Int. J. Indust. Entomol. Vol. 19, No. 1, 2009, pp. 175~180

International Journal of
Industrial Entomology

Single Oral Dose Toxicity Study of an Alcohol Extract of *Bombus ignitus* pupae in Rats

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(Received 26 May 2009; Accepted 24 July 2009)

Recently, as the male silkworm pupae, bee pupae have the potential that strengths men's vitality on vascular endothelial nitric oxide in endothelial cells. Especially we prepared alcohol extract of pupae of bumblebee, native bee named Hobakbul, *Bombus ignitus*. The alcohol extract of pupae of *B. ignitus* was administered to rats at doses of 0, 0.04, 0.2, 1 or 2 g/kg as a single oral dose. There were no observed clinical signs or deaths related to treatment in all the groups tested. Therefore, the approximate lethal dose of the alcohol extract *B. ignitus* pupae was considered to be higher than 2 g/kg in rats. Mild decreases in body weight gain in male were observed dose-dependently within *B. ignitus* pupae alcohol extract treated groups in dose response manner over 2 weeks. Throughout the administration periods, no significant changes in diet consumption, ophthalmologic findings, clinical pathology (hematology, clinical chemistry and coagulation) or gross pathology were detected. Minor changes in male and female rats were found in hematological parameters for all or partial of *B. ignitus* pupae extract treated groups but all the changes observed were within the physiological range. From these results, it was concluded that there was no-evidence of specific toxicity related to the ingestion of alcohol extract of *B. ignitus* pupae.

Key words: *Bombus ignitus* pupae extract, Single oral dose toxicity test

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Introduction

The main differences between larva and pupa could be distinguished from the changes of age and fat accumulation. According to bee larva became to be bee pupa, the fat was accumulated, moisture was dried and some chemical component may be destroyed or changed to some other chemicals. A few report about bumble or honeybee pupae physiological activity or medicinal use; phenoloxidase activity in *Apis mellifera* honey bee pupae (Zufelato *et al.*, 2004). *Bombus ignitus*, otherwise known as the Bumblebee is found dominant in Korea and is mass-produced worldwide for use as a pollinator (Yoon *et al.*, 2003). Nowadays, the medicinal and nutritional uses of honey and other hive products including bee larva, have been potential bumblebee products as domestic foods or medicinal drugs with the potential for prevention and curing efficacy (Meda *et al.*, 2004; Ahn *et al.*, 2009).

Honeybee larvae have been used for the treatment of male impotence and infertility (Meda *et al.*, 2004). In our subsequent study, Bumble bee, which are recognized as exerting many health benefits, have recently attracted the attention of the pharmaceutical preparation for the development of nutraceuticals (Ahn *et al.*, 2009).

However, safety evaluation data on these bumblebee pupae extracts are scare, so that we assessed the acute toxicity of an alcohol extract of *B. ignitus* pupa alcohol extract.

Hence, the present investigation was undertaken to assess the acute toxicity of *B. ignitus* pupae alcohol extract by orally administering doses of 0, 0.04, 0.2, 1 or 2 g/kg on a single dose.

Material and Methods

Extraction of hobakbul

The dried Hobakbul, *B. ignitus* pupae (500 g) supplied by the

Department of Agricultural Biology, National Academy of Agricultural Science, Korea, was soaked and extracted three times with EtOH by ultrasonification for 30 min. The extracts obtained were dried on a rotary evaporation and freeze-dried as a *B. ignitus* pupae alcohol treatment extract (BIPE).

Test preparation of *B. ignitus* pupa extract

Dried *B. ignitus* pupae alcohol treatment extract was homogenized in a blender to a powder, stored at 4°C, dissolved in phosphate Buffered saline (Sigma-Aldrich Inc., St. Louis, MO), and then orally administered at doses of 0, 0.04, 0.2, 1 or 2 g/kg on a single dose.

Analysis of mineral content and amino acid composition

Mineral content was analyzed by atomic absorption spectroscopy and phosphorus content was determined by colorimetric method, which utilizes ammonium molybdate, hydroquinone, and sodium sulfate (Kim *et al*., 2001) Amino acid compositional analysis was carried out by derivatization of the first N-terminal amino acids with phenylisothiocyanate (PITC) followed by RP-HPLC (Williams *et al*., 1998).

