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Evaluation of Compounds Extracted from Eight Genera of Wild Mushrooms from Nigeria for Anti-cell Proliferation Activity in Vitro

Adongbede, Erute Magdalene[1]*, Aduralere Israel Temitope[2]
Department of Botany, University of Lagos, Akoka, Yaba, Lagos, Nigeria

Abstract
Mushrooms have bioactive compounds that have antimicrobial, anti-cancer and antioxidant activities among other medicinal benefits. In the present study, we examined the anti-cell proliferation activities of mushrooms from eight genera obtained from the wild in Nigeria. *Saccharomyces cerevisiae* was used as a model organism to screen mushroom extracts for anti-cell proliferation activity. Polyphenols, high molecular weight polysaccharides and low molecular weight compounds from aqueous extracts were obtained from the test mushrooms using methanol and water respectively. The extracts were screened in vitro at different concentrations of extracts with the CyQuant cell proliferation assay. The high molecular weight polysaccharides from tested mushrooms reduced cell proliferation (96.79% inhibition in *Ganoderma multipileum* Ding Hou to 66.71% inhibition in *Coltricia perennis* (L.) Murrill at 10.00mg/ml). Percentage inhibition caused by low molecular weight compounds varied from 94.22% (*Ganoderma multipileum*) to 76.19% (*Coltricia perennis)* at 10mg/ml. Percentage of inhibition with the polyphenols varied from 94.12% (*Microporus xanthopus* Fr) Kuntze) to 79.82% (*Coltricia perennis*) at high doses. High molecular polysaccharides, low molecular weight compounds and polyphenols from mushrooms have anti-cancer properties. The CyQUANT assay proliferation kit was a very efficient tool for screening extracts from wild mushrooms for anti-cell proliferation activities. Medicinal mushrooms in Nigeria show a lot of promise as a reservoir for drug discovery particularly in the area of cancer research.

Keywords: *Saccharomyces cerevisiae,* Percentage inhibition, Polysaccharides, Polyphenols, Cell proliferation.

Introduction

Cancer is one of the leading causes of death among people of all ages around the world [1]. The search for prevention and cure of the disease has led to a lot of resources and research been put into drug discovery from various sources. The treatment of cancer involves taking drugs for prevention and the use of chemotherapy, all of which have serious adverse effects [1]. Researches into new drugs for cancer target and remove cancer cells with compounds that act as immunopotentiators, immuno-iniators and biological response modulators [1]. Mushrooms are an unlimited source of compounds which are modulators of tumour growth and as such highly favoured for cancer prevention and treatments [2]. Compounds extracted from mushrooms have been used to successfully inhibition cell proliferation and reduce or stop progression of different types of cancers. Screening compounds extracted from wild mushrooms harnessing the potential benefits nutritionally healthy and medicinal mushroom species not yet studied have high potentials for drug discovery.

Mushrooms have been used from ancient times in Nigeria for medicinal purposes. The first record of mushroom used as a hallucinogenic agent was credited to the Yoruba tribe in Nigeria [3]. The use of mushrooms in traditional medical practice is therefore not new and a common practice in Nigeria [4]. Mushrooms abound in the wild in Nigeria and are yet to be properly studied or studied at all. Edible and non-edible mushrooms have gained popularity for their medical importance [1]. These

*Email: e.adongbede@gmail.com

medicinal mushrooms possess an unlimited source of polysaccharides and polysaccharide-protein complexes in their tissues [5] [6]. These polysaccharides are biologically active as anti-tumour and immuno-stimulative agents. Of particular interest are high molecular weight polysaccharides from mushrooms because they enhance innate and cell-mediated immune responses and have a proven record of anti-tumour activities in labouratory animals and humans [7]. High molecular weight polysaccharides like lentinan, schizophyllan and krestin from different mushrooms are known anticancer agents [1]. Records show that low molecular weight compounds like isoflavones, catechols, amines, triglycerols and sesquiterpenes extracted from mushrooms have anti-tumour activities too [2]. These low molecular weight compounds from mushrooms target processes such as angiogenesis, apoptosis, cell cycle regulation and signal transduction cascades [7]. Polyphenol extracts of gilled mushrooms like *Pleurotus eryngii* have potentials to inhibit cell proliferation of human colon cancer cells without any cytotoxic effects [8]. Mushrooms therefore are potential sources of new drugs for the prevention and treatment of cancer.

