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Subacute Oral Toxicity of the Methanol Extract from *Phellinus pini* in Rats

Yun Jung Hong, Hyun Jin Jang, and Ki Sook Yang*

College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

Abstract – The present investigation evaluated the safety of the methanol extract from the fruit body of *Phellinus pini* Ames (PPA) by determining its potential toxicity after a subacute administration in rats. The extract was orally administered in doses of 1 g/kg, 2 g/kg, and 4 g/kg daily for 14 days to rats. Body weight, biochemical, and hematological parameters were determined at the end of 14 days of daily administration. The no-observed adverse effect levels (NOAEL) of the extract were 4 g/kg, when given by gavage routes. Daily oral administration of PPA extract for up to 14 days did not result in the death of significant changes in the body weight, hematological, and mainly biological parameters. In biological analysis, some significant changes occurred, including triglyceride and blood urea nitrogen (BUN), indicating that the PPA extract has liver and kidney-modulating activity. The PPA extract was found to be low or non-toxic in rats.
Keyword – *Phellinus pini*, subacute toxicity, rat, biologocal

Introduction

Phellinus pini Ames (Hymenocaetaceae) is a white-rot fungus that frutifies over the stems of Pinaceae, Cupressaceae etc. This mushroom has been known for its immunomodulating (Jeong *et al.,* 2004), hypolipidemic (Yang *et al.,* 2002), and anti-inflammatory (Jang and Yang, 2011) activities. The antitumor activities and immunostimulating activities of the polysaccharide fraction of this mushroom have been investigated (Ikegawa *et al.,* 1968). Despite the side use of the Phellinus species in the East Asian countries, very few investigations on acute oral toxicity (Han *et al.,* 2001) have been published in the literature about its toxicological profile. Therefore, the purpose of this study was designed to determine the subacute oral toxicity of the methanol extract from the PPA in rats.

Experimental

Plant material – The dried fruit body of the PPA was obtained from a traditional herbal market, located in Seoul. A voucher specimen was deposited at the herbarium in the College of Pharmacy, Sookmyung Women's University.

Preparation of PPA extract – The fruit bodies (1 kg) of the mushroom were ground and extracted with methanol by boiling under reflux for 4 hours. The refluxes obtained were concentrated under reduced pressure and then dried to yield approximately 5.9% (w/w) of the methanol extract, which was stored at -20 °C until used.

Experimental animal – Healthy Sprague-Dawley (SD) rats, weighing 150 - 170 g, were housed in plastic cages (5 to a cage). They were maintained in a room temperature, were photoperiod at 12 h, and were given frequent air changes. The rats had free access to water and food, except for a fasting period before the treatment.

The animals were randomly assigned into three groups, with 5 rats in each group, and their weights were recorded. PPA extract was diluted with distilled water containing 0.5% sodium carboxymethylcellulose and administered daily by gavage for 14 days to the groups of rats at doses 1, 2, and 4 g/kg, while the control rats received the distilled water containing only 0.5% sodium carboxymethyl cellulose. The animals were continuously observed for general behavioral changes, signs of toxicity, and mortality for 1 hour after treatment, then intermittently for 4 hours, and thereafter over a period of 24 hours (Twaij *et al.,* 1983). All animals were supplied with food and water *ad libitum* during the testing periods. Blood samples were obtained by the retro-orbital puncture under diethylether anesthesia with anticoagulant. Blood with the anticoagulant was used immediately for the determination of hematological parameters, while blood without the anticoagulant was centrifuged at 4000 rpm for

*Author for correspondence
Tel: +82-2-710-9578; E-mail: ksyang@sookmyung.ac.kr

10 min at 4 °C, and the serum obtained was stored at −20 °C until analyzed for biological parameters. The care and handling of rats were in accordance with the internationally accepted guidelines for the use of animals, and the protocol was approved by our university committee on animal care following the Sookmyung Women's University specifications for the production, care, and use of the laboratory animals.

Observation and examination methods – Clinical signs of the rats were observed daily for physiological and behavioral changes through the 14 days of dosing. The toxic manifestation and mortality were also monitored once a day. At the end of each 14-day period, body weight, water, and food intakes were recorded.

