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Immunomodulatory Properties of Polysaccharopeptides from an Indigenous Isolate of *Auricularia Polytricha* In Vivo

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ABSTRACT

Many mushrooms have polysaccharides, terpenoids, lectins and fungal immunomodulatory protein which help to activate immune cells e.g. macrophages, natural killer (NK) cells which in turn increase cytokine for immune response. The immunomodulatory effects are different from one mushroom to another depending on their chemical structures. In this study the serum interferon – alpha properties of polysaccharopeptides of isolated species of *Auricularia polytricha* were investigated with 150mg/g yield was obtained. Sixty (60) Adult female Swiss albino mice weighing 28-35 g were used for the study. Low dose PSP (30 mg/kg) significantly increases serum IFN- α to 19.87pg/ml as against the control ($p<0.05$), while high dose (20mg/kg) reduces it below cutoff. There was no death of the experimental animals or evidence of any acute sickness after 24hrs in the mice given 303.75mg/kg or less of this mushroom extract. This shows that the LD_{50} of the PSP is above the highest concentration set for the experiment. Therefore, doubling the doses of the extracellular PSP (30, 60 and 120mg/kg) was proposed to determine their effects on endogenous production of IFN-α in mice. The intraperitoneal (i.p) administration of crude PSP at 30mg/kg thrice weekly for five consecutive weeks positively affected the body weight of mice and increased their serum IFN-α response, while a high dose (120mg/kg) of the PSP inhibited interferon production. The high dose was mildly toxic causing a low survival rate of 33.3% at the end of the treatment ($P<0.001$, log rank test). Statistical comparisons between groups were carried out employing analysis of variance (ANOVA) and Tukey's multiple comparison tests to determine the effect of the PSP on the animals. Pearson's correlation coefficient was used to assess the relation between serum interferon concentration and PSP doses; Statistical analysis tools used are Graphpad Prism v8, and SPSS version 25. A value of $P<0.05$ was taken as significant probability level.

Keywords: Immunomodulatory properties, polysaccharopeptides (PSP), intraperitoneal and *Auricularia polytricha*

1.0 Introduction

Auricularia polytricha offers many potential health benefits due to its antioxidant, anti-inflammatory, antitumor and immunodulatory activities (Yoon *et al.,* 2003; Hongwei *et al.,* 2019). It has been reported that *Auricularia* extract prevents blood clotting (Harding, 2008) strokes, heart attack and also it is effective in the treatment of diabetes (Gulati *et al.,* 2019). It is also known to control cholesterol, protects the liver and kidneys and even promotes the brain health (Yi *et al.,* 2003; Chen *et al.,* 2008). The mushroom is also popular in Nigeria, especially the southern part where the climate is more suited for its growth. *Auricularia* species is the fourth highest produced worldwide after *Agaricus, Lentinus* and *Pleurotus* species among all

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industrially cultivated culinary and medicinal mushrooms (Yan *et al.,* 2004; Osemweigie and Dania, 2016).

Auricularia species has been used in the 19th century for treating health complaints such as sore throats, sore eyes and jaundice, and as an astringent and used in Ghana, as a blood tonic (Apertorgbor *et al.,* 2005). In recent researches it was discovered that *A. auricula-judae* has antitumour, hypoglycaemic and anticoagulant properties. It was discovered too that the anticoagulant activity was due to catalysis of thrombin inhibition by anti thrombin (Yoon *et al.,* 2003), and cholesterol-lowering properties and triglycerides (Yi *et al.*, 2005; Chen *et al.*, 2008, Seo and Chao, 2021). As a result of these properties they have been used for treatment of hemorrhoids, hemoptysis, angina, diarrhoea and gastrointestinal upsets, healing of colds and fever in Chinese medicine (Harding, 2008; Grupta *et al.,* 2019). *Auricularia* species contain polysaccharide which is known to be immunostimulatory and inhibitory towards cancer cells, antiviral, antibacterial and cholesterol lowering effects (Chen *et al.,* 2008). *Auricularia auricular* polysaccharide anti- inflammatory activity can be compared with *Auricularia auricula's* traditional use for soothing irritated or inflamed mucous membranes (Yi *et al,* 2005). Some reputable researchers have indicated anticancer potential as well as anticoagulant and hypoglycemic effects (Dong and Chungsun, 2021).

This study aims to evaluate the sub-chronic immunomodulatory effects of PSP from an indigenous Nigerian isolate of *A. polytricha* specifically on endogenous IFN- α production and survival in Swiss albino mice.