Animals

Specific pathogen free SD rats (5 weeks old, weighing 165±5 g, male and female), purchased from Samtako Co. Ltd. (Osan, Korea), were housed in an environmentally-controlled room with 23±1°C, relative humidity of 55±10%, air ventilation of 10~18 times/hr, a 12-hr light/dark cycle of 150~300 lux, and feed and water available *ad libitum*.

All procedures were conducted in accordance with the Korean Food and Drug Administration (KFDA) "Testing Guidelines for Safety Evaluation of Drugs"(Notification No. 2005-60, issued by the KFDA on Oct 21, 2005). Rats were kept for one week under normal physical conditions (23±2°C, 55±10%, humidity and a regular day/night cycle, air ventilation of 10~18 times/hr, a 12-hr light/dark cycle of 150~300 lux) and fed with standard diet (Samtako Co. Ltd., Osan, Korea), and water *ad libitum* before repeated-dose toxicity study testing began. Five animals of both sexes in each group were weighed, and administered with BIPE at a dose of 0.04, 0.2, 1 or 2 g/kg by a single oral dose treatment. The test parameters were clinical signs and mortality, body weight, food consumption, hematological analysis, serum biochemical analysis and ophthalmic observation findings.

Body weight

Animals were observed three times daily for clinical signs. Changes in body weight were recorded per three days and group means were calculated. Rat body weights were measured at the initiation of treatment and then per three days until autopsy at 14 days post-treatment initiation.

Food consumption

Daily food consumption was determined by subtracting leftover feed from provided feed. Food consumption was measured daily for the 1st week and per three days thereafter. Again differences between that food supplied and remaining were regarded as daily consumption (g/rat/day).

Blood sampling and plasma assay

After 14 days of treatment, blood (~3 ml) was collected from posterior vena cava under light CO_2 inhalation and used for serum chemistry measurements. The parameters examined included total protein, albumin, total bilirubin, glucose, glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), alkaline phosphatase (ALP), lactic dehydrogenase (LDH), total cholesterol, blood urea nitrogen (BUN), creatinine, triglyceride, uric acid, sodium, potassium and chloride. All were evaluated using an autoanalyzer (Hitachi 7060 automatic clinical analyzer, Tokyo).

Statistical analysis

Mean and standard errors of all parameters were determined for each of the 5 animals. The Student's *t*-test was used to establish the significances of differences between the control and treatment groups. $p<0.05$ was considered statistically significant.

RESULTS

Mineral content of *B. ignitus* pupae extract (BIPE)

The protein fat, ash and mineral content of BIPE were

Table 1. Chemical composition and Amino acid content of *B. ignitus* pupa extract used in this study (%)

Component percentage (%)								Metal content (ppm)			
Protein	Fat	Ash	Ca	P	K	Na	Mg	Fe	Mn	Zn	Cu
39.78	30.32	7.25	0.07	1.44	2.45	0.07	0.13	88.85	1.86	49.55	14.16
Amino acid			Cys	Met	Asp	Thr	Ser	Glu	Gly	Ala	Pro
Content (%)			0.242	0.635	2.228	1.177	1.272	5.147	1.684	2.125	2.867
Amino acid			Val	Ile	Leu	Tyr	Phe	Lys	His	Arg	
Content (%)			1.498	1.290	2.201	2.319	1.198	1.890	0.747	1.366	

Table 2. Mortality of Sprague-Dawley rats treated orally by pupa extract of *B. ignitus* bee on single-dose toxicity

Sex	Dosage (g/kg)	Days after treatment						Final mortality
		0	1	4	7	12	14	
Female	0	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	0.04	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	0.2	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	1	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	5	5/5	5/5	5/5	5/5	5/5	5/5	0/5
Male	0	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	0.04	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	0.2	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	1	5/5	5/5	5/5	5/5	5/5	5/5	0/5
	5	5/5	5/5	5/5	5/5	5/5	5/5	0/5