Hematological and biological analysis – On day 14, all surviving animals were fasted overnight, and anesthetized afterwards for blood collection from the right ventricle. Blood samples were collected into two tubes: (1) heparinized centrifuge tubes and (2) dry non-heparinized centrifuge tubes. The heparinized blood was used for hematological determination using the automatic hematocyte analyzer (Bechman Coulter Company, USA) with included white blood cell (WBC), red blood cell (RBC), hemoglobin concentration (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), and reticulocyte (RET).

The non-heparinized blood was allowed to coagulate before being centrifuged and the serum separated. The serum was assayed for total protein (TP), albumin (ALB), total bilirubin (TBL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin/globulin ratio (A/G), glucose (GLU), total cholesterol (TCHO), triglyceride (TG), alkaline phosphate (AP), blood urea nitrogen (BUN), creatinine (CREA), creatinine phosphokinase (CK), calcium (Ca), and inorganic phosphate (IP) using the automatic blood analyzer (Hitachi 7600-110).

Statistical analysis – All results were presented as the mean value ± standard deviation (S.D.). Within-group comparisons were performed using the ANOVA test. Significant differences between the control and the experimental groups were assessed by the Student's t-test. Results were considered significant at $p < 0.05$.

Results

General signs, body weight, food and water intake – No deaths with significant changes in general behavior of other physiological activities were observed at any point (Table 1). Changes in body weight in the control and the PPA extract treated rats are presented in Table 2. Rats gained weight with time, with no significant difference in weight gain at the end of the 14-day treatment between the controls and the rats treated with 1 g/kg, 2 g/kg, and 4 g/kg PPA extract. Other parameters, such as food and water intake, did not show any significant differences in either the control or treated group (data not shown).

Hematological and biochemical parameters – The effect of the PPA extract on the hematological parameters of the experimental and control rats are presented in Table 3.

The results indicated that all the hematological parameters measured (white blood cell, red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, and reticulocyte) remained within physiological range throughout the treatment period (14 days). The data for the biochemical parameters in the treated and control rats are presented in Table 4. Subacute oral administration of the PPA extract did not indicate any significant changes in several biochemical parameters, including serum total

Table 1. Effect on death and symptoms in rats after oral administration of PPA extract

Group	Dose (g/kg)	D/T	Symptoms
Control	–	0/5	None
PPA extract	1	0/5	None
	2	0/5	None
	4	0/5	None

SD rats (n = 5/group) were administered PPA extract orally in daily doses of 1 g/kg, 2 g/kg, and 4 g/kg for up to 14 days. D/T, No. of dead rats/No. of treated rats. None, no symptoms observed during the observation period.

Table 2. Effect on body weight in rats after oral administration of PPA extract

	Dose (g/kg)	D_0	D_7	D_{14}
Control	–	158.2 ± 7.8	197.0 ± 11.8	240.8 ± 15.6
PPA extract	1	155.4 ± 6.9	209.6 ± 5.8	257.6 ± 11.4
	2	161.3 ± 7.7	213.5 ± 11.5	253.7 ± 26.3
	4	161.6 ± 0.7	198.1 ± 6.4	220.8 ± 9.5

SD rats (n = 5/group) were administered PPA extract orally in daily doses of 1 g/kg, 2 g/kg, and 4 g/kg for up to 14 days. Data are expressed as mean ± S.D. No statistical difference between the control and PPA extract.

Table 3. Effects of daily oral administration of PPA extract for up to 14 days on the hematological parameters of rats

Parameters (units)	Control D_{14}	Dose (g/kg)		
		1	2	4
WBC (10^3/mm^3)	3.87 ± 0.53	4.33 ± 0.33	4.60 ± 0.08	4.13 ± 0.95
RBC (10^6/mm^3)	6.84 ± 0.15	6.84 ± 0.06	6.80 ± 0.19	6.66 ± 0.42
HGB (g/dl)	16.0 ± 0.2	16.1 ± 0.1	15.9 ± 0.3	15.7 ± 0.7
HCT (%)	44.2 ± 0.8	43.9 ± 0.7	43.9 ± 1.6	40.7 ± 2.2
MCV (fl)	62.9 ± 1.4	63.7 ± 0.3	63.2 ± 0.7	61.6 ± 0.6
MCH (pg)	22.1 ± 1.0	23.3 ± 0.5	23.7 ± 0.5	23.8 ± 0.3
MCHC (g/dl)	37.0 ± 1.3	37.3 ± 0.2	37.1 ± 0.1	38.6 ± 0.6
PLT (10^3/mm^3)	81.4 ± 5.1	87.6 ± 2.6	88.7 ± 5.8	82.1 ± 5.7
RET (10^3/mm^3)	5.70 ± 0.49	5.93 ± 0.41	5.80 ± 0.59	6.43 ± 1.18