2.0 Materials and Method

Extraction of protein bound polysaccharides (po lysaccharopeptides) from isolates

The isolated mushroom species were cultured in a broth medium composed as follows; (g/L distilled water) peptone (1.0), yeast extract (2.0), K_2HPO_4 (1.0), $MgSO_4.7H_2O$ (0.2), $(NH_4)_2SO_4$ (5.0) and glucose (6.0) and the pH was adjusted to 6.0 according to Refaie, *et al.,* 2009 methods. The medium was transferred to 2L conical flasks and was sterilized at 121° C for 15min.The sterilized medium was inoculated with 1cm^2 of fresh culture of sample and incubated in a shaker incubator at 25°C for 12 -15 days under aerobic conditions at 200rpm.

The mycelia biomass was separated from broth and was concentrated in rotator evaporator. The biomas was put in 95% ethanol (1:1) overnight at 4°C to precipitate the extracellular polysaccharopeptides (PSP). The suspension was centrifuged at 5,000 rpm for 30 min. and the wet precipitate was freeze – dried into powder (Christ, Alpha.1.2 LD plus USA). The powder was extracted in hot water (80°C) for 3 hr, and centrifuged at 5,000 rpm for 30 min to remove insoluble matter. The clear filtrate was precipitated in 95% ethanol (1:1, v/v) overnight at 4°C. It was centrifuged and wet precipitate was freeze-dried (Christ, Alpha.1.2 LD plus USA).

Polysaccharides Characterization by Colorimetric (Phenol-Sulfuric Acid Method)

The content of polysaccharides in the extracts was determined by Phenol-Sulfuric acid method using Yoon *et al.,* 2003 methods with slight modification. Two millilitres of standard grade sugar solutions (ranging from 10 and 100 µg/ml concentrations of sugar) and 2 ml of purified polysaccharides from *Auricularia* at 100 µg/ml concentrations was pipette into a test tube, and 1 ml of 5% phenol solutions was added. Then 5 ml of concentrated sulfuric acids was added rapidly. The tubes were shaken and placed in water bath at 30 °C before readings procedure was taken. The colorimetric analysis of all solutions was carried out using a UV–Vis spectrophotometer. The absorbance of the characteristic yellow-orange colour was measured at 390 nm for hexose monosaccharide and 360 nm for pentose monosaccharide using UV spectrophotometer (VWR. UV6300pc). Blanks were prepared by substituting distilled water for the sugar solution. The amount of polysaccharides in fungal extracts was determined and expressed as amounts of hexose and pentose sugars by using constructed standard curves of each standard sugar (Dubois *et al*., 1956).

Fourier transforms infrared spectroscopy measurements (FTIR)

Fourier transforms infrared spectroscopy measurements (FTIR) were carried out using the method of Ravishanker *et al.* (2011) to determine the different functional groups present in the produced nanoparticles.The residual solution after the nanoparticle reaction was centrifuged at 10,000 rpm for 15 minutes. This process was repeated for three times, after which the purified suspension was washed with deionized water to obtain pure protein/enzyme free sample. The sample was completely dried at 60° C. Finally the dried nanoparticles were analoged by FTIR (Thermo Nicolet nexus 670 spectrometer of resolution 4cm -1). The Fourier transforms infrared spectroscopy (FT-IR) spectrum of the sample was recorded on Perkin Elmer spectrum 100 FT-IR spectrometer using KBr.

Toxicological studies

Determination of acute median lethal dose (LD_{50}) of PSP

Sixty (60) Adult female Swiss albino mice weighing 28-35 g provided by an animal breeder in Abeokuta in Ogun state were kept in a two ventilated cage covered with sawdust. The mice were provided with feeds and saline water to acclimatize to the condition of the environment. Two days after, they were used to determine the acute LD_{50} of PSP by intraperitoneal (i.p.) administration of the polysaccharides. Initially, animals were randomly grouped into six equally sized (10mice/dose) and administered with PSP at increasing doses: 40, 60, 90, 135, 202.5 and 303.75 mg/kg (increasing factor: 1.5). The mortality of animals was recorded after 24 h of injection.

Animal treatment and blood collection

A total of 24 adult female Swiss albino mice weighing 28 -35g were allocated in equality to four groups as follows: Group I, normal controls: normal mice injected intraperitoneally (i.p) with saline three times per week for five consecutive weeks; Group II, low dose: mice injected intraperitoneally.with 30 mg/kg of PSP three times per week for five weeks; Group III, intermediate dose: mice injected intraperitoneally with 60 mg/kg of PSP three times per week for five weeks; and Group IV, high dose: mice injected intraperitoneally with 120 mg/kg of PSP three times per week for five weeks.