Saccharomyces cerevisiae is a known and accepted eukaryote model used for investigating various molecular and cellular biological functions [9]. Studies indicate that 30% of known genes in humans that can cause disease, have a counterpart in yeast [10]. Yeast is used as a simplified model to screen extracts that have potentials to inhibit or stop cell proliferation. The screened compounds can then be taken for further studies with proper cell lines for individual cancer related diseases. The use of yeast for screening extracts is justified by the fact that it is a unicell and a eukaryote and can, therefore, bridge cell and organism-based assays. The ease of manipulation of yeast cells for the study of both biochemical and genetic analysis has made them invaluable tools for cancer research. It is therefore possible and convenient to screen extracts for cancer relevant fundamental biological processes like cell cycle progression, DNA replication and segregation using the yeast system [9]. Yeast cells are well adapted for growth in microtiter plates as it has a 90 minutes doubling time which makes it possible to complete investigation within a short time while examining exposure to drug or extract. The budding yeast *S. cerevisiae, therefore,* presents itself as a rapid, inexpensive and efficient screening tool. It has been used in the past to screen world leading compounds used in therapeutic applications for quite a number of human cancers [11]. Yeast cells have assisted in the determination of the sensitivity or resistivity of cancer cells to anticancer drugs. The use of yeast cells for the identification and production of new drugs and targets is, therefore, imperative [12]. The practice of using yeast cells as a research tool has its drawbacks as they are not capable of modeling aspects of tumour progression such as angiogenesis, tissue invasion and metastasis. Never the less yeast screenings are fast and powerful tools for screening compounds and basic mechanisms in anticancer research [13].

The CyQUANT cell proliferation assay is a very sensitive fluorescence-based microtiter assay used for evaluating inhibition of cell proliferation. The assay makes use of the CyQUANT GR, which produces fluorescence after binding to cellular nucleic acids; whose activity can be measured using standard fluorescein excitation and emission wavelengths. The assay measures cell proliferation and is therefore used for determination of inhibition. The assay is simple, fast and convenient for measuring a large number of samples within a short time [14].

This study investigated the anti-cell proliferation activities of the polyphenol, polysaccharide and low molecular weight compounds in the aqueous extracts of ten different species of mushrooms obtained from the wild in Lagos, Nigeria viz- *Crepidotus applanatus* (Pers.) P. Kumm*, Neonothopanus nambi* (Speg) R.H Peterson *&* Krisai*, Gymnopilus lepidotus* Hesler*, Ganoderma multipileum* Ding Hou*, Microporus xanthopus* (Fr) Kuntze, *Trametes pubescens (*Schumach.) Pilåt*, Trametes ochracea* (Pers.) Gilb & Ryvarden*, Trametes suaveolens* (L) Fr.*, Coltricia perennis* (L.) Murrill and *Lentinus squarrosulus* (Mont.) using *Saccharomyces cerevisiae* as the model organism.

Materials and Methods

Collection and drying of mushroom specimens.

Fresh sporophores of mushrooms were collected from the Lekki Conservation Center (NCF) Lagos, Nigeria and University of Lagos Campus at Akoka, Yaba, Lagos. The collection locations and GPS coordinates are stated in Table-1.