SD rats (n = 5/group) were administered PPA extract orally in daily doses of 1 g/kg, 2 g/kg, and 4 g/kg for up to 14 days. Data are expressed as mean ± S.D. No statistical difference between the control and PPA extract**.**
WBC: White blood cell, RBC: Red blood cell, HGB: Hemoglobin concentration, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet, RET: Reticulo-cyte.

Table 4. Effects of daily oral administration of PPA extract for up to 14 days on the biochemical parameters of rats

Parameters (units)	Control D_0	Dose (g/kg)		
		1	2	4
TP (g/dl)	6.23 ± 0.05	6.60 ± 0.29	6.13 ± 0.05	7.57 ± 0.87
ALB (g/dl)	3.27 ± 0.05	3.34 ± 0.05	3.27 ± 0.05	3.33 ± 0.12
TBL (mg/dl)	0.30 ± 0.01	0.37 ± 0.03	0.33 ± 0.05	0.36 ± 0.09
AST (IU/l)	165 ± 2	171 ± 26	187 ± 31	192 ± 40
ALT (IU/l)	51.0 ± 2.9	51.3 ± 4.1	62.0 ± 11.5	69.0 ± 13.4
AP (IU/l)	457 ± 64	428 ± 65	398 ± 48	390 ± 70
A/G (ratio)	1.07 ± 0.05	0.93 ± 0.05	1.00 ± 0.14	0.80 ± 0.14
GLU (mg/dl)	113 ± 22	90.7 ± 22.2	88.0 ± 17.7	76.0 ± 4.9
TCHO (mg/dl)	77.0 ± 7.1	80.3 ± 8.7	87.7 ± 19.4	81 ± 20
TG (mg/dl)	84.0 ± 13.9	63.4 ± 11.8	56.3 ± 8.34	42.7 ± 11.6*
BUN (mg/dl)	17.0 ± 2.3	16.0 ± 0.7	15.6 ± 1.4	12.0 ± 1.3*
CREA (mg/dl)	0.60 ± 0.08	0.53 ± 0.05	0.53 ± 0.09	0.4 ± 0.12
CK (IU/l)	723 ± 42	812 ± 247	792 ± 207	857 ± 208
Ca (mg/dl)	11.1 ± 0.4	11.3 ± 0.3	12.1 ± 1.7	14.2 ± 1.8
IP (mg/dl)	14.9 ± 0.7	14.7 ± 0.9	14.9 ± 1.1	13.9 ± 1.3

SD rats (n = 5/group) were administered PPA extract orally in daily doses of 1 g/kg, 2 g/kg, and 4 g/kg for up to 14 days. Data are expressed as mean ± S.D. Statistically significant compared with control (* $p < 0.01$)**.**
TP: Total protein, ALB: Albumin, TBL: Total bilirubin, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, AP: Alkaline phosphatase, A/G: Albumin globulin ratio, GLU: Glucose, TCHO: Total cholesterol, TG: Triglyceride, BUN: Blood urea nitrogen, CREA: Creatinine, CK: Creatinine kinase, Ca: Calcium, IP: Inorganic phosphate

protein, albumin, TBL, AST, ALT, AP, A/G ratio, glucose, total cholesterol, creatinine, creatinine kinase, calcium, and phosphorus.

However, the levels of triglyceride and blood urea nitrogen decreased (as compared to the control D_0 rats treated with the vehicle) in the control D_{14} rats and the PPA extract treated rats ($p < 0.05$).

For the subacute toxicity of the PPA extract with a very high dose level 4 g/kg, no significant changes in hematological and biochemical profile were observed, except for a significant decrease in the level of triglycerides and urea nitrogen, indicating the PPA extract was relatively low or non-toxic under the study conditions.