On the penultimate day of the last injection of PSP, animals in all groups were weighed, and then blood samples were taken from the retro-orbital venous plexus in a glass capillary tube under light ether anesthesia. About 1.5 mL of blood was collected into sample tubes containing a separating gel for biochemical parameters. Serum was prepared by centrifuging blood samples at 5,000 rpm for 10 min. Serum samples were then aliquoted and stored at -20° C until analysis. The percentage of survival of animals was calculated for all groups studied in order to assess the toxic effects of the PSP doses.

Determination of serum interferon alpha (IFN-α) level

IFN-α level in serum was assayed by the protein specific Mu-IFN-α ELISA kit (sandwich technique procured from Biomedical lab, Germany) at the Nigerian Institute of Medical Research, Yaba, Lagos. The IFN-α titer (pg/ml) in the samples was determined from the standard curve of IFN-α (12.5-500 pg/ml).

ELISA protocols

Sample proteins are pre-mixed with enzyme conjugate in a non-treated 96-well plate using Hongbao, *et al.,* 2006 method. 50µl of the mixture was added to the ELISA 96-well plate in a two-fold serial dilution manner and in duplicate rows with the aid of a multi pipettor. The plate was sealed with nylon-like cellotape and incubated in a 5% CO_2 incubator (37°C). After incubation, the ELISA plate was washed generously with working wash buffer diluted in distilled water. After each washing, the plate was allowed to dry by draining the water onto a tissue paper. The reservoir containing the mixture was also washed to prevent contamination with the remaining samples. After this, substrate was added to the plate. The colorless substrate will be converted to a colored chemical by any enzyme conjugate bound to the ELISA plate. The plate was incubated for 1hr and stop solution was added to the samples in the plate after observing color change in the most concentrated standards. The plate was slide into the plate reader to take

readings of the absorbance of the samples at wavelength of 450 nm. The standards were placed in the first two columns and the replicates of the samples in the remaining ten (10) columns.

Statistical Analysis

The data obtained from the experiment were used to determine the descriptive statistics. Statistical comparisons between groups were carried out employing Analysis of variance (ANOVA) and Tukey's multiple comparison tests to determine the effect of the PSP on the animals. Pearson's correlation coefficient was used to assess the relation between serum interferon concentration and PSP doses; Statistical analysis tools used are Graphpad Prism v8, and SPSS version 25. A value of $P<0.05$ was taken as significant probability level.

3.0 Results and Discussion

The Physiochemical studies of extracellular Polysaccharopeptide (PSP) properties of Auricularia polytricha.

The characterized crude extracellular PSP from the mycelia broth of *Auricularia polytricha* by ethanol precipitation was carried out. The PSP weight was 1.5g/L mycelia broth with 150mg/g yield. The results from the physiochemical studies showed the presence of carbohydrate content (84.18%) and low amount of soluble protein (4.81%). The sugar component and their ratios on the thin layer chromatogram revealed eight spots with migration distances and color reactions to galatose, sucrose, maltose, lactose, xylose, arabinose and mannose standards. Glucose was a major monosaccharide (69.1mg/100 dry weight), galatose, sucrose, maltose, lactose, xylose and arabinose as minor ones (1.98, 8.18, 8.75, 4.79,1.83, 1.55 and 3.67 mg/100 dry weight respectively) (Figure 3).

The elemental analysis of PSP in this study revealed that C=36.15%, H=5.96%, O= 56.70%, S=0.41% and N=0.7%. Nitrogen value was very low (0.7%) but this indicated that there are protein bound to the polysaccharide. The empirical formular of the PSP can be deduced to be $(C_6H_{12}O_7)$ x with molecular weight is 196.16g/mol.

Fourier Transforms Infra Red Spectroscopy measurement (FT-IR)

FT-IR study revealed absorption bands at 3380, 2921, 2839, 1658, 1083 and 610cm $^{-1}$ showing O-H $_{(stretching)}$, C-H$_{(stretching)}$, Aldehyde, C=O$_{(stretching)}$, Amide, N-H$_{(stretching)}$, Amide, C-Cl and Alkylhalide respectively indicating the functional groups reducing the silver nitrate to silver ion (Fig 4.).

