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Clastogenicity Potential Screening of *Pleurotus pulmonarius* and *Pleurotus ostreatus* Metabolites as Potential Anticancer and Antileukaemic Agents Using Micronucleus Assay

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Abstract: Development of anticancer agents that will selectively destroy cancer cells without injury to normal cells has led to the discovery of novel immunotherapeutic agents such as *Pleurotus pulmonarius* and *Pleurotus ostreatus* metabolites. This study is to screen the agents of dreadful side effects of causing mutation after a prolonged use. Clastogenicity potential of the novel anti-cancer and antileukaemic agents *Pleurotus pulmonarius* and *Pleurotus ostreatus* metabolites was evaluated in this study. Wister rats were grouped into four with the test groups inoculated intraperitoneally at doses 64 and 16 mg/kg as 12.8 and 3.2% of the LD_{50} into the high and low dose rat groups respectively with each metabolite in a separate experiment. The treated rats were sacrificed after 24, 48 and 72 h post treatment. Cyclophosphamide (clastogen) was inoculated into the positive control group at doses 112 and 28 mg/kg while saline was used for the negative control group. In all the treatment groups, only the rats in the positive control group formed micronuclei in their bone marrow cells. There was only an increase in the formation of normochromatic and polychromatic erythrocytes in rat groups inoculated with *Pleurotus ostreatus* metabolites. There is no statistically significant difference ($p>0.05$) between the 3 post treatment sacrificing periods. Similar result was also obtained for *Pleurotus pulmonarius* group. The chromosomal damaging potential screening reveals that the *Pleurotus ostreatus* and *Pleurotus pulmonarius* metabolites are not clastogenic (genotoxic) that is, unlikely to cause cancer producing mutations, but rather enhanced erythropoiesis. They could therefore be useful anticancer agents when the potential is fully explored.

Key words: Clastogen, genotoxicity, normochromatic, polychromatic erythrocytes

INTRODUCTION

Cancer can be described as a neoplastic growth disorder seen in cells, which escape normal regulatory mechanism and establish an autonomous clone known as tumor. In malignant tumor, the cells show varying degree of incomplete differentiation, there is rapid growth rate, and cells undergoing mitosis are seen within them on microscopy. The most important attribute of malignant tumors is their invasiveness; they grow into the surrounding tissues in a destructive manner and the advancing edge of growth poorly delineated. A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of a normal tissue and which persist in the same excessive manner after the cessation of the stimulus, which evokes the change. Cancer can occur in any part of the body including tissues and organs. An example of cancer that occurs in tissue is leukaemia (Stass *et al*., 2000).

Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis. Substances that cause DNA mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Particular substances have been linked to specific types of cancer. Tobacco smoking is associated with many forms of cancer (Sasco *et al*., 2004), and causes 90% of lung cancer (Biesalski *et al*., 1998).

Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen

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(Seitz *et al*., 1998). Such chemicals may promote cancers through stimulating the rate of cell division. Faster rates of replication leaves less time for repair enzymes to repair damaged DNA during DNA replication, increasing the likelihood of a mutation (English *et al*., 1997; Feychting *et al*., 2005).

Clastogenicity describes the process by which certain substances cause one or more types of structural changes in chromosomes of cells. These substances called clastogens are agents that cause breaks in chromosomes that result in the gain, loss, or rearrangement of chromosomal segments. The genetic damage that results in chromosomal breaks, structurally abnormal chromosomes, or spindle abnormalities leads to micronucleus formation. The incidence of micronuclei serves as an index of this type of chromosomal damage. It has been established that essentially all agents that cause double strand chromosomes breaks (clastogens) induce micronuclei (Garriot *et al*., 1995).

Clastogens can also cause sister chromatid changes which are homologous chromatid strand, interchanges and reunion, which occur during DNA replications. Structural damages that are caused by clastogens to DNA of cells may persist if the cells divide before the damaged is repaired and may subsequently develop into cancer. This cancer is as a result of abnormal proliferation of cells leading to the production of defective cells (Kumar *et al*., 1997).