Table 1-Test Mushrooms and Collection Sites with GPS coordinates

	Test Mushrooms	Location	GPS Coordinates
1.	*Crepidotus applanatus*	Lekki Conservation Centre, Lagos, Nigeria	Latitude: 6.4384 Longitude: 3.5368
2.	*Neonothopanus nambi*	Lekki Conservation Centre, Lagos, Nigeria	Latitude: 6.4411 Longitude: 3.5352
3.	*Gymnopilus lepidotus*	Lekki Conservation Centre, Lagos, Nigeria.	Latitude: 6.4382 Longitude: 3.5368
4.	*Ganoderma multipileum*	High Rise, University of Lagos, Lagos, Nigeria.	Latitude: 6.4231 Longitude: 3.3836
5.	*Microporus xanthopus*	Lekki Conservation Centre, Lagos, Nigeria.	Latitude: 6.4368 Longitude: 3.5357
6.	*(a)Trametes pubescens*	Lekki Conservation Centre, Lagos, Nigeria.	Latitude: 6.4383 Longitude: 3.5367
	(b)Trametes ochracea	Lekki Conservation Centre, Lagos, Nigeria	Latitude: 6.4413 Longitude: 3.5352
	(c)Trametes suaveolens	Lagoon front, University of Lagos, Lagos, Nigeria.	Latitude: 6.5146 Longitude: 3.3976
7.	*Coltricia perennis*	Lekki Conservation Centre, Lagos, Nigeria	Latitude: 6.4414 Longitude: 3.5353
8.	*Lentinus squarrosulus*	High Rise, University of Lagos, Lagos, Nigeria.	Latitude: 6.5085 Longitude: 3.3963

The mushrooms were carefully cleaned and dried using a dehydrator (Stockli Dorrex Dehydrator, Switzerland) at a temperature of 40^0C for 12hours. Voucher specimens of the test mushrooms were deposited in the University of Lagos, Herbarium. The larger mushrooms were cut in bits before drying in the dehydrator. The specimens for the study were then milled to fine powder with a Kenwood Multi-Mill (Kenwood Ltd., UK) and stored at 4^0C until they were used.

Preparation of Mushroom Extracts (Extraction of Polyphenols, High molecular Polysaccharides and Low Molecular Weight Compounds)

The polyphenols and polysaccharides were extracted from the pulverized mushroom specimens (10g) using acidified methanol (200ml) following a modified version of Bridgers *et al.,* [15] and distilled water (200ml) [16] respectively. The polyphenols were extracted for 1hr with beaker on magnetic stirrer at 250rpm at room temperature. The extract was recovered by filtering with Whatman No. 4 paper to remove tissues. The extraction process was repeated twice. The supernatants were pooled together and concentrated in a rotary evaporator at 40^0C under reduced pressure. The supernatant was evaporated to dryness and the dried extract was dissolved in 70% methanol to make a stock solution of 50mg/ml and stored at 4^0C. Polysaccharides were extracted with boiling water in a water bath with shaker at 150rpm and tissues were recovered from the suspension by filtering with Whatman No. 4 filter paper. The extraction process was also repeated twice, and the supernatant pooled together and concentrated in a rotary evaporator under pressure at 60^0C. the concentrated solution was lyophilized and resuspended in distilled water by boiling for 1hour. Polysaccharides were precipitated out with 80% ethanol at 4^0C for a period of 6-8hrs (left in the fridge overnight). The precipitate was recovered by centrifuging at 10,000rpm for 10 minutes and the supernatant decanted into falcon tubes. The obtained precipitate was further purified using a DEAE cellulose tube and high molecular weight carbohydrates of 12,000 Da was dialyzed with distilled water for 72h to further remove low molecular weight carbohydrates [16]. Dried extracts were dissolved in the extracting solvent (hot water for 1hour). The supernatant of the precipitated polysaccharide, which contained low molecular weight components (mono- and disaccharides, oligosaccharides, amino acids and some phenols) [16] were concentrated in a rotary evaporator (Crude Low Molecular Weight Compounds/Extracts).

Methanol extracts (polyphenols) were dissolved in 70% methanol and high molecular weight polysaccharides were dissolved by boiling in hot water for one hour. The low molecular weight compounds dissolved in 70% ethanol. All extracts were standardized to a stock solution of 40mg/ml for the assay.

