Acute Dermal Toxicity and Repeated Dose 90-Day Oral Toxicity Studies of the Bioinsecticide from *Stemona curtisii* Hook. F.

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ABSTRACT

A formulation of bioinsecticide from Stemona curtisii Hook. F. (Family Stemonaceae), marketed in Thailand as Biopes, is used in agriculture by the farmers in the northern area of Thailand. Subsequently, this insecticide used for insect control has become common and may lead to pesticide toxicity to both consumers and farmers. The aim of the present study is to evaluate the safety of Biopes (containing 20% w/w of Stemona crude extract) to mammals. Safety assessments included an acute oral toxicity test and a repeated dose 90-day oral toxicity test in Sprague-Dawley rats, and acute dermal irritation test in guinea pigs. In the acute oral toxicity test, Biopes showed lethal effect with the LD50 of 1,078.95 and 630.96 mg/kg-body weight in male and female rats, respectively. In the repeated dose 90-day oral toxicity test with the doses of 80 and 140 mg/kg-body weight (50 and 100 folds to the concentration used in agriculture), there were minimal but significant differences in body weight gains, some values of hematology, blood biochemical indices and organ weights between control and treated groups. The histopathology findings indicated small toxic effects of Biopes on gastrointestinal tract, lung and liver of the treated rats. In dermal irritation test, the direct exposure to non-diluted Biopes caused irritation on the skin, and death in two female guinea pigs on day10 and day14. Therefore, bioinsecticide Biopes should be used with caution because it might be harmful to the users when being directly exposed. The diluted solution of Biopes at the concentration used in agriculture slightly caused dermal irritation but it was improved within a short period.

Keywords: Acute toxicity, Subchronic toxicity, Dermal toxicity, Bioinsecticide, *Stemona curtisii*

INTRODUCTION

According to the increase of interest for human and environmental safety, there has been a renovated concern in the use of natural products (such as plant bioactive compounds) as insecticides and pesticides. Nowadays, many naturally-occurring insecticides (bioinsecticides) have been used as active control agents for a variety of insect pests. They are available in the local markets.

Stemona curtisii Hook. F. is an herbaceous plant found in the south and north-east regions of Thailand. This plant is named as "Non Tai Yak" in Thai, and belongs to the Family Stemonaceae, which consists of about 25 species (Gagnepain, 1934; Konoshima, 1973; Kaltenegger et al., 2003). Plants in this family contain an interesting group of alkaloids, also called as *Stemona* alkaloids, which constitute a unique chemical character (pyrrolol [1,2-a] aqepinecore) and are not detected in any other plant family (Greger, 2006). The roots of various Stemonaceae species have long been prescribed in traditional Thai, Japanese and Chinese medicine as insecticidal and antitussive agents (Jiangsu New Medical College, 1986; Philli and Ferreira de Oliveira, 2000; Tsi and Duyfjes, 2000). Moreover, the extracts from roots of these plants have been used for respiratory disorders, including pulmonary tuberculosis and bronchitis, and externally used against different insect pests (Xu, 2000; Brem et al., 2002). *Stemona* alkaloids are postulated to be involved in apoptotic effects of chemo-resistant cancer cells (Rinner et al., 2004). In Thailand, the insecticidal properties of several Stemona species have been known for centuries. The use of *Stemona* species (such as *S. collinsae, S. tuberosa*, and *S. curtisii*) for insect control is now of interest to Thai farmers and leads to the bioinsecticide development. Many bioinsecticide products have been marketed by shedding the highlight on an active ingredient; *S. curtisii.* Recently, Biopes is a bioinsecticide from *S. curtisii* marketed in Thailand, has been developed and formulated by Department of Biology, Faculty of Sciences, Chiang Mai University, Thailand. It is used for insect control in agriculture, especially in fruit and vegetable plantation, by Thai farmers in the northern Thailand.

So far, it has long been postulated that the chemical insecticides (e.g., organophosphates and carbamates) are claimed to be more toxic than herbal insecticides (e.g., bioinsecticides). However, it should be kept in mind that not all of bioinsecticides are non-toxic. It has long been known that the use of insecticides has not only influenced the level of agricultural production and its sustainability but also affected the health of users (mainly farmers), who are living near farms and consumers of agricultural products. Deaths were not only due to occupational poisoning but included the cases of self ingestion (suicide), accidental ingestion and homicides. The present research aimed to evaluate the safety of natural product; Biopes to mammals. Safety assessments included an acute and subchronic oral toxicity tests in rats, and acute dermal irritation test in guinea-pigs.

MATERIALS AND METHODS

Materials

The formulation of bioinsecticide Biopes (patent no. 3863), containing 20% w/w of S. curtisii crude extract, was kindly provided by Assoc. Prof. Dr. Araya Jatisatienr, the Department of Biology, Faculty of Science, Chiang Mai University, Thailand. This formulation was freshly prepared by dissolving in distilled water before use.