A mushroom is a macrofungus with a distinctive fruiting body, which can either be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked with the hand (Chang and Miles, 1992). The knowledge about the great potential of microscopic fungi for the production of bioactive metabolites eg penicillin, aspergillus etc, the experience in ethnomedicinal use of mushrooms, the ecologic need of fungi to produce bioactive secondary metabolites and the improved possibilities for genetics, pharmacological and chemical analysis, assumes that mushroom have a great potential for successful bioprospecting (Lindequist *et al*., 2005).

The *Pleurotus* specie comprises of a group of edible ligninolytic mushrooms with medicinal properties and important biotechnological and environmental applications (Badole *et al*., 2006). The bioactive molecules isolated from the different fungi are polysaccharides (Reshetnikov *et al*., 2001). Examples of the *Pleurotus species* are *Pleurotus ostreatus*, *Pleurotus florida*, *Pleurotus pulmonarius* etc. These species are promising as medicinal mushrooms, exhibiting haematological, antiviral, antibacterial, antibiotic, antifungal, hypocholesterolic, antioxidant, antitumor, anticancer, immunomodulatory, anti-inflammatory, hepatoprotective, antidiabetic, hypolipedemic, antithrombotic and hypotensive activities (Wasser and Weis, 1999).

Pleurotus pulmonarius has a high medicinal value and the compounds extracted from it exhibit activity against various diseases including hypertension (Ajith and Janardhanan, 2007). The medicinal beneficial effect was discovered independently in different countries and the awareness came not only from Asia but from Central Europe, South America and Africa (Zaidman *et al*., 2005). It is edible, nutritious and possesses significant antioxidant, anti-inflammatory and antitumor activities (Badole *et al*., 2006). It contains a pharmacological active polysaccharide that can be derived from its fruit bodies which is xyloglucan and xylanprotein (Reshetnikov *et al*., 2001).

The cytotoxicity and clastogenicity side effects of most previously used anticancer agents had led to the attempt of using *Pleurotus plumonarius* and *P. ostreatus* metabolites, as more readily available non-chemical agent to prevent or cure cancers. The aim of this work is therefore to absolve the potential anticancer agents from being clastogenic with the objective of determining the LD_{50} of the metabolites and carrying out micronucleus assay using wister rats.

MATERIALS AND METHODS

Study site: The study was conducted between December, 2009 and February, 2010 at the Animal house and pharmacology laboratory of Ladoke Akintola University of Technology, College of health sciences (Mercy land wing) Osogbo. Osun state, Nigeria.

Reagents: Fetal calf serum (Sigma, USA C8056). Methanol (CH_3OH, mol wt. 32.04), Giemsa stain (Gurr Microscopy materials, Prod 34034 both of BDH chemicals Ltd Poole England. Positive product control (Cyclophosphamide 500mg); Normal saline; *Pleurotus plumonarius* and *Pleurotus ostreatus* metabolites.

Determination of acute toxicity: This experiment demonstrates the method by which the median lethal dose (LD_{50}) was determined.

Method: Mice were placed into 7 different groups of eight per group of 2 replicates of 4 each and allowed to acclimatize for 7 days in the animal house. The mice were fasted for 24 h after which each of the mice was weighed. The mice used were of the same sex and of approximately the same weight. 20mg.ml^{-1} of *Pleurotus plumonarius* and *P. ostreatus* metabolites aqueous solution were prepared and orally administered to the mice based on their weight. Groups I to VII were administered with dose of 70,140,280,500,560,640 and 760, respectively. They were then fasted again for another 24 h after which LD_{50} was determined by the number of deaths recorded within 24 h post treatment (Table 1) (Rang *et al*., 2003).

Table 1: Summary of LD_{50} determination

Dose (mg/kg)	No. of animals	No. of deaths	Death (%)
70	8	NIL	0
140	8	NIL	0
280	8	NIL	0
500	8	4	50
560	8	Died instantly	100
640	8	Died instantly	100
760	8	Died instantly	100

Micronucleus assay:

Animal preparation: Wister rats used for this assay were allowed to acclimatize to the laboratory environment of the study. They were fed with conventional laboratory feeds and were given unlimited drinkable water. The rats were grouped into four with the treatment in each group replicated.

