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Immunomodulatory Effects of Aqueous Extracts of *Auricularia* sp and *Pleurotus* sp Mushrooms in Cyclophosphamide-Immunosuppressed Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all the authors. Authors ENM and GN wrote the main research grant winning proposal that formed the main idea for the work of this paper. Authors AHK and PEO carried out the laboratory experiments, performed the statistical analysis, and wrote the first draft of the manuscript. Authors ENM and CO managed the literature searches and supervised all the experiments. All the authors read and approved the final manuscript.

Research Article

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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 and Therapeutics, College of Health Sciences and Division of Pharmacology, Department of Physiological Sciences, School of Veterinary Medicine, Makerere University, between August 2010 and December

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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 respectively and Groups IV &V received 400mg/kg & 800mg/kg *Pleurotus* sp extract respectively. *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 to determine the total and differential WBC counts. Data is presented as mean±SEM and analyzed using one-way ANOVA followed by a student's t-test for statistical significance. Mean values are compared with initial values and the control group (Group VIII).
Results: No mortality of *Wistar* rats was observed over the 28-day experimental period. Cyclophosphamide though caused statistically significant ($p<0.05$) reduction in 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 14, & 4.12±0.22 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 & *Pleurotus* sp mushrooms significantly ($p<0.05$) moderated the reductions in total & differential WBC on day 14 and 28 as compared to the control group. The mushroom extracts also increased total and differential WBC in normal rats as compared to the normal group (Group VIII).
Conclusion: Aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms moderated cyclophosphamide-induced reduction in WBC in *Wistar* rats indicating potential benefit in chemotherapy induced immunosuppression. Application of these mushrooms in immune suppression research appears to be new as reflected in the literature. These are however preliminary data to be more completely documented by further experiments, possibly investigating also some aspect of immune cell functions (e.g. cytotoxicity or cytokine production).

Keywords: Immunomodulatory; aqueous extract; immunosuppression; wistar rats; wild edible mushrooms.

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 [1]. 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 [2]. Immunosuppression caused by cyclophosphamide and other anticancer drugs significantly complicates the course of cancer chemotherapy and worsens the condition 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 [3]. In fact, attempts are being made to incorporate traditional medicines with cancer chemotherapy to reduce the side effects of anticancer drugs through this immunomodulation [4,5]. 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 (including those of the genera Pleurotus and Auricularia) which are popular for their nutritional and medicinal properties have recently been extensively investigated for their anticancer and immunomodulatory effects [6,7]. Mushrooms from the genera Pleurotus and Auricularia are reported to possess antibacterial, anti-tumour activity, antioxidant, anti-hypercholesteremic and immunomodulatory effects [8,9,10,11]. There are, however, various species of mushrooms in these two genera which are yet to be identified and their medicinal potential profiled. Moreover, in many tropical countries, mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products and they represent an unlimited source of polysaccharides with antitumour and immunostimulating properties [12]. In Uganda, *Auricularia* sp (wood ear) and *Pleurotus* sp (oyster) mushrooms which naturally grow on decaying logs in the rainforests are believed to be traditionally used for medicinal purposes by local communities for treatment of various ailments. Polysaccharides, proteins and other compounds previously isolated from mushroom species of these two genera have been found to stimulate immune cells both *in vitro* and in vivo [13]. Indeed, there is a great deal of evidence that species from these two genera might be a potential source of immunomodulatory compounds that can benefit patient care. In this study, we investigated the potential benefits of the aqueous extracts of a *Pleurotus* sp. and *Auricularia* sp. wild mushrooms on markers of cyclophosphamide induced immunosuppression in using male *Wistar* rat model.

2. MATERIALS 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 ± 1ºC 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 [14] and [15]. 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 s tirring 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.

2.3 Experimental Design

The immunosuppression model for cyclophosphamide developed by [2], 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 III: 600mg/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.

Selection of the two doses of mushroom extracts corresponded to doses that were 1/32 and 1/16 of the LD50 values (i.e. 9638.4mg/kg and 11641mg/kg for *Auricularia* and *Pleurotus* respectively) calculated from the acute toxicity study we conducted on the same mushrooms.