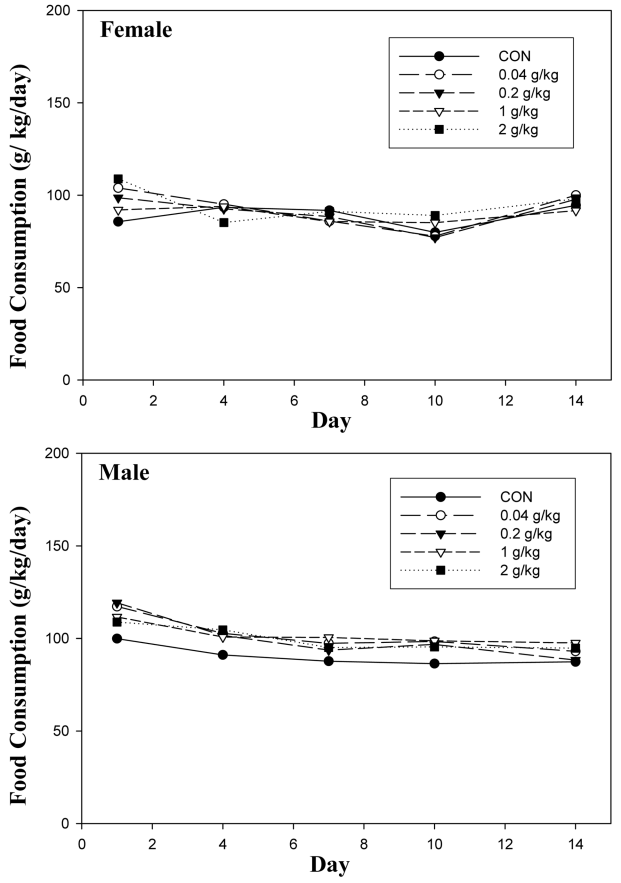

Fig. 1. Food consumption of SD rats, treated orally with *B iginitus* pupa extract on a single dose.

investigated. *B. ignitus* pupae extract contained more proteins and minerals and each minerals and protein contents are as follows: protein (39.8%), Ca (0.07%), P (1.44%), K (2.45%), Na (0.07%), Mg (0.13%), Fe (88.85 ppm), Mn (1.86 ppm), Zn (49.55 ppm) and Cu (14.16 ppm) (Table 1). Amino acid content of *B. ignitus* pupae extract showed no

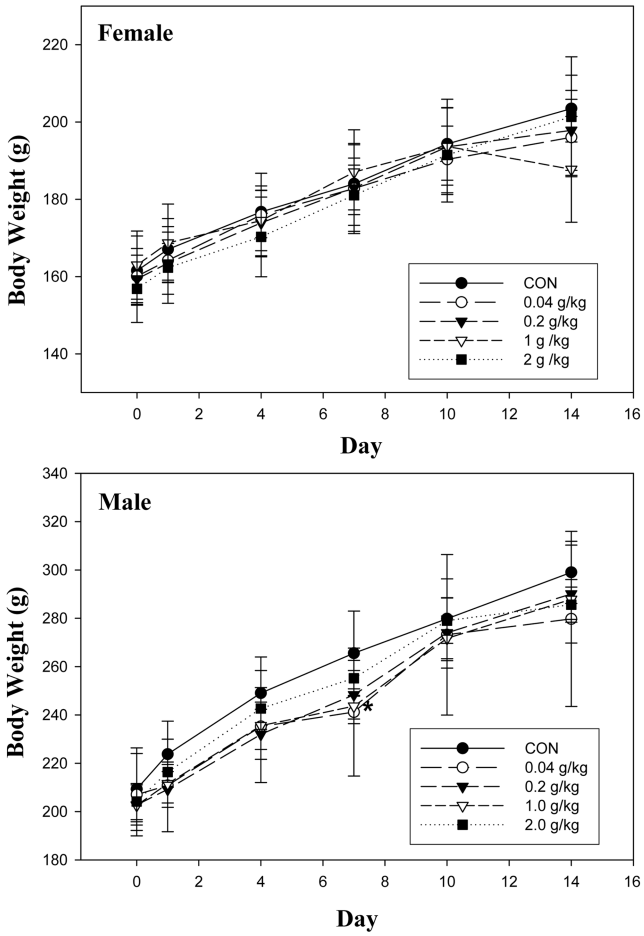

Fig. 2. Body weight increases of male and female SD rats, treated orally with *B iginitus* pupae extract on a single dose
*Significantly different from the untreated controls ($P<0.05$).

obvious trend in the changes of protein and mineral contents in the statistical perspective (Table 1).