Test condition: 0.2 g of each metabolite was dissolved in 10.0 mL of distilled water, and 100 mg of cyclophosphamide was dissolved in 5.0 mL of distilled water.

Administration of doses: The first group was administered with *Pleurotus pulmonarius* metabolites, the second group was given *Pleurotus ostreatus* metabolites, the third group was given cyclophosphamide (positive control) while the last group (negative control) was given normal saline. The doses were administered to the animals intraperitoneally.

Bone marrow preparation: After administration of appropriate doses of each metabolite (64 and 16 mg/kg as high and low dose respectively), some of the rats were sacrificed after 24 h, some were sacrificed after 48hours and the last sacrifice was done after 72 h. After sacrificing the rats, their femurs were dissected by removing the femur caps and then flushing the marrow out with 0.5 mL was then spun at 1000 rpm for 5 min. After spinning, the tubes were decanted and the cell pellets were smeared onto glass slides. The smears fetal calf serum into centrifuge tubes. The flushed marrow were air-dried. After 24 h, the slides were fixed in absolute methanol for 5 min and dried. All the slides from bone marrow smears were stained with 5% Giemsa solution and viewed under microscope using oil immersion with x100 objectives.

RESULTS AND DISCUSSION

According to the result obtained from this experimental work, there was no micronucleus formed when the two agents were inoculated into the rats. Only the rats given cyclophosphamide (clastogen) which was the positive control formed micronuclei, (Table 2a, b and 3a, b). Also, the rate of micronuclei formation decreases every 24 h. This is in consonance with the report of a work done by Isai *et al*., (2009), on the effect of an extract of the Oyster Mushroom, *Pleurotus ostreatus* in an experimental Animal model. In the experiment, the mean±S.D. value of polychromatic erythrocytes of the animals given the metabolites of *Pleurotus ostreatus* for the duration of 24 h was compared with those given for the period of 48 and 72 h and there was a significant difference ($p<0.05$) at 24 h compared with 48 h and also 24 h compared with 72 hours but no difference ($p>0.05$) when 48 h was compared with 72 h. Also, the result of mean±S.D value obtained for Normochromatic erythrocytes counted for the animals given *Pleurotus ostreatus* metabolites at time intervals compared for 24 and 48 h was statistically significant and also at 24 h compared with 72 h was also significant but that of 48 hours compared with 72 h was not statistically significant. This implies that there was increase in the number of polychromatic erythrocytes and Normochromatic

Table 2a: Number of Micronuclei (MN) in 1000 Polychromatic Erythrocytes (PCE) and 1000 Nomochromatic Erythrocytes (NCE) at high dose of Pleurotus pulmonarius metabolites and cyclophosphamide

			MN in PCE (h)			MN in NCE (h)			PCE/NCE (h)		
Group	Dose (mg/kg)	No. of Rats	24	48	72	24	48	72	24	48	72
A	64	8	-	-	-	-	-	-	-	-	-
B	64	8	-	-	-	-	-	-	-	-	-
C	64	8	-	-	-	-	-	-	-	-	-
Negative control	28.8	4	-	-	-	-	-	-	-	-	-
Positive control	112	4	22	18	15	19	21	14	1.16	0.86	1.07

Table 2b: Number of Micronuclei in 1000 Polychromatic Erythrocytes (PCE) and 1000 Nomochromatic Erythrocytes (NCE) at low dose of Pleurotus pulmonarius metabolites and Cyclophosphamide

			MN in PCE (h)			MN in NCE (h)			PCE/NCE (h)		
Group	Dose (mg/kg)	No. of Rats	24	48	72	24	48	72	24	48	72
A	16	8	-	-	-	-	-	-	-	-	-
B	16	8	-	-	-	-	-	-	-	-	-
C	16	8	-	-	-	-	-	-	-	-	-
Negative control	28.8	4	-	-	-	-	-	-	-	-	-
Positive control	28	4	11	09	06	10	07	06	1.10	1.29	1.00

