Immunomodulatory effects of aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms in cyclophosphamide-immunosuppressed Wistar rats

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ABSTRACT

Aims: To determine the immunomodulatory effect of aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms using an immunosuppression animal model.
Study design: Pre-clinical experimental study.
Place and Duration of Study: Department of Pharmacology & Therapeutics, College of Health Sciences and Division of Pharmacology, Department of Physiological Sciences, School of Veterinary Medicine, Makerere University, between August 2010 and December 2011.
Methodology: A total of 80 Wistar rats divided into 8 groups (n=10) were used in the experimental study. Cyclophosphamide (10mg/kg) was administered orally (p.o) to fifty (50) Wistar rats in the first 5 groups for 28 days. In addition, rats in group I received distilled water, groups II & III received 300mg/kg & 600mgkg of *Auricularia* sp extract and groups IV &V received 400mg/kg & 800mg/kg Pleurotus sp extract. Wistar rats in group VI received only 300mg/kg *Auricularia* sp extract, group VII received 400mg/kg *Pleurotus* sp extract and group VIII received only distilled water. Blood samples were collected on days 0, 14 and 28 determine the total and differential WBC counts. Data was presented as mean±SEM and analyzed using one-way ANOVA followed by student's t-test for statistical significance. Mean values were compared with initial values and the control group.
Results: No mortality of Wistar rats was observed over the 28-day experimental period. Cyclophosphamide caused significant reduction total WBC on day 14 and 28 compared with day 0 in control group from 11.26±0.59 on day 0 to 6.11±0.41 day ($p<0.05$) 14 & 4.12±0.22 ($p<0.05$) on day 28. Lymphocytes and Neutrophil counts were also significantly reduced in control group by day 28 compared to mushroom extract treated rats. Results show that aqueous extracts of *Auricularia* sp extract & *Pleurotus* sp mushrooms moderated the reductions in total & differential WBC on day 14 and 28 ($p<0.05$) as compared with control group. The mushroom extracts also increased total & differential WBC in normal rats as compared to the normal group (group VIII).
Conclusion: Aqueous extracts of *Auricularia* sp & *Pleurotus* sp mushrooms moderated cyclophosphamide-induced reduction in WBC in Wistar rats indicating potential benefit in chemotherapy induced immunosuppression.

Keywords*: Immunomodulatory, Auricularia, Pleurotus, aqueous extract, immunosuppression, Wistar rats*

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1. INTRODUCTION

Cyclophosphamide is probably one of the most prescribed anticancer drugs used for treatment of various forms of cancers. It is nitrogen mustard whose mode of action involves addition of alkyl groups to DNA thus slowing or stopping tumour growth (Bauman, 2001). Besides the cytotoxic effects of cyclophosphamide towards tumour cells, it also affects other cell types in the body most notably the immune cells which protect the body from harmful agents (Hou et al., 2007). Immunosuppression caused by cyclophosphamide and other anticancer drugs significantly complicates the course of cancer chemotherapy and contributes to the agony of the patients.

In regard to the immunosuppressive effects of anticancer chemotherapy, the stimulation of production of immune cells in an immunosuppression model has been classified as immunomodulation (Vigila, 2008). In fact, attempts are being made to incorporate traditional medicines with cancer chemotherapy to reduce the side effects of anticancer drugs through this immunomodulation (Gupta *et al*., 2010; Shukla, 2010). There is growing interest among biomedical scientists in the ability of some natural products to stimulate the production of immune cells in immunosuppressed animal models. Several sources including mushrooms are being screened for immunomodulatory compounds that can be used to enhance cancer chemotherapy.

Mushrooms which are popular for their nutritional and medicinal properties have recently been extensively investigated for their anticancer and immunomodulatory effects (Wasser et al., 2010). In Uganda, *Auricularia* sp (wood ear) and *Pleurotus* sp (oyster) mushrooms which naturally grow on decaying logs in rain forest are traditionally used for medicinal purposes by local communities for treatment of various ailments. These two species of mushrooms are reported to possess antibacterial, anti-tumour activity, antioxidant, anti-hypercholesteremic and immunomodulatory effects (Zhang et al., 2011). Polysaccharides, proteins and other compounds previously isolated from these two species of mushrooms have been found to stimulate immune cells both in vitro and in vivo (Synistya, et al., 2008). There is a great deal of evidence that these two species are a potential source of immunomodulatory compounds that can benefit patient care. In this study, we investigated the potential benefits of the aqueous extracts of the two mushroom species on markers of cyclophosphamide induced immunosuppression in using male Wistar rat model.