Table 2-Yield of polyphenols, high molecular weight polysaccharides and low molecular weight compounds from Tissues of Test Mushrooms

Test Mushrooms	Yield of Polyphenols (%)	Yield of Polysaccharide (%)	Yield of Low Molecular Weight Compounds (%)
1. Crepidotus applanatus	5.96±0.41[a]	6.36±0.09[a]	6.32±0.93[a]
2. Neonothopanus nambi	8.55±0.29[a]	5.64±0.20[a]	6.23±0.75[a]
3. Gymnopilus lepidotus	6.14±0.92[a]	6.53±0.39[a]	12.40±0.63[b]
4. Ganoderma multipileum	7.21±0.20[a]	11.93±0.84[b]	6.17±0.48[a]
5. Microporus xanthopus	9.44±0.72[ab]	12.23±0.49[b]	11.76±0.94[b]
6. (a).*Trametes pubescens*	8.37±0.21[ab]	18.27±0.53[c]	5.93±0.72[a]
(b). *Trametes ochracea*	5.450.32[a]	5.31±0.03[a]	11.76±0.54[b]
(c). *Trametes suaveolens*	5.72±0.38[a]	5.83±0.59[a]	6.01±0.31[a]
7. Coltricia perennis	9.22±0.92[ab]	6.28±0.50[a]	5.65±0.63[a]
8. Lentinus squarrosulus	5.67±0.49[a]	5.78±0.68[a]	5.51±0.45[a]

*Superscripts with different letters are significantly different at P≤0.05 with the Duncan's multiple range test

Standardization of *Saccharomyces cerevisiae (*Yeats Cell Culture)

Saccharomyces cerevisiae (ATCC 204508 (S288C)) was obtained from the ATCC (American Type Culture Collection). The cell cultures of the *Saccharomyces cerevisiae* were standardized to a cell concentration of 1.5 x10^5 by serial dilution. A concentrated yeast cell suspension was made using the Difco™ Yeast Extract-Peptone-Dextrose (YPD) Broth as the growth medium following manufacturer's instruction. Serial dilutions were made in the first two rows of wells of the 96 well plate such that the cell number ranged from (150,000 to 3,750) (Figure-1) for standard curve generation and control treatments.

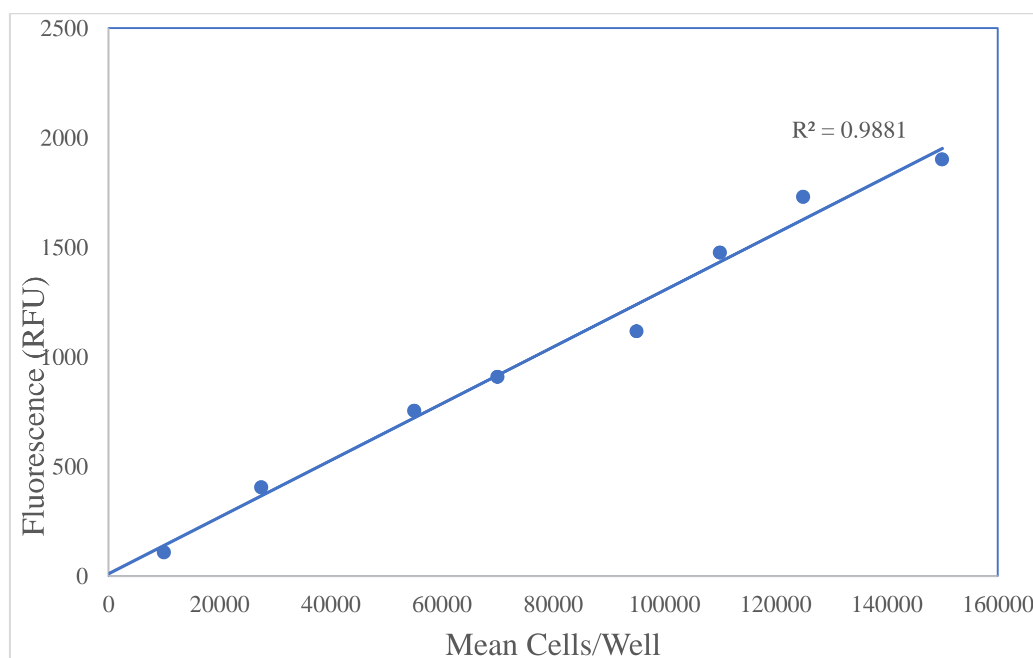

Figure 1-Standard Curve for quantification of *Saccharomyces cerevisiae* cells using CyQuant Cell Proliferation Assay