Table 3a: Number of Micronuclei in 1000 Polychromatic Erythrocytes (PCE) and 1000 Nomochromatic Erythrocytes (NCE) at high dose of Pleurotus ostreatus metabolites and Cyclophosphamide

			MN in PCE (h)			MN in NCE (h)			PCE/NCE (h)		
Group	Dose (mg/kg)	No. of Rats	24	48	72	24	48	72	24	48	72
A	64	8	-	-	-	-	-	-	-	-	-
B	64	8	-	-	-	-	-	-	-	-	-
C	64	8	-	-	-	-	-	-	-	-	-
Negative control	28.8	6	-	-	-	-	-	-	-	-	-
Positive control	112	6	20	17	15	22	16	17	0.90	1.06	0.88

Table 3b: Number of Micronuclei in 1000 Polychromatic Erythrocytes (PCE) and 1000 Nomochromatic Erythrocytes (NCE) at low dose of Pleurotus ostreatus metabolite and Cyclophosphamide

			MN in PCE (h)			MN in NCE (h)			PCE/NCE (h)		
Group	Dose (mg/kg)	No. of Rats	24	48	72	24	48	72	24	48	72
A	64	8	-	-	-	-	-	-	-	-	-
B	64	8	-	-	-	-	-	-	-	-	-
C	64	8	-	-	-	-	-	-	-	-	-
Negative control	28.8	6	-	-	-	-	-	-	-	-	-
Positive control	28	6	12	08	06	10	07	04	1.20	1.14	1.50

Table 4: Comparison of two agents - Pleurotus ostreatus and Pleurotus pulmonarius with a negative control

	A		B		C	
	Control	Agent	Control	Agent	Control	Agent
No. of rats	4	4	4	4	4	4
Time	24	24	48	48	72	72
Pleurotus ostreatus						
PCEmean±SD	13.75±4.78	10.25±1.71	25.25±5.06	27.10±6.33	27.75±4.27	33.75±2.22
NCEmean±SD	9.00±1.41	5.75±2.06	19.75±3.86	23.00±3.74	24.25±4.92	24.00±4.76
Ratiomean±SD	1.51±0.39	1.34±0.35	1.27±0.47	1.21±0.28	1.56±0.13	1.46±0.37
Pleurotus pulmonarius						
PCEmean±SD	8.25±2.87	10.25±1.71	11.75±1.71	12.25±4.79	9.50±3.51	9.50±3.50
NCEmean±SD	8.75±1.50	5.75±2.06	12.75±2.06	9.00±2.94	8.75±2.22	8.25±2.22
Ratiomean±SD	0.93±0.19	1.34±0.35	0.95±0.30	1.50±0.72	1.26±0.49	1.23±0.49

Table 5: Comparison of the effect of Pleurotus ostreatus metabolites on PCE and NCE at different time intervals

	Time (h)	PCE	t, p-value	NCE	t, p-value	PCE/NCE	t, p-value
Agent A	24	5.11	0.00	8.08	0.00*	0.58	0.58
Agent B	48						
Agent A	24	16.79	0.00	7.04	0.00*	0.48	0.65
Agent C	72						
Agent B	48	0.20	0.09	0.33	0.75	1.10	0.31
Agent C	72						

PCE: Polychromatic erythrocytes, NCE: Normochromatic erythrocytes, *: $p>0.05$

erythrocytes of animals used after 24 h of administration of *Pleurotus ostreatus* but there was no further increase after 24 h of administration which means that time was not a factor to the effect of *Pleurotus ostreatus* metabolite on polychromatic erythrocytes and Normochromatic erythrocytes of animals (Table 4).

Also, for the metabolite of *Pleurotus pulmonarius* given to the animals, in all the time intervals there was none that shows statistically significant difference ($p>0.05$) (Table 5).