Laboratory animals

Sprague-Dawley rats of both sexes, 7-8 weeks of age (weighing 180-200 g), and albino guinea-pigs of both sexes (weighing 250-300 g) were purchased from the National Laboratory Animal Center (NLAC), Salaya, Mahidol University, Nakorn Pathom. All animals were kept in a room maintained under automatically-controlled conditions of 24±1°C and 12 h light-12 h dark cycle. They were fed a standard laboratory diet (Pokphand Animal Feed Co. Ltd., Bangkok, Thailand) and water and were acclimatized at least 1 week before starting the experiment. The experimental protocol was approved by the Animal Ethics Committees in accordance with the guidelines for the care and use of laboratory animals set by the Faculty of Medicine, Chiang Mai University, Thailand.

Acute dermal toxicity test

The procedure was performed according to the OECD guideline number 404 (OECD, 2002) with slight modification. Both sexes of albino guinea pigs with healthy intact skin were used. One day prior to commencing the study, fur was removed by shaving from the back to expose an area of approximately 10% of the total body surface. Only those animals without injury or irritation of the skin were used and divided into 2 groups (the control group and Biopes-treated group). On the test day, non-diluted Biopes as well as 0.05% (w/w) Biopes (the concentration of Biopes used in agriculture) were applied on the shaved skin, and then covered with a clean gauze dressing. The dressing was removed 4 h later and the skin was then cleaned of residual Biopes with distilled water. Skin reactions as well as signs and symptoms of toxic effects were assessed approximately 0, 1 and 24 h after removal of the dressings. All animals were then observed daily for 14 days. Erythema and edema were scored on a scale of 0-4, with 0 showing no effect and 4 representing severe symptoms. The results were compared to those of the control animals which received distilled water.

Acute oral toxicity test

Acute toxicity study was done in accordance with the Organization for Economic Co-operation and Development (OECD) guideline number 420 for the acute oral toxicity testing in rodents (OECD, 2001). Sprague-Dawley rats of both sexes were divided into six groups of five females and five males. The first group was received distilled water orally served as control. The other groups were received Biopes at the doses of 300, 500, 750, 1,000 and 2,000 mg/kg in a single oral dose by gavage. All rats were fasted overnight prior to substance oral

administration. The toxicity was assessed on the basis of mortality.

Observations were made and recorded systematically at 1, 2, 4 and 6 h after test substance oral administration. The number of survivors was noted after 24 h and then maintained for a further 14 days with a once-daily observation. At the end of the experiment, all surviving animals were sacrificed and the internal organs were examined.

Repeated dose 90-day oral toxicity (Subchronic oral toxicity) test

This study was conducted in accordance with OECD guideline number 408 for the subchronic oral toxicity testing in rodents (OECD, 1998). Both sexes of Sprague-Dawley rats were divided into 3 groups (10 rats/sex/group). One group of animals was served as control and received the vehicle (distilled water). The second group and third group were oral administered with Biopes at the dose of 80 and 140 mg/kg/day (50 and 100 folds dilution to the concentration used in agriculture) for 90 days. The toxic manifestation such as signs of toxicity, mortality and the body weight change were monitored daily.

Blood analysis and histopathology

Rats were anesthetized with 100% ether on day 91. The heparinized blood samples were taken for determining complete blood count, red blood cell count, platelet count and red cell indices. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis by using an automated chemistry analyzer (Olympus AU400, Olympus, Tokyo, Japan).

All rats were sacrificed after blood collection. The viscera and some tissues were weighed to determine relative organ weights and observed for gross lesions. All tissues were preserved in 10% (v/v) buffered formadehyde solution for histological examination (Wongcome et al., 2007).

Statistical analysis

All values are expressed as mean ± S.E.M. Student's t-test, one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test were used to determine significant differences between groups. Data were analyzed using SPSS version 10 for windows computer software. P-values < 0.05 were considered significant.

RESULTS

Acute dermal toxicity test

The results show that non-diluted Biopes caused erythema and edema on the skin of guinea pig and more severe than those caused by 0.05% (w/w) Biopes, especially in female guinea pig. The 0.05% (w/w) Biopes slightly caused erythema on the guinea pig skin within one hour after removing the gauze soaked with the substance from the skin. The skin recovered to the normal state within one day. Additionally, one female guinea pig which was applied with non-diluted Biopes died on day 10 and another one showed fatigue and convulsion on day 14 (data not shown).