Discussion

A World Health Organization survey indicated that about 70-80% of the world's population rely on non-

conventional medicine, mainly of herbal source, in their primary healthcare (WHO, 2007). This is especially the case in developing countries where the cost of consulting a Western-trained doctor and the price of medication are beyond the means of most people (Dyson, 1998; Chan, 2003). Although medicinal plants may produce several biological activities in humans, very little is known about their toxicity and the same applies for Phellinus species. Safety should be the overriding criterion in the selection of medicinal plants for use in the healthcare system (Tomlinson and Akereke, 1998). In addition to the use of historical documentation on *Phellinus pini,* one should also have a formal toxicological evaluation of this plant to optimize its safety use as a medicine.

The use of the extract of *Phellinus pini* in this study was prompted by the fact that this type of plant medicine closely mimics the traditional dosage form, and that it provides a convenient system that can be standardized in terms of its chemical or physical constituents, and that it is easily storable. Thus, the assessment of the safety of this dosage form of PPA extract appears to be biologically essential.

The subacute toxicity of PPA extract with three dose levels of 1 g/kg and 2 g/kg does not show toxicity effects in rats. However, the high dose (4 g/kg day) in the subacute study was applied because human exposure indicates the use of a high dose level in accordance with the subacute guideline (OECD, 2001). A lower dose of 1 g/kg day was used to determine dose related toxic effects. In this subacute toxicity study, it appears that the PPA extract at these doses did not produce the marked changes in rats, as evidenced by the absence of toxic symptoms.

Changes in body weight have been used as an indicator of the adverse effects of drugs and chemicals (Hilaly *et al.,* 2004). Since no significant changes were observed in the general behavior and the body weight of rats in the treated groups compared to the control group after a 14-day period of daily treatment, it suggests that with oral administration, the PPA extract had no effect on the normal growth of rats. The hematopoietic system is one of the most sensitive targets for toxic chemicals and an important index of physiological and pathological status in humans and animals.

The data of the hematological parameters showed no significant differences between the control and the treated groups, indicating that the PPA extract did not have effects on the circulating blood cells or on their production. However, the data of the triglyceride and BUN are lower than the control group, indicating that the PPA extract may have some effects on the liver and kidney, and it could decrease during a long administration. Further study is needed to look into the causes.

In this study, there were no adverse effect on the usual markers of liver and kidney toxicity (the plasma levels of liver enzymes, ALT and AST, creatinine, and BUN). We may conclude that the PPA extract did not induce significant damage to these organs. Indeed, the transaminases (AST and ALT) are well-known enzymes used as good indicators of liver functioning as biomarkers predicting possible toxicity (Hilaly *et al.,* 2004). Generally, any damage to the parenchymal liver cells results in the elevations of both transaminases in the blood. AST found in the serum is of both mitochondrial and cytoplasmic in origin, and any rise can be taken as a first sign of cell damage that leads to the outflow of the enzymes into the serum. Therefore, no changes in ALT and AST activities suggest that the subacute administration of PPA extract did not alter the hepatocytes function and metabolism. Equally, there was also no marked damage to the functional nephrons (Lameire *et al.,* 2005).

Thus, the results in this study suggest that PPA extract did not affect renal function. Clearly, this only serves as a preliminary test and that for a better estimation of renal function, a creatinine clearance test is required. The levels of triglyceride markedly decreased in the dose groups compared to the control group, indicating that the PPA extract had lipid-modulating and hypoglycemic activity, and confirming a previous similar report of the pharmacological activity (Li *et al.,* 2008b). The liver is the site of cholesterol disposal or degradation and the major site of synthesis.

Since dose 4 g/kg of PPA extract is considered a very high dose for humans and no significant changes were observed in the cholesterol levels in this study, it suggests that the PPA extract had no effects on the cholesterol metabolism of the rats. Except for a significant decrease in the levels of plasma BUN, no significant changes in hematological and biochemical profiles were observed, indicating that the PPA extract was relatively low or non-toxic under the study conditions.

These results provide valuable data on the subacute toxicity profile of the methanol extract of *Phellinus pini* that should prove useful for future research in vivo and clinical study of this plant medicine. PPA extract was found to be low or non-toxic when oral subacute toxicities in rats were performed.

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