In the comparison of *Pleurotus ostreatus* with *Pleurotus pulmonarius* metabolites, the mean±S.D value of *Pleurotus ostreatus* metabolites on both polychromatic erythrocytes and Normochromatic erythrocytes of the animals shows higher values compared to that of *Pleurotus pulmonarius,* the difference which is statistically significant ($p<0.05$) (Table 4). From all the results gotten from this experimental work, it shows that both *Pleurotus ostreatus* and *Pleurotus pulmonarius* metabolites are not clastogenic. This also supports what Isai *et al.* (2009) obtained from the test for the clastogenic effect of *Pleurotus ostreatus*, which shows that it has none.

In addition to the fact that edible mushrooms have high nutritional value, a work done on the effect of mushroom on the nutritional balance of mushroom eaters reveals that mushroom eaters met the Daily Recommended Intake (DRI) and also the Recommended Daily Allowance (RDA) for 19 nutrients which include: calcium, copper, iron, magnesium, phosphorus, zinc,

Table 6: Comparison of the effect of Pleurotus pulmonarius metabolites on PCE and NCE at different time intervals

	Time (h)	PCE	t, p-value	NCE	t, p-value	PCE/NCE	t, p-value
Agent A	24	0.79	0.46	1.81	0.12	0.41	0.69
Agent B	48						
Agent A	24	0.26	0.81	1.65	0.15	0.37	0.72
Agent C	72						
Agent B	48	0.84	0.43	0.41	0.70	0.64	0.55
Agent C	72						

PCE: Polychromatic erythrocytes, NCE: Normochromatic erythrocytes

Table 7: Comparison of the effect of *Pleurotus ostreatus* with *Pleurotus pulmonarius* metabolites on polychromatic erythrocyte and normochromatic erythrocytes

	PCE Mean ± SD	NCE Mean± SD	Ratio Mean± SD
Pleurotus ostreatus	23.67, 10.93	17.58, 9.37	1.33, 0.32
Pleurotus pulmonarius	10.75, 3.42	7.67, 2.64	1.36, 0.50
F (ANOVA)	15.26	12.46	0.02
p-value	0.00*	0.00*	0.90

PCE: Polychromatic erythrocytes, NCE: Normochromatic, *: $p<0.05$

foliate, niacin, riboflavin, thiamin, vitamin A, B6, B12, C and E, energy, carbohydrate, fiber and protein. To support the reason for the nutritional boosting effect of mushroom particularly *Pleurotus ostreatus* and *Pleurotus pulmonarius* some works were done on the chemical composition of these two mushrooms. The results show that both mushrooms do not possess clastogenic properties but rather immune boosting effect on their eaters (Yitzhak *et al*., 1986; Sueli *et al*., 2002).

This experimental work reveals that the metabolites of mushroom screened *Pleurotus ostreatus* and *Pleurotus pulmonarius* have not demonstrated clastogenic properties and may not cause chromosomal damage in patients as they are not genotoxic.

Separate experiment was set up for *Pleurotus pulmonarius* and *P. ostreatus* metabolites. Thus, the LD_{50} obtained is 500 mg/kg for both metabolites (Table 1).

No MN was seen in both agents and negative control, but it was seen in positive control. The rate of MN formation however decreases every 24 h (Table 2, 3).

Table 4 shows that in the rats given *Pleurotus ostreatus* there was an increase in the polychromatic erythrocytes as the time increases compared with the control and also in rats given *Pleurotus pulmonarius* there was an increase in polychromatic erythrocytes but in the normochromatic erythrocytes in the two agents there was no increase compared to the control.

Table 5 shows the p-value and the values of student t-test when the time intervals were compared and there was a significant difference in the NCE at 24 and 48 h comparisons and also at 24 and 72 h comparison ($p<0.05$) but not at 48 and 72 h comparisons. PCE-Polychromatic erythrocytes and Nomochromatic erythrocytes.

Table 6 shows the p-value and student t-test values. When the time intervals were compared there was no significant difference between the PCE and NCE.

Table 7 shows the difference in polychromatic and nomochromatic erythrocytes when the *Pleurotus ostreatus* and *Pleurotus pulmonarius* metabolites were given to the rats. There is significant difference in the polychromatic and nomochromatic erythrocytes at $p<0.05$.

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