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2. MATERIAL AND METHODS

2.1 Experimental animals

One hundred (100) healthy male Wistar albino rats of approximately 8 weeks of age were purchased from the Faculty of Veterinary Medicine, Makerere University and maintained at a temperature of 25 ± 1 ℃ and relative humidity of 45 to 55% under 12-hr light : 12-hr dark cycle. The animals were allowed a 1 week acclimatization period with free access to food pellets and water *ad libitum*.

2.2 Mushroom samples and preparation of mushroom aqueous extract

The fruiting portion of the *Auricularia* sp*.* and *Pleurotus* sp mushrooms were collected from decaying logs and tree branches in Mabira and Mpanga Forest reserves in Uganda. Identification and authentication of specimens was done by a mycologist at the Department of Botany, Makerere University. Aqueous extracts were prepared from air-dried mushrooms using the methods described by Badole et al., (2009) and Mengyao et al., (2009). Five hundred (500g) of the air-dried mushroom samples were powdered mechanically and mixed into 1L of distilled water. The mixture was boiled for 1hr at 100℃ with frequent stirring and then left to cool. The extract was then filtered and concentrated using a freeze drier. The resulting brown concentrate was then reconstituted using distilled water for a final weight per volume of 100mg/mL and stored in a refrigerator at 4℃ until when it was required for use in the experiments.

2.4 Experimental design

The immunosuppression model for cyclophosphamide developed by Hou et al., (2007), in Wistar albino rats was used to evaluate the immunomodulatory effect of the mushroom extracts. Eighty (80) healthy male *Wistar* albino rats were randomized into eight groups (n=10). Wistar rats from 5 groups had induction of immunosuppression using 10mg/kg body weight cyclophosphamide and then received either mushroom extracts or distilled water as follows;

Group I: 2ml of distilled water + cyclophosphamide (10mg/kg b.w)
Group II: 300mg/kg Auricularia sp extract + cyclophosphamide (10mg/kg b.w)
Group II: 300mg/kg Auricularia sp extract + cyclophosphamide (10mg/kg b.w)
Group IV: 400mg/kg Pleurotus sp extract + cyclophosphamide (10mg/kg b.w)
Group V: 800mg/kg Pleurotus sp extract + cyclophosphamide (10mg/kg b.w)
Group VI: 300mg/kg Auricularia sp extract only
Group VII: 400mg/kg Pleurotus sp extract only
Group VIII: 2ml distilled water only
All treatments were administered via oral intra-gastric tubing.

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Selection of the two doses of mushroom extracts corresponded to doses that were 1/32 and 1/16 of the LD50 value calculated from a previous the acute toxicity study we conducted on the same mushrooms.

2.4.1 Animal monitoring

On experimental days 0, 14 and 28, whole blood samples were drawn from the tail vein of each Wistar rat into EDTA containers (1mL) and processed for total and differential WBC. Body weights were recorded weekly throughout the experimental 28 day period.

2.5 Statistical analysis

Data was presented as mean±SEM and analyzed for differences using One way ANOVA followed by a Student-Neumann-Keuls t-test. Comparison of mean WBC counts was done for test group with initial and the control group. The p-values <0.05 were considered statistically significant at 95% confidence level using Graph Pad Prism software, version 5.0.

2.6 Ethical issues

The experimental animals were handled in accordance with the OECD guidelines for testing chemicals and were allowed free access to food and clean water ad libtum. The experimental protocol was approved by the Makerere University, College of Health Sciences, Research and Ethics Committee.

3. RESULTS AND DISCUSSION

Wistar rats treated with cyclophosphamide alone (group I) had significant reduction in total (Table 1) and differential white blood cell counts on days 14 and 28 compared to day 0 (Table 2& 3). In addition to cyclophosphamide, *Auricularia* sp (group II & III) and *Pleurotus* sp (group IV&V) extract treated rats had moderate reductions in total and differential white cell counts on days 14 and 28 compared to day 0. The mean WBC counts in extract treated rats were all greater than those of group I at day 14 & 28 (Table 1). The rise in the total WBC count lowered by cyclophosphamide in Wistar rats was observed at 300 mg/kg and 600mg of *Auricularia* sp while 400mg/kg and 800mg/kg for *Pleurotus* sp extract. The rats treated with mushroom extracts had their white cell counts restored to almost near initial levels recorded on day 0 which were significantly greater than those observed in the control group. In normal Wistar rats, the mushroom extracts (i.e group VI for *Auricularia* sp and group VII for *Pleurotus* sp) there was a significant increase in total and differential white cell counts compared to control group. Results are presented as mean± SEM, in the tables 1, 2 & 3 below;

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Table 1. Mean total WBC of Wistar rats on day 0, 14 & 28