CyQUANT Cell Proliferation Assay

The CyQuant cell proliferation assay (Thermo-Fisher Scientific) was performed according to manufacturer's (Life Technologies) protocol.

The effects of the three extracts from each of the ten mushrooms on yeast cell proliferation was tested in the microtiter plates. The treatments involved plating cells at (1x 10^5) 50µl, 100µl of YPD broth and 50µl of each extract into each well of the 96well microplate in triplicates for each of the extract of the ten test mushrooms. The plates were incubated for 48hours at 30ºC and after incubation, the medium was removed from the wells leaving the cells behind after spinning down.

The cells in the microplate were then frozen at -70^{0}C overnight, the freezing of the cells is important for efficient cell lysis in the CyQuant assay. After freezing, the plates were thawed at room temperatures and then 200 µL of the CyQuant GR dye previously prepared was added to each sample well to stain the DNA following manufacturer's instructions. The plates were incubated at room temperature for 2-5 minutes and read using a fluorescence the BioTek Synergy microplate reader with excitation at ~480 nm and emission at ~520 nm. The fluorescence data were converted to relative live cell numbers using the standard curve of cells prepared during the assay. The percentage inhibition of cell proliferation was calculated from the concentrations of cells got the extracts and control wells using the formula

$$\text{Percentage Inhibition} = \frac{\text{Number of Cells in Control} - \text{Number of Cells in Treatment}}{\text{Number of Cells in Control} + \text{Number of Cells in Treatment}} \text{ x } 100$$

Results

The extracts from the eight different mushrooms were active and affected cell proliferation in varying degrees. The CyQuant Cell Proliferation kit measured effect of the extracts on cell proliferation by the model organism within a period of 48hours after extraction of the various compounds from the ten test mushrooms.

Inhibition of Cell Proliferation by Polyphenol Extracts from Test Mushrooms

The polyphenol extracts of all tested mushrooms as shown in Figure-2 inhibited cell proliferation of the model organism with percentage inhibitions varying between 94.12% in *Trametes pubescens* to 79.82% in *Coltricia perennis* at 10mg/ml. Inhibition of yeast cell proliferation by polyphenol extracts was dose dependent. The inhibition of yeast cell proliferation at 5.0mg/ml was above 50% inhibition in two tested mushrooms -*Gymnopilus lepidotus* and *Trametes pubescens* with the polyphenol extracts. All tested mushrooms recorded over 50% inhibition of cell proliferation at 7.5-10.00mg/ml concentrations with the polyphenolic extracts. Percentage inhibition of yeast cell proliferation by polyphenol at the 2.50mg/ml was less than 50% for all test mushrooms.