Determination of acute median lethal dose (LD_{50}) of PSP

There was no death of the experimental animals or any evidence of acute signs of sickness after 24 hr, in mice given 303.75mg/kg or less of mushroom extract. Sample count for all treatments against the PSP concentrations is shown in Table 1. This shows that the LD_{50} of the polysaccharopeptide is above the highest concentration set for the experiment. The probabilities of increase deliterous effect increased with increase in the concentration of PSP. Therefore, doubling the doses of extracellular PSP (30, 60 and 120 mg/kg) were proposed to determine their effects on endogenous production of IFN-α in mice (Table 2).

Dose response curve of serum IFN-α level after PSP treatment

Results obtained show that there was no mortality within the first two weeks. However, there was death in animal group treated with high dose in the third week. Those fed with intermediate dose recorded death on the fourth week and there was death recorded on the fifth week for those fed with low dose.

It is noteworthy that the low dose (30 mg/kg) group lost one animal after 4 weeks, attaining a survival rate of 83.3% at the end of the experiment (5weeks), while the intermediate dose (60 mg/kg) group exhibited a lower survival rate of 66.67% at the end of the experiment. This pin points to the mild toxicity of low dose of PSP on sub-chronic administration. The low rate of mortality (10.00%) in the low dose group suggested further investigation. The high dose was mildly toxic causing a low survival rate of 16.67% at the end of the treatment ($P<0.001$, log rank test).

Determination of serum interferon alpha (IFN-α) level

In this study the standard dose response curve of endogenous serum IFN-α production (in pg/ml) at absorbance of 450 nm (Fig 1) and its corresponding cell growth in logarithm form (Fig 2). The curve showed a linear equation graph. It also showed $R^2 = 0.9807$ depicting a good model for future studies. Hence, the equation was used to generate the sub-lethal dose values of PSP for doubling dose experiment.

The changes in body weight of mice treated with sub-lethal doses of PSP are shown in Figure 3. Sub-chronic administration of mice (5 weeks) with low (30 mg/kg), intermediate (60 mg/ kg) and high (120 mg/kg) doses of PSP, thrice weekly, recorded a notable increase in the body weight of experimental animals at the end of the treatment period. An increase of 12.9% was observed for the low dose, 18.89% increase in the intermediate dose at P<0.0001 compared to normal control 28.45%, the high dose 9.30 % (Fig 5). The normal control was healthy and active with no death was recorded. The dose response curve of IFN-α production level after administration of the three doses of PSP for 5 weeks was carried out (Fig 6). It depicted that the treatment with low and intermediate doses of PSP for five consecutive weeks manifested higher serum IFN-α level, (of the average 19.87 pg/ml and 9.72 pg/ml respectively), than the cut-off value (7.94 pg/ml) calculated as mean+2SD. In the high dose group, the only surviving animal showed serum IFN-α level (0.92pg/ml) below the cut-off value.

There is a strong negative correlation existing between the serums IFN-α production and the doses of PSP administered (Figure 7). A strongly significantly negative correlation coefficient (r= -0.97935, P=0.072) between the PSP dose and serum IFN-α concentration was observed.

Table 1: Cell counts and residuals

Number	Conc	No of subjects	Observed response	Expected response	Residual	Probability
1	1.602	10	0	.0165	-.165	.017
2	1.778	10	0	.297	-.297	.030
3	1.954	10	1	.529	.471	.053
4	2.130	10	1	.924	.076	.092
5	2.306	10	2	1.567	.433	.157
6	2.483	10	2	2.531	-.531	.253

Table 2: Logit model of PSP concentration to determine LD50 of Auricularia polytricha on mice

Parameter		Estimate	Std Error	Z	Sig	95% Confidence interval Lower Bound	Upper Bound
LOGIT[a]	Conc	3.414	1.843	1.852	.64	-.198	7.026
	Intercept	-9.556	4.179	-2.286	.022	-13.736	-5.377

a. LOGIT model: LOG (p/(1-p)) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm).

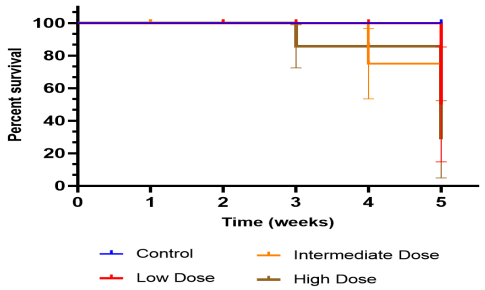

Figure 1: Survival curves of mice treated with doubling doses of polysaccharopeptides (PSP) [n= 10 mice/group] for 5 weeks. The control group received saline, and it was represented as100% survival.