Group	Day 0	Day 14	Day 28
Group I	11.26±0.59	6.11±0.41**	4.12±0.22**
Group II	10.17±0.56	8.56±0.41[a]	8.77±0.85[a]
Group III	9.82±0.36	8.69±0.34[a]	8.41±0.23[a]
Group IV	10.07±0.74	7.07±0.38[a]	6.01±0.48**
Group V	10.52±0.44	8.76±0.36[a]	8.93±0.20[a]
Group VI	10.28±0.28	11.95±0.42[a]	12.15±0.72[a]
Group VII	10.91±0.31	11.44±0.32[a]	11.58±0.21[a]
Group VIII	10.77±0.21	10.75±0.32[a]	10.67±0.38[a]

***p<0.05 compared with initial values at day 0 in same group, [a]p<0.05 compared with group I*

Table 2. Mean lymphocyte counts of Wistar rats on day 0, 14 & 28

Group	Day 0	Day 14	Day 28
Group I	44.83±4.11	27.76±2.40**	26.42±2.65**
Group II	41.18±1.95	32.04±1.55**[a]	37.97±0.97[a]
Group III	40.70±1.60	39.93±0.34[a]	41.47±1.96[a]
Group IV	39.90±1.39	31.25±1.50**[a]	31.91±1.16**[a]
Group V	42.83±2.07	34.99±2.40*[a]	35.69±1.49[a]
Group VI	40.61±1.82	41.26±1.42[a]	46.82±1.63[a]
Group VII	40.10±1.43	41.19±0.89[a]	41.60±1.15[a]
Group VIII	38.56±1.63	37.64±1.51[a]	39.27±1.48[a]

***p<0.05 compared with initial values at day 0 in same group, [a]p<0.05 compared with group I*

Table 3. Mean Neutrophil counts of Wistar rats on day 0, 14 & 28

Group	Day 0	Day 14	Day 28
Group I	48.01±1.80	37.80±2.78**	37.14±5.15**
Group II	48.17±0.82	43.50±3.56**[a]	40.77±1.97[a]
Group III	48.93±1.60	45.48±3.56[a]	48.00±2.38[a]
Group IV	50.33±1.61	37.57±1.41**[a]	37.29±1.91**[a]
Group V	49.60±0.86	45.20±2.83*[a]	40.91±1.24[a]
Group VI	52.55±2.34	51.39±1.53[a]	51.44±0.74[a]
Group VII	49.23±1.47	51.20±0.74[a]	50.72±2.12[a]
Group VIII	49.66±1.26	49.08±2.23[a]	48.98±1.14[a]

***p<0.05 compared with initial values at day 0 in same group, [a]p<0.05 compared with group I*

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In our study, administration of cyclophosphamide at 10mg/kg to daily to Wistar rats successfully caused significant immunosuppression as previously described in a similar animal model (Hou et al., 2007). Both total and differential WBC counts were severely reduced in Wistar rats receiving cyclophosphamide only on days 14 and 28 owing to the effects of the drug on the bone marrow. The bone marrow has a high rate of cell proliferation and this makes it a sensitive target for cyclophosphamide cytotoxicity (Shukla et al., 2010). Destruction of stem cells in the bone marrow results into leucopoenia manifested as reduced levels of total and differential WBC in Wistar rats (Ghule et al., 2006).

The stimulation of production of White blood cells (WBC) in an immunosuppressed animal model has classified as an immunomodulatory effect (Vigila et al., 2008; Shukla et al., 2010). Aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms moderated the immunosuppressive effects of cyclophosphamide in male Wistar rats at doses that were far below the estimated lethal doses. This effect was considered a significant immunomodulatory effect of the two mushroom extracts in cyclophosphamide immunosuppressed Wistar rats. The extracts of *Auricularia* sp and *Pleurotus* sp mushrooms were found to increase total and differential WBC which was reduced by cyclophosphamide in Wistar rats. Both mushroom extracts were used at doses 1/16 and 1/32 levels below the estimated LD_{50} values of each mushroom species.

The present data demonstrates that the aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms can stimulate the activity of bone marrow to produce WBC. In normal Wistar rats, both extracts increased the total and differential WBC at doses 1/32 of their LD_{50} values. This observation may explain the observed restoration of WBC levels in immunosuppressed Wistar rats by the mushroom extracts on day 14 and 28. The results also suggest that of *Auricularia* sp mushroom extractives may possess greater immunomodulatory effects than *Pleurotus* sp extractives. This is based on the observation that *Auricularia* sp mushrooms was used at a lower dose than *Pleurotus* sp mushroom extracts doses used for the immunomodulatory experiments in which.