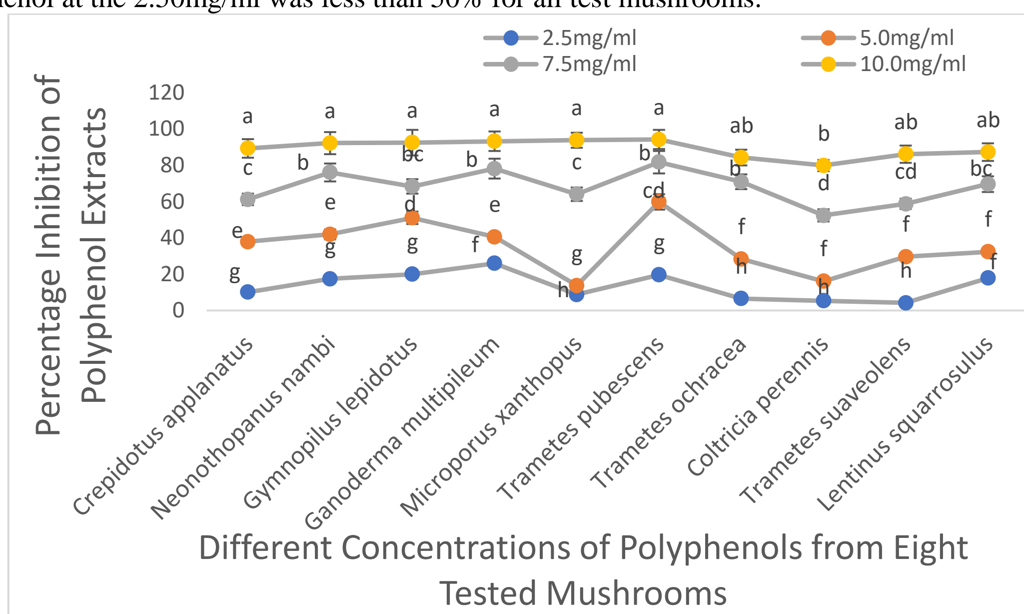

Figure 2-Effect of Different Concentrations of Polyphenol Extracts of Eight Tested Mushrooms on Yeast Cell Proliferation

*Superscripts with different letters are significantly different at P≤0.05 with the Duncan's multiple range test

Inhibition of Cell Proliferation by High Molecular Weight Polysaccharides from Test Mushrooms

The high molecular weight polysaccharides of all the tested mushrooms inhibited cell proliferation in varying degrees particularly at 10.00mg/ml (Figure-3). Inhibition of yeast cell proliferation was dose dependent with percentage inhibition by the high molecular weight polysaccharides varying from 92.79% in *Ganoderma multipileum* to 66.71% in *Coltricia perennis* (Figure-3) at the highest concentration (10mg/ml).

The percentage inhibition of all test mushrooms at the doses 2.5-5.0mg/ml recorded less than 50% activity with the high molecular weight polysaccharides (Figure-3)

Seven out of the ten test mushrooms evaluated recorded percentage inhibitions that were higher than 50% at higher doses (7.5-10mg/ml) with the high molecular weight polysaccharides (Figure-3).