Clinical sign and food consumption

No deaths or adverse clinical signs were observed due to the ingestion of BIPE at a dose of 0.04, 0.2, 1.0 or 2.0 g/kg (Table 2). Food consumption levels were similar for all study groups, though it was more or less differences between control and treated group (Fig. 1).

Body weight changes

There were no toxicologically significant differences in mean body weight between any of the treatment groups (Fig. 2). During the 14-day administration after a single dose treatment, the body weights of the male and female SD rats in the 4 treatment groups were comparable across the control and treated groups. The mean per 3 days body weights versus time are presented in Fig. 2. In the male rats, there was statistically a significant difference in body weight gain between the BIPE treated groups (1.0 g/kg

Table 3. Hematological finding of the *B. ignitus* pupa extract treated groups

Dosage (g/kg)	Item	Unit	CON [a]	0.04	0.2	1	2
Female	WBC	10^3/mm^3	8.5±2.5	7.8±2.4	6.2±0.7*	7.2±1.1	6.5±0.8
	RBC	10^6/mm^3	7.1±0.3	7.5±0.7	7.7±0.5	7.2±0.7	7.3±0.2
	Hgb	g/dl	14.3±0.3	14.9±0.9	15.3±1.2	14.3±1.1	14.6±0.7
	Hct	%	44.3±1.0	46.8±2.7	47.9±3.8	44.9±4.1	46.2±1.6
	MCV	fl	62.1±0.9	63.0±2.9	62.6±1.2	62.5±1.5	63.2±0.9
	MCH	pg	20.1±0.5	20.1±0.9	19.9±0.4	19.9±0.4	20.0±0.6
	MCHC	g/dl	32.4±0.7	31.8±0.1	31.8±0.1	31.9±0.7	31.7±0.5
	PLT	10^3/mm^3	1042.3±219.1	713.3±322.3	978.7±143.9	872.0±81.3	965.2±198.1
Male	WBC	10^3/mm^3	8.4±5.4	10.5±1.8	12.8±0.9	12.8±3.1	11.5±3.4
	RBC	10^6/mm^3	8.1±1.3	7.8±0.1	7.5±1.1	8.3±0.3	8.3±0.3
	Hgb	g/dl	16.0±2.3	15.7±0.6	15.1±1.6	16.6±0.4	16.2±0.8
	Hct	%	52.4±8.0	50.5±2.0	48.7±6.1	54.4±1.6	53.2±2.6
	MCV	fl	65.1±0.9	65.0±0.9	65.1±1.1	65.3±2.1	64.3±1.5
	MCH	pg	20.0±0.4	20.2±0.7	20.2±0.7	19.9±0.6	19.6±0.5
	MCHC	g/dl	30.7±0.7	31.0±0.3	31.1±0.6	30.5±0.3	30.4±0.1
	PLT	10^3/mm^3	978.0±115.0	1120.5±132.9	1147.0±91.9	1319.0±181.6*	1231.3±206.4

[a]CON : PBS (as a vehicle) treated with murine normal diet
Each value represents mean±S.D.
Statistically significant from control (*P <0.05)