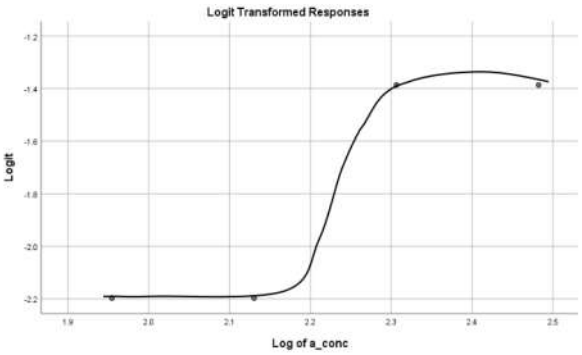

Figure 2: Logarithmic concentration of A. polytricha's polysaccropeptide

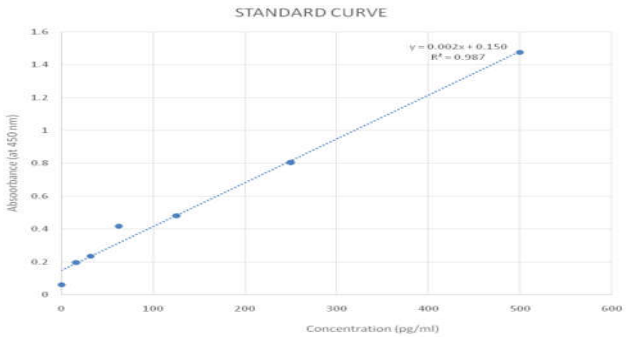

Figure 3: Standard curve of ELISA using absorbance against serum concentration

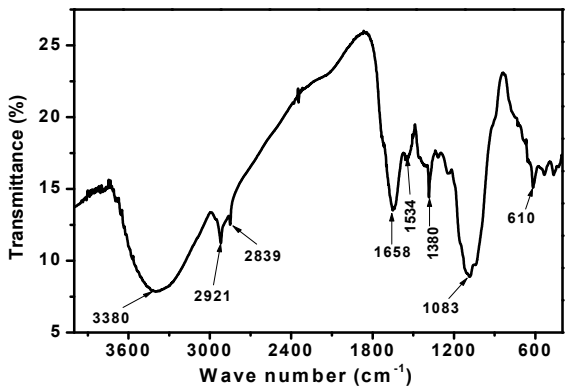

Figure 4: The FTIR of silver nanoparticle synthesized by *Auricularia polytricha*

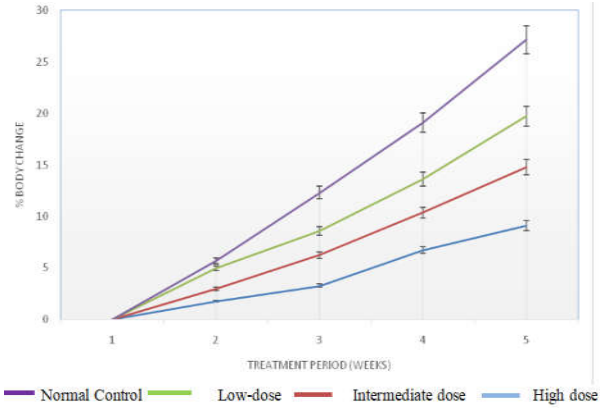

Figure 5: Changes in the body weight gain (%) of mice received low (30 mg/kg), intermediate (60 mg/kg) and high (120 mg/kg) doses of polysaccharopeptides (PSP) throughout the experimental period (5 weeks) compared to their initial body weights. Each point represents mean±S.D. of 2-5 value

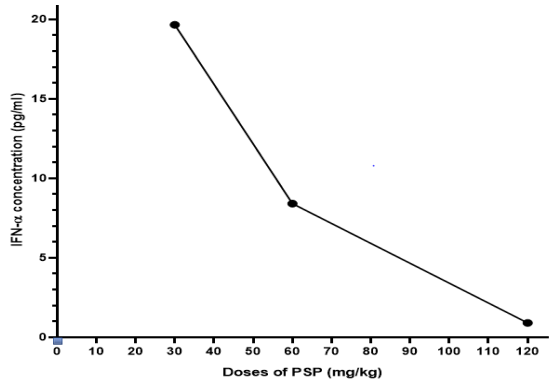

Figure 6: Dose response curve of serum IFN-α level at doubling doses of polysaccharopeptides (PSP). Each point represents mean±SD of 3-9values