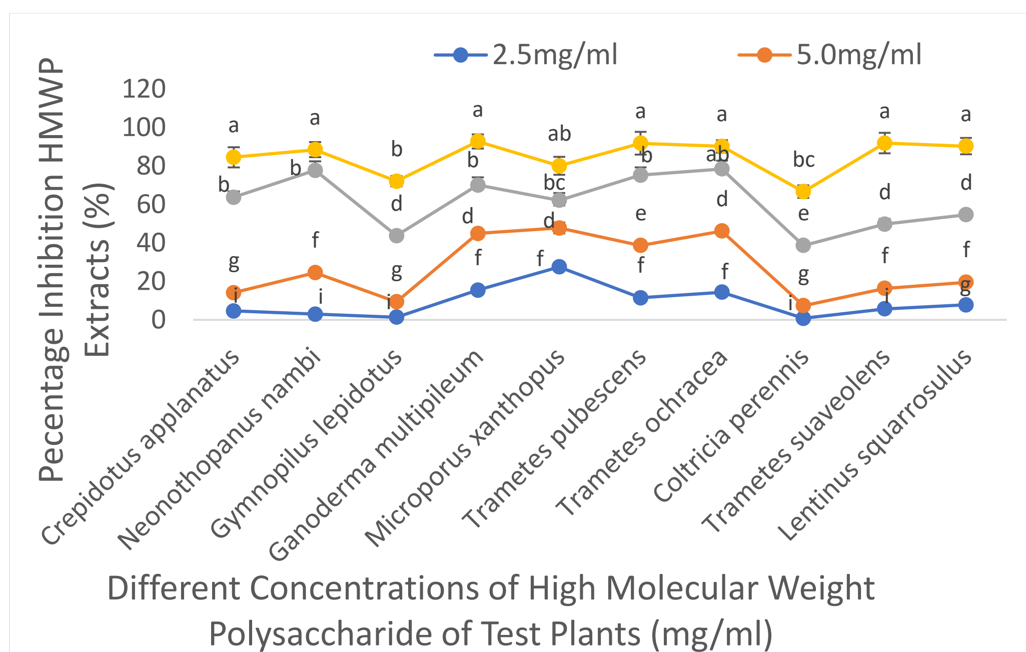

Figure 3-Effect of High Molecular Weight Polysaccharide extracts of ten test mushrooms on yeast cell proliferation

**Superscripts with different letters are significantly different at P≤0.05 with the Duncan's multiple range test

Inhibition of Cell Proliferation by Low Molecular Weight Compounds from Aqueous Extracts of Test Mushrooms

All the low molecular weight compounds from the aqueous extracts of the eight tested mushrooms inhibited cell proliferation of the model organism at 10.00mg/ml. Percentage inhibition of cell proliferation varied from 94.22% in *Ganoderma multipileum* to 76.19% in *Coltricia perennis* at this dose (Figure-4). Inhibition of yeast cell proliferation by the low molecular weight compounds were also dose dependent (Figure-4). Percentage inhibition of cell proliferation at 2.5-5.0mg/ml were all below 50% inhibition for all tested mushrooms. Inhibitory effects of seven tested mushrooms at 7.5mg/ml varied between 20-80% inhibition (Figure-4). There was no significant difference between anti-cell proliferation effect of the 7.5mg/ml dose of *Crepidotus applanatus, Ganoderma multipileum* and the 10.00mg/ml dose of the test mushroom *Coltricia perennis* with the low molecular weight compounds at p≥ 0.05 using the Duncan's multiple range test (Figure-4).

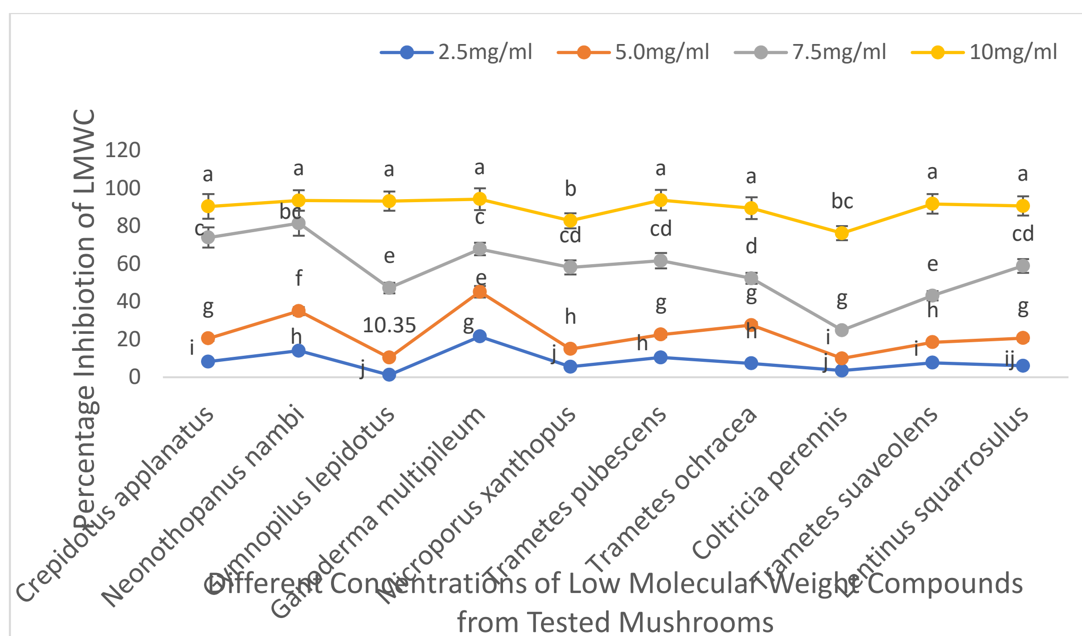

Figure 4-Effect of Low Molecular Weight Compounds from Eight Tested Mushrooms on Yeast Cell Proliferation