Table 4. Biochemical serum values of female rats treated orally with BIPE

Item	g/kg	CON[a]	0.04	0.2	1	2
Toal protein	g/dL	6.4±0.3	6.5±0.2	6.3±0.3	5.9±0.3	6.0±0.2
GPT	IU/L	43.0±4.4	36.3±6.9	37.8±7.2	35.0±1.8	35.0±5.4
GOT	IU/L	125.2±12.2	114.8±26.2	105.5±21.8	103.3±17.6	95.8±16.2
ALP	IU/L	264.4±27.9*	193.3±4.2	245.8±49.2	213.8±39.4	216.0±71.1
Glucose	mg/dL	167.6±13.6	183.5±74.6	159.5±57.4	211.5±53.4	174.0±7.9
Cholesterol	mg/dL	76.4±6.7	75.8±11.4	78.0±8.4	75.5±11.5	73.5±8.1
Bilirubin	mg/dL	0.2±0.6	0.1±0.1	0.1±0.0	0.1±0.1	0.8±0.1
BUN	mg/dL	21.1±3.6	18.8±2.2	19.1±4.7	19.4±3.8	18.5±6.2
Creatine	mg/dL	0.5±0.1	0.6±0.1	0.5±0.1	0.5±0.1	0.5±0.0
Triglyceride	mg/dL	120.6±12.1	109.0±45.3	101.0±26.4	161.5±18.6	80.3±18.8
Uric acid	mg/dL	3.7±1.2	4.7±1.2*	4.9±0.7	5.3±1.2*	4.6±1.4
K	mmol/L	19.7±1.9	19.5±2.5	19.3±1.6	17.0±1.9	17.4±2.3
Na	mmol/L	136.0±32	136.8±3.6	136.3±7.2	134.5±5.3	138.3±3.2
Ca	mmol/L	10.7±0.5	11.0±0.9	10.7±0.3	10.7±0.4	10.2±0.2
CK	IU/L	491.8±138.4	571.3±252.6	438.5±195.7	377.8±134.5	308.3±80.1
HDL	IU/L	24.4±3.8	21.3±3.4	3.3±5.2	23.5±3.0	23.0±2.0

[a]CON : PBS (as a vehicle) treated with murine normal diet
Each value represents mean±S.D.
Statistically significant from control (*P <0.05, **P <0.01)

dose) and the control group (group, $p>0.05$); however, no statistically significant differences were observed between the other (0.04, 0.2, or 2.0 g/kg) BIPE-treated group and the control group. Furthermore, such a decrease in body weight gain was not found in the female BIPE treated rats.

Hematology and blood chemistry

Minor changes in male rats was found in hematological parameters for the 1.0 g/kg *B. ignitus* pupa extract treated groups (platelet level increase of 34.9%), but all changes were within physiological range (Table 3 and Table 4). In the female rat sera of the BIPE treated groups, the level of total alkaline phosphatase was significantly lower than in the control and uric acid was significantly higher than in the control after 2 weeks. A significant increase in the uric acid was observed in the 0.04 g/kg, and 1.0 g/kg group for only the females (controls, 3.7±1.2 mg/dL; 0.04 g/kg, 4.7±1.2 mg/dL; 1.0 g/kg, 5.3±1.2 mg/dL). The alkaline phosphatase levels of the treated groups were reduced in 0.04 g/kg in the females (control, 264.4±27.9 nmol/L; 0.04 g/kg, 193.3±4.2 nmol/L) (Table 3 and Table 4).

In the male sera of the BIPE treated groups, the level of potassium ion was significantly lower than in the control after 2 weeks. A significant increase in the triglyceride was observed in the 0.04 g/kg group for only the males (controls, 87.6±21.9 mg/dL; 0.04 g/kg, 145.8±14.5 mg/dL). The potassium ion levels of the treated groups were reduced in dose dependent manners in the males (control, 18.5±5.1 nmol/L; 1.0 g/kg, 10.4±3.5 nmol/L; 2.0 g/kg, 8.4±0.4 nmol/L), whereas the HDL (high density lipoprotein) level increased or decreased (male: control, 19.4±2.7 nmol/L; 0.2 g/kg, 23.0±2.1 nmol/L; 2.0 g/kg, 15.0±2.3 nmol/L, the calcium ion levels of the treated groups were increased in dose dependent manners in the males (control, 11.0±1.0 nmol/L; 2.0 g/kg, 12.34±0.56 nmol/L), and sodium ion levels also increased compared to control group (control, 140.2±3.3 nmol/L; 2.0 g/kg, 151.4±1.7 nmol/L) (Table 5).

Some significant differences were observed between the treated and control groups with respect to the hematological parameters at the end of the experiment. At the end of the administration period, as a coagulation parameter, an increase in platelet count was observed in the male rats (control, 978.0 g/dl; 1.0 g/kg, 1319.0 g/dl) in all of the treated groups; as an indicator of RBC function, we found that MCV, MCH and MCHC were not significantly different between the treated groups and the control group.

