms.
Values are means of replicates (n=3). LHE: hexane extract of *L. betulina*, LEA: ethyl acetate extract of *L. betulina*, LET: ethanol extract of *L. betulina*, THE: hexane extract of *T. vesicolor*, TEA: ethyl acetate extract of *T. vesicolor*, TET: ethanol extract of *T. vesicolor*, CHE: hexane extract of *C. polyzona*, CEA: ethylacetate extract of *C. polyzona* and CET: ethanol extract of *C. polyzona*.

The ability of extracts to scavenge for DPPH radicals is presented in Table 1. The extracts displayed concentration dependent DPPH scavenging activity. The scavenging activity of extracts at 1000µg/mL ranged from 14.65% to 77.51%. Ethanolic extracts displayed better DPPH scavenging capacity when compared with the other extracts. This may be as a result of higher phenolic contents of ethanolic extracts (29.88 µgGAE/mL to 60.54µgGAE/mL). It had been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioglu *et al*., 1998). In this study, the correlation coefficient of DPPH activity of extract with the phenolic content was found to be 0.996.

The inhibition of the formation of thiobarbituric acid reactive species (TBARS), a measure of lipid peroxidation was concentration dependent and it ranges from 7.97% to 91.18% at 1000µg/mL. Generally, the ethanolic extracts exhibited higher TBARS inhibition (85.46% to 91.18%) when compared to the other extracts (Table 1). The higher TBARS inhibition exhibited by ethanolic extracts may be as a result of higher phenolic content present in it. In general, a correlation between higher antioxidant activity and larger amount of total phenolics was found in the mushroom extracts. The inhibition of the formation of TBARS by ethanolic extracts was higher than the second positive control, tocopherol (63.78%).

Table 1. Scavenging of DPPH radicals (%)* and inhibition of the production of TBARS (%)*in rat brain homogenate by Wild Macrofungi Extracts

	DPPH Scavenging			Inhibition of TBARS production		
Extracts	**10µg /ml**	**100 µg /ml**	**1000 µg /ml**	**10µg /ml**	**100 µg /ml**	**1000 µg /ml**
LHE	0.68	2.95	34.19	0.00	0.00	7.97
LEA	3.14	3.55	39.89	0.00	0.00	25.91
LET	2.37	6.54	70.99	0.00	2.78	90.90
THE	1.45	1.84	14.65	0.00	0.00	18.68
TEA	1.31	3.68	25.44	0.00	4.77	58.07
TET	2.98	10.12	43.77	0.00	1.91	91.18
CHE	0.87	2.85	21.28	0.00	0.00	11.13
CEA	3.38	11.61	44.20	8.37	11.97	75.81
CET	4.55	17.16	77.51	0.00	16.08	85.46
BHT	43.42	87.87	88.22	96.26	97.00	97.90
Tocopherol	35.89	90.02	90.71	63.78	98.54	98.80

Growth inhibition of human cancer cell lines by extracts is presented in Table 2. The inhibition of cell lines varies from one extract to the other. However, the following extracts, THE, CHE, TEA, CEA and TET exhibited significant inhibition of the various cell lines. Overall, ethanol extract of *Trametes vesicolor* (TET) showed the highest cytotoxic activity with 100% inhibition of HCT-15, MCF-7 and SKLU-1 cancer cell lines. A *β*-glucans, krestin from cultured mycelia biomass of *Trametes versicolor* (Turkey Tail) had earlier been reported to possess antitumour activity (Ikekawa 2001; Wasser, 2002). The least cytotoxic effect was exhibited by ethyl acetate extract of *Lenzites betulina* (LEA). Growth inhibition of K562 was the lowest when compared to the inhibition of other cell lines by the extracts.

Table 2. Cytotoxic Effects (%)* of Wild Macrofungi Extracts on Human Cancer Cell lines.

Extracts	U251	PC-3	K562	HCT-15	MCF-7	SKLU-1
LHE	NA	NA	41.85	17.65	37.93	25.15
LEA	NA	23.55	NA	NA	NA	NA
LET	NA	11.65	3.50	22.00	22.31	14.28
THE	13.38	51.95	22.28	41.45	63.78	25.15
TEA	45.46	78.80	NA	76.77	92.26	98.12
TET	46.15	84.97	12.77	100	100	100
CHE	12.90	37.77	16.58	61.12	52.23	56.47
CEA	76.68	59.99	NA	95.13	74.80	92.74
CET	NA	18.77	28.50	15.70	21.00	12.90

*Values are mean of replicates (n=3). NA: No Activity. U251:human glioblastoma, PC-3:human prostatic adenocarcinoma, K562: human chronic myelogenous leukemia, HCT-15: human colorectal adenocarcinoma, MCF-7: human mammary adenocarcinoma and SKLU-1: human lung adenocarcinoma.

In conclusion, significant DPPH scavenging effect, inhibition of the formation of TBARS and growth inhibition of cancer cell lines demonstrated by extracts indicates that these wild macrofungi contain bioactive compounds that may be used in ameliorating the problem of free radicals and cancerous cells. Further works of isolation, purification, identification and bioassay of specific bioactives from these macrofungi will be the next focus of this research.

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