Discussion

The CyQuant cell proliferation assay kit was used to successfully screen the extracts of the ten mushrooms investigated in this study with the model organism-*Saccharomyces cerevisiae*. The test mushrooms screened for anti-cell proliferation activity showed strong inhibitory effects at higher doses. Polyphenols, high molecular weight polysaccharides and low molecular weight compounds had inhibitory effects against yeast cell proliferation as previously recorded for some of these mushrooms and their extracts in a dose dependent manner [17]. Polyphenols, high molecular weight polysaccharides and the low molecular weight compounds extracted from the test mushrooms all had percentage inhibition of over 50% at the highest dose (Figures 2-4). Medicinal mushrooms like *Ganoderma* and *Trametes* species have been proven to have anticancer activities against cancer tested cell lines [18], [19], [20], [21]. Some extracts from *Trametes* species have gone into clinical trials for the treatment of breast cancer with positive results [22]. The test mushroom *Gymnopilus lepidotus* grows abundantly in Nigeria and is used locally as a medicinal mushroom. In the current study *G. lepidotus* recorded highly degree of percentage inhibition against cell proliferation with the three extracts tested especially with the polyphenol and low molecular weight compounds Figure-(2, 4). This report agrees with previous studies by Haiying and Yu, [23] with the human breast cancer cell line (MCF-7).

In their study cell proliferation was inhibited completely (100%) by extracts from *Gymnopilus* sp. There are different high molecular weight polysaccharides that have been extracted from different mushrooms with anti-tumour activities. Some of the high molecular weight polysaccharides that have been extracted from different species of mushrooms with proven anti-tumour activities include - homoglucans and heteroglucans like lentinan, Schizophyllan, Xyloglucan, Xylogalactoglucan [24]. Other high molecular weight polysaccharides like the glycans have also been shown to have anti-tumour activities and examples of such high molecular weight polysaccharides include fucogalactan, Xylan, mannan, galacto-glucomannan from different mushroom species [24]. The low molecular weight compounds separated from the aqueous extracts of the test mushrooms also showed strong inhibitory activity against cell proliferation in the model organism. The low molecular weight compounds in this complex include monosaccharides, disaccharides and compounds like quinones, amines, sesquiterpenes and steroids. Some of these low molecular weight compounds extracted from mushrooms have been proven to have anti-tumour effects. The compounds that have been implicated in anti-tumour activity and of mushroom origin include Panepoxydone, ergosterol, lucidenic acid, genistein [7]. The gilled mushroom *Lentinus squarrosulus* had strong anti-cell proliferation activities with the model organism. Extracts from this mushroom has also shown a lot of promise in previous

researches by Prateep et al., (2017) [25]. Extracts from *L. squarrosulus* have been used to induce apoptosis in human cell lung cancer cells [25]. The polyphenol extracts from the test mushrooms show great potentials as anti-cell proliferation agents. Polyphenols extracted from mushrooms have had been used to inhibit proliferation of cancer cell lines [26], [27]. Some of the polyphenols extracted from mushrooms with proven antitumour activities include hispolon [17] [28] [29].

Conclusion

In the current research, the CyQuant cell proliferation assay was used to successfully screen various extracts from ten test mushrooms within a short time. The compounds screened were high molecular polysaccharides, low molecular weight compounds and polyphenols. The extracts concentrations at 7.50mg/ml and 10.00mg/ml showed a lot of promise for anticancer activity. These extracts can be further purified and analyzed for further test using human cell lines. The use of yeast as a model organism and the CyQuant cell proliferation assay for screening compounds extracted from biological systems for anti-cell proliferation activity was quite efficient and fast. These methods help determine if a particular natural product has potentials as new drugs

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