2.3.1 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.

2.4 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 for Windows, version 5.0 (Graph Pad Software Inc., San Diego, CA, 2005).

2.5 Ethical Issues

The experimental animals were handled in accordance with the Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals and were allowed free access to food and clean water *ad libitum*. The experimental protocol was examined and approved by the Makerere University, College of Health Sciences, Research and Ethics Committee. All authors hereby declare that all experiments were examined and approved by the appropriate ethics committee and were therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

3. RESULTS AND DISCUSSION

Wistar rats treated with cyclophosphamide alone (Group I) had significant reduction in total white blood cells (WBC) ($p < 0.001$; Table 1) and differential white blood cell (i.e. Lymphocyte and Neutrophils) 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 WBC 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, and 400mg/kg and 800mg/kg for *Pleurotus* sp extract. Hence, both extracts had a dose dependent increase in stimulation of WBC although *Auricularia* sp extract had higher total WBC compared to *Pleurotus* treated rats. The *Wistar* rats treated with both mushroom species aqueous 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. There was a significant increment in total and differential white cell counts in normal *Wistar* rats treated with 300mg/kg *Auricularia* extract (i.e. Group VI; $p< 0.001$) and 400mg/kg *Pleurotus* sp extract (i.e. Group VII; $p < 0.05$) compared to those in the control group (Tables 1, 2, & 3). Elsewhere, aqueous and ethanolic extracts from *Pleurotus* fruiting bodies powder have been reported to have an *in vitro* lymphoproliferative-stimulating response [16].

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*

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 [2]. 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 [5]. Destruction of stem cells in the bone marrow results into leucopoenia manifested as reduced levels of total and differential WBC in *Wistar* rats [17].

The increased WBC number as demonstrated in this study would be an important contributing factor to reduce the risk of various infectious diseases in immunosuppresed patients consuming these two studied mushroom species [6]. The stimulation of production of White blood cells (WBC) in an immunosuppressed animal model has been classified as an immunomodulatory effect [3,5]. 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 (i.e. 9638.4mg/kg and 11641mg/kg for *Auricularia* and *Pleurotus* respectively). The increased neutrophils (Table 3) in the immunesuppressed organisms is crucial for their survival as they make the innate immune system, and mount an immediate non-specific response to foreign microbial agents [18].

The present results demonstrate that the aqueous extracts of *Auricularia* sp and *Pleurotus* sp mushrooms can stimulate the activity of bone marrow to produce WBC. It also demonstrates that there are more species of mushrooms in the genera *Pleurotus* and *Auricularia* that have medicinal values and are yet to be tested. 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 aqueous extracts of the studied *Auricularia* sp mushrooms may possess greater immunomodulatory effects than those of *Pleurotus* sp. This is based on the observation that extracts of *Auricularia* sp mushrooms were used at a lower dose than for the *Pleurotus* sp mushroom in the immunomodulatory experiments.

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 [19,20,11]. The immunostimulant action of studied Pleurotus sp and Auricularia sp mushrooms suggest that they may be enhancing the humoral and cellular immune responses by either enhancing cytokine secretion or by directly stimulating B- or T-Lymphocytes [21]. Elsewhere, some mushroom species of the genus *Auricularia* have been shown to produce many different proteins and polysaccharides that stimulate the immune system in humans or in some cases cause the production of interferon and interleukins that then stop the proliferation of cancer cells [22,23]. 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. Our findings suggest that oral administration of *Pleurotus* sp and *Auricularia* sp aqueous extracts would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of the immune function [10]. 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 as practiced by many local communities in Uganda.

4. 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. Application of these mushrooms in immune suppression research appears to be new as reflected in the literature. These are however preliminary data to be more completely documented by further experiments, possibly investigating also some aspect of immune cell functions (e.g. cytotoxicity or cytokine production).

CONSENT

Not applicable.

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COMPETING INTERESTS

The authors declare that there are no competing interests.

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