Ophthalmologic findings

No significant treatment-related ophthalmologic findings were observed. Any minor changes were few and dose independent.

Table 5. Biochemical serum values of male rats treated orally with BIPE

Item	g/kg	CON[a]	0.04	0.2	1	2
Toal protein	g/dL	6.9±0.3	6.4±0.2	6.3±0.5	6.6±0.3	6.9±0.2
GPT	IU/L	46.0±9.1	46.8±5.1	49.0±3.4	47.0±4.5	46.4±5.0
GOT	IU/L	127.8±9.3	123.8±7.1	124.8±13.3	104.8±18.8	108.8±8.1**
ALP	IU/L	297.0±71.0	399.8±23.3	358.8±122.5	273.4±54.2	219.6±28.7
Glucose	mg/dL	249.2±182.3	207.5±109.1	216.0±151.2	294.8±179.4	310.8±83.7
Cholesterol	mg/dL	77.2±10.2	79.3±4.3	73.2±8.0	80.4±8.0	74.2±4.6
Bilirubin	mg/dL	0.1±0.0	0.1±0.1	0.0±0.1	0.0±0.1	0.0±0.1
BUN	mg/dL	23.0±6.8	21.8±4.0	19.8±4.3	18.7±1.8	21.7±0.7
Creatine	mg/dL	0.7±0.8	0.6±0.1	0.6±0.1	0.6±0.1	0.7±0.0
Triglyceride	mg/dL	87.6±21.9	145.8±14.5*	101.2±3.3	99.4±13.8.	67.4±9.8
Uric acid	mg/dL	5.7±4.4	4.4±2.6	6.5±4.9	6.8±2.8	7.5±0.9
K	mmol/L	18.5±5.1	14.0±3.5	10.4±2.7	10.4±3.5**	8.4±0.4*
Na	mmol/L	140.2±3.3	144.8±2.2**	144.0±3.2	145.6±3.8	151.4±1.7**
Ca	mmol/L	11.0±1.0	10.45±0.76	10.94±1.24	11.94±1.45	12.34±0.56*
CK	mmol/L	613.4±257.0	759.5±253.1	784.4±381.6	434.4±80.4	401.2±46.9
HDL	IU/L	19.4±2.7	26.3±2.6	23.0±2.1**	19.0±2.3	15.0±2.3**

[a]CON : PBS treated with murine normal diet

Each value represents mean±S.D.

Statistically significant from control (*$P<0.05$, **$P<0.01$)

Discussion

There are many endeavors that utilize bee food or drugs with compositions comprising larvae or pupae of bee or extract thereof (Ahn, 2006). In many countries, some peoples have eaten bee larva or pupae as protein sources for shortage of food and crude insect drug. Especially native bee, Bumblebee pupae including larvae can be produced all the year around by development of rearing skills. To investigate of domestic food or medicinal crude drug with the potential for prevention and curing efficacy, the ethanol extract of bumblebee pupae did not induce any remarkable acute toxic responses in single oral toxicity study in SD rats. Throughout the administration period, no significant changes in diet consumption, ophthalmologic findings, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis), and gross pathology were detected. Therefore, the approximate lethal oral dose of *B. ignitus* pupa ethanol extract was considered to be higher than 2 g/kg in rats.

The potassium levels in 14th day- rat serum after single-dose treatment was reduced in a dose dependent manner in male rats; the potassium level of the extract was also higher (1.44%) than other cation level (Ca, 0.07%; Na, 0.07%; Ma, 0.13%). as like the alcohol extract of *B. ignitus* larvae. These results, however, were not sufficient to deem BIPE a diuretic agent (Furukawa *et al*., 1997) but the data obtained can be also interpreted within the context of a *B. ignitus* extract preparation study as a medicine or food (Hu *et al*., 2009; Ahn *et al.,* 2009). In male rats, there were statistically only a significant difference in body weight gain between the BIPE (1.0 g/kg dose) treated groups and the control group (group, $p>0.05$); however, no statistically significant differences were observed between BIPE-treated group and the control group in the female rats. From these results, the approximate lethal oral dose of the ethanol extract from pupae of *B. ignitus* was concluded to be higher than 2 g/kg in rats.

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