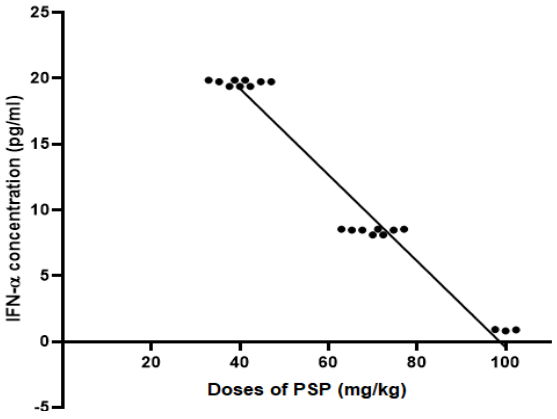

Figure 7: Negative correlation coefficient between doubling concentrations of polysaccharopeptides (PSP) and serum IFN-α concentration

There are several reports in literature that suggest the therapeutic values (anti cancer, anti diabetics, anti inflammatory and immune modulatory properties) of many mushroom species (Seo and Chao, 2021) . The medicinal properties of these mushroom species have been linked to their polysaccharide and polypeptides content. In this study, the immune modulatory properties of the isolated *Auricularia* species were investigated. Polysaccharopeptide content of the isolated species was extracted and their immune regulatory properties evaluated. There was no mortality with animals treated with 303.75 mg/kg or less of the extracted polysacchropeptide after 24 hr. This agrees with Refaie *et al.* (2009) who noted that PSP of 854mg/kg or less of *Pleurotus ostreatus* showed no lethal effect after 24 hr of administration to mice. This implies that the LD_{50} of the polysaccharopeptide of our mushroom extract is above the highest concentration set for the experiment indicating that it is safe for animal consumption.

The doubling dose of polysaccharopepptide (PSP) (30, 60 and 120mg/kg) were investigated to determine their effect on endogenous production of IFN-α in mice. There was no death recorded for first and second weeks when the dosage of PSP administered to the animals was observed. However, there was death in animal group treated with high dose in the third week. The high dose was toxic to animal to caused death of animals having 16.67% survival rate. The two animals administered with i.p 60 mg/kg (intermediate dose) died in the fourth week (with 66.67%

survival rate) and in the low dose in the fifth week lost an animal (with 83.3% survival rate) at the end of the experiment. Interestingly, Refaie *et al.* (2009) recorded survival rates 87.5 and 75% in their study at the end of the experiment. There is a notable increase in the body weight gain of experimental animals at the end of the treatment period.; 12.9% increase in the low dose, 18.89% increase in the intermediate dose in comparison to normal controls 28.45% and 9.30% increase in the high dose at $P<0.01$. This is in agreement with the report of Refaie *et al.* (2009) who reported 14.3% and 14.57% body weight increase on intraperitoeal adinistered at low and intermediate doses. However, the report of Bobek *et al.* (1997) who observed that there was no body weight gain on rat who were fed with 5% powder dried oyster mushroom which is in contrast with our finding. Oral administration of *Lentinula edodes* for 30 days was not sufficient to reduce body weight in a high fat diet feeding group Spim *et al*. (2017).

The PSP has been known to have ability to induce interferon and interferon production helps to boost immune system response of animals. However, the doubling dose of PSP was to determine which sublethal dose of PSP will produce the immune response. In this study, the low and intermediate doses manifested high serum IFN-α level of 19.87pg/ml and 9.72pg/ml respectively, than cut off value of 7.94pg/ml. High dose has 0.92pg/ml which was below cut off. A similar result was reported by Won *et al*. (1992) that low range dose 1 -5 mg/kg of *Ganoderma tsugae* mycelia endopolysaccharides was the optima concentration for endogenous IFN production for all the mice. There is a strong negative correlation existing between serum IFN-α concentration and the dose of PSP administered. This suggested that low dose PSP of 30 mg/kg *Auricularia polytricha* that was administered intraperitoneally for five consecutive weeks boosted the production of serum IFN-α and high dose inhibited interferon production.

Conclusion

Intraperitoneal (i.p) administration of crude polys accharopepptides (PSP) from broth culture of *Auricularia polytricha* mycelium at low dose positively affected body weight of experimental animals and increased their serum IFN-α response. This suggested that low dose of PSP of *Auricularia polytricha* will boost the production of serum IFN-α and high dose of PSP will inhibit it. Further studies should evaluate other cytokines (IL-6, TNK-α, IFN-ϒ) and immune cells population (NK, Macrophages) to fully characterize immunomodulation.

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