Acute oral toxicity test

By the oral route, Biopes at a dose of 300 mg/kg did not induced the death or any toxic effects. Biopes at doses of 500, 1,000 and 2,000 mg/kg caused the death in male rats with 20, 40 and 80%, respectively. In the female animals, Biopes at doses of 500, 750 and 1,000 mg/kg caused death with 20, 80 and 100%, respectively (data not shown). Observable changes in behaviour after the lethal dose oral administration were decrease in motor activity and respiratory rate and violent clonic convulsion. Death was due to asphyxia from respiratory arrest. The median lethal dose (LD50) of the Biopes was found to be 1078.95 mg/kg and 630.96 mg/kg in male and female rats, respectively. All rats were sacrificed and the gross examination showed that two treated rats exhibited some lesions of gastric wall. Out of these treated rats, there were no changes in size or color of internal organs when compared with those of the control rats.

Subchronic oral toxicity test

Oral administration of Biopes into the rats at the doses of 80 and 140 mg/kg/day for 90 days caused significant difference in body weight gain of male rats when compared with that of the control group. However, in female rats, the body weight gain of treated and control groups was similar (data not shown). Laboratory clinical tests, gross lesion incidence and organ-weight data did not suggest a compound-related effect (data not shown).

Blood analysis and histopathology

Only in the female Biopes-treated groups, the red blood cell (RBC), hematocrit (HCT), hemoglobin (HGB) and mean corpuscular volume (MCV) values were significantly different from those of the control group (Tables 1 and 2). However, these hematological values of the female treated groups were in the range of those of the normal control group.

Table 3 and Table 4 demonstrate the differential white blood cell (WBC) count. The oral administration of the Biopes at the doses of 80 and 140 mg/kg/day for 90 days caused significant increase in WBC count and the percentage of neutrophil in female rats, but these changes were within the normal limit.

As shown in Table 5 and Table 6, significant difference among the experimental groups was evident when blood glucose, blood urea nitrogen (BUN) and albumin values were analyzed. However, these blood biochemistry levels were in the range of those of the normal control groups. Regarding to hepatotoxicity of Biopes, 90 days of repeated oral administration of Biopes at the doses of 80 and 140 mg/kg/day showed increase in alkaline phosphatase in male rats. However, the change was less than 1 fold, and the histopathological study did not show any correlation with the changes of liver enzymes.

In histopathological study, all control animals did not show any abnormalities. There were slightly differences of histology for the tissues taken from the died animals during the experiments. Although the stomach and the intestine were dilated, only erosion of the epithelial surface was noted. The congested livers and spleens revealed the intact tissue architectures. Neither cellular necrosis nor

portal inflammation was noted. Some animals displayed mucous retention in the bronchioles and peribronchilar lymphoid infiltration.

Table 1. Hematological values of female rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
RBC ($x10^6$/µl)	7.10 ± 0.12	7.9 2 ± 0.19*	7.04 ± 0.07
HGB (g/dl)	15.22 ± 0.19	16.20 ± 0.37*	15.65 ± 0.15
HCT (%)	44.71 ± 0.75	48.33 ± 1.33*	46.50 ± 0.50
MCV (fl)	63.07 ± 0.16	60.90 ± 0.73*	62.75 ± 0.85
MCH (pg)	21.44 ± 0.23	20.45 ± 0.21	21.10 ± 0.00
MCHC (g/dl)	34.03 ± 0.35	33.58 ± 0.27	33.70 ± 0.40
Platelet ($x10^5$/µl)	7.44 ± 0.16	8.07 ± 0.74	7.52 ± 0.04

Data are expressed as mean ± S.E.M. (n = 10/sex)
RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration
Significantly different from control: *P<0.05

Table 2. Hematological values of male rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
RBC ($x10^6$/µl)	6.14 ± 0.95	6.75 ± 1.04	8.46 ± 0.20
HGB (g/dl)	18.61 ± 1.51	18.23 ± 0.75	17.12 ± 0.55
HCT (%)	39.14 ± 3.92	40.42 ± 5.60	49.60 ± 1.63
MCV (fl)	71.90 ± 10.03	66.57 ± 8.10	58.76 ± 0.52
MCH (pg)	47.33 ± 20.61	45.40 ± 21.24	20.26 ± 0.59
MCHC (g/dl)	55.00 ± 13.09	57.37 ± 16.16	34.46 ± 1.02
Platelet ($x10^5$/µl)	12.60 ± 2.85	10.94 ± 1.27	8.43 ± 0.27

Data are expressed as mean ± S.E.M. (n = 10/sex)
RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration
Significantly different from control: *P<0.05

Table 3. Differential white blood cell count of female rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
White blood cell (x10^3/µl)	2.57 ± 0.34	5.05 ± 1.11*	4.28 ± 1.35*
Neutrophil (%)	9.86 ± 1.22	23.17 ± 6.15*	20.50 ± 5.50*
Lymphocyte (%)	80.86 ± 1.91	72.33 ± 5.88	77.50 ± 5.50
Monocyte (%)	6.28 ± 1.13	3.17 ± 0.94	2.00 ± 0.00
Eosinophil (%)	3.00 ± 0.58	1.33 ± 0.80	0.00 ± 0.00
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± S.E.M. (n = 10/sex)
Significantly different from control: *P<0.05