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The mechanisms through which *Auricularia sp* and *Pleurotus sp* mushrooms stimulate production of WBC in immunosuppressed rats was not explored in this study. However, we hypothesize that the observed immunomodulatory effect of these mushrooms may be related to compounds like proteins and polysaccharides previously isolated from mushrooms and reported to have immunomodulatory potential both *in vivo* and *in vitro* elsewhere (Zuzek et al., 2006; Liao et al., 2006 & Zhang et al., 2011). On the basis of the current data, we demonstrated that both *Auricularia* sp and *Pleurotus* sp mushrooms may be of potential benefit in anticancer-drug induced immunosuppression. This may be important in enhancement of cancer chemotherapy through reduction of side effects particularly the associated immunosuppression. Our extraction method of boiling corroborates the traditional methods of cooking the mushrooms for food and medicinal purposes by local communities.

2.6 CONCLUSION

Aqueous extracts of *Auricularia* sp and *Pleurotus* sp from Ugandan rain forests increased total and differential WBC counts in cyclophosphamide immunosuppressed Wistar rats. This effect was considered an immunomodulatory effect and shows the potential benefit of the mushrooms in enhancement of cancer chemotherapy through reduction of side effects of anticancer drugs especially immunosuppression.

2.7 ACKNOWLEDGEMENT

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2.8 COMPETING INTERESTS

The authors declare that there are no competing interests.

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REFERENCES

Badole, S.L., & Bodhankar, S.L. (2007). Interaction of aqueous extract of Pleurotus pulmonarius (Fr.) Quel.-Champ with acarbose in alloxan induced diabetic mice. Applied Biomedicine., 5,157–166.

Baumann, F. & Preiss, R. (2001). Cyclophosphamide and related anticancer drugs. J. Chromatogr. B. Biomed. Sci. Appl., 764,173-192.

Ghule, B.V., Murugananthan, G., Nakhat, P.D., & Yeole P.G. (2006). Immunostimulant effects of *Capparis zeylanica* Linn. leaves. J Ethnopharmacol.,108, 311–315

Gupta A., Guatam, M.K., Singh, R.K., Kumar, M.V., & Rao, C.H. (2010). Immunomodulatory of Moringa Oleifera Lam on cyclophosphamide induced toxicity in mice. Indian J Experimental Biology 14: 1157-1160.

Hou, F.X., Hui, F.Y., Tao, Y., & Wei C (2007). The immunosuppressive effects of 10 mg/kg cyclophosphamide in Wistar rats. Enviro Toxicol and Pharmacol., 24, 30-36.

Liao CH, Hsiao YM, Hsu CP, Lin MY, Wang JC, Huang YL, (2006). Transcriptionally mediated inhibition of telomerase of fungal immunomodulatory protein from Ganoderma Mushroom tsugae in A549 human lung adenocarcinoma cell line. Mol Carcinog., 45, 220–229.

Mengyao, Y., Xiaoyan, X., Yuan, Q., Xia, L., Zirhong, Y., & Linyong, Z (2009). Isolation of an anti-tumor polysaccharide from Auricularia polytricha (jew's ear) and its effects on macrophage activation. Eur Food Res & Techn., 228, 477-485.

Shukla, S.H., Saluja, A.K & Pandya, S.S (2010). Modulating effect of *Gmelina arborea* Linn. on immunosuppressed albino rats. Pharmacognosy Res., 2, 359–363.

Synytsya, A., Mčov · K., Jablonsk, I., Slukov · M., & Colov J. (2008). Mushrooms of genus Pleurotus as a source of dietary fibres and glucans for food supplements. Czech J Food Scie., 26, 441–446.

Vigila, G.A. & Baskaran, X. (2008). Immunomodulatory Effect of Coconut Protein on Cyclophosphamide Induced Immune Suppressed Swiss Albino Mice. Ethnobot Leaflets 12., 1206-1212.

Wasser, S.P. (2010). Medicinal mushroom science: history, current status, future trends, and unsolved problems. Int J Med Mushrooms, 12(1):1-16.

Zuzek, M.C., Peter, M., Kristina, S., Vojteh, C., & Robert, F., (2006). Toxic and lethal effects of ostreolysin, a cytolytic protein from edible oyster mushroom (Pleurotus ostreatus), in rodents. Toxicon 48: 264–271

Zhang, H., Wang, Z., Yang, L., Yang, X., Wang, X & Zhang, Z. (2011). In Vitro Antioxidant Activities of Sulfated Derivatives of Polysaccharides Extracted from Auricularia auricular. Int. J. Mol. Sci., 12, 3288-3302

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