Table 4. Differential white blood cell count of male rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
White blood cell (x10^3/µl)	4.32 ± 0.31	6.22 ± 0.44*	7.04 ± 0.77*
Neutrophil (%)	14.43 ± 3.08	14.14 ± 2.19	22.40 ± 2.50*
Lymphocyte (%)	77.71 ± 3.56	78.14 ± 3.24	69.80 ± 3.51
Monocyte (%)	5.86 ± 1.40	5.57 ± 0.95	5.40 ± 1.29
Eosinophil (%)	2.00 ± 0.72	2.14 ± 0.63	2.40 ± 0.81
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Data are expressed as mean ± S.E.M. (n = 10/sex)
Significantly different from control: *P<0.05

Table 5. Blood chemistry values of female rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
Glucose (mg/dl)	124.71 ± 2.64	141.00 ± 6.40*	122.00 ± 3.00
BUN (mg/dl)	20.57 ± 0.65	21.33 ± 0.91	26.50 ± 1.50*
Creatinine (mg/dl)	0.36 ± 0.02	0.40 ± 0.02	0.40 ± 0.00
Total protein (g/dl)	5.20 ± 0.09	5.48 ± 0.16	5.75 ± 0.45
Albumin (g/dl)	3.78 ± 0.07	3.40 ± 0.08*	3.90 ± 0.40
Total bilirubin (mg/dl)	0.27 ± 0.02	0.22 ± 0.02	0.56 ± 0.05
Direct bilirubin (mg/dl)	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
SGOT (U/l)	142.43 ± 7.55	127.50 ± 14.56	136.50 ± 15.50
SGPT (U/l)	38.71 ± 2.89	38.17 ± 3.83	35.00 ± 2.00
Alkaline phosphatase (U/l)	32.28 ± 1.23	64.17 ± 16.65	27.50 ± 1.50

Data are expressed as mean ± S.E.M. (n = 10/sex)
BUN: blood urea nitrogen; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase
Significantly different from control: *P<0.05

Table 6. Blood chemistry values of male rats in subchronic oral toxicity of Biopes.

Parameter	Control	Biopes	
		80 mg/kg/day	140 mg/kg/day
Glucose (mg/dl)	128.71 ± 5.00	129.14 ± 3.59	130.40 ± 2.79
BUN (mg/dl)	20.86 ± 0.40	21.14 ± 0.46	22.80 ± 0.97*
Creatinine (mg/dl)	0.38 ± 0.01	0.40 ± 0.05	0.42 ± 0.02
Total protein (g/dl)	5.47 ± 0.10	6.13 ± 0.23	6.10 ± 0.43
Albumin (g/dl)	3.50 ± 0.05	3.31 ± 0.18	3.30 ± 0.10
Total bilirubin (mg/dl)	0.18 ± 0.04	0.22 ± 0.03	0.26 ± 0.04
Direct bilirubin (mg/dl)	0.00 ± 0.00	0.18 ± 0.18	0.58 ± 0.58
SGOT (U/l)	148.86 ± 8.05	187.28 ± 51.57	155.60 ± 8.60
SGPT (U/l)	42.71 ± 2.02	61.00 ± 20.68	49.40 ± 8.20
Alkaline phosphatase (U/l)	59.57 ± 1.56	84.00 ± 10.18*	98.00 ± 10.64*

Data are expressed as mean ± S.E.M. (n = 10/sex)
BUN: blood urea nitrogen; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamic pyruvic transaminase
Significantly different from control: *P<0.05

DISCUSSION

The use of insecticides in agriculture has long been known to affect the health of users, mainly farmers. Each year, tens of thousands of farmers, especially in developing countries, are affected by exposure to insecticides (Dharmani and Jaga, 2005; Konradsen, 2006). Drinking water and food crops, especially fruits and vegetables are also contaminated by insecticides, which can cause a serious health hazard to consumers. Although deaths from the exposure of insecticides are uncommon, the increased mortality and morbidity of human beings due to this problem is reported (Dharmani and Jaga, 2005; Eddleston et al., 2006; Konradsen, 2006). As reported by World Resources Institute (WRI, 1998), the pesticide use by the farmers is increasing rapidly in the developing world, and these farmers also apply insecticides that are more toxic than those used in developed countries.

Organophosphates and carbamates are synthetic compounds widely used as insecticides, thereby protecting livestocks, crops and communities. However, organophosphate and carbamate poisoning is an important clinical problem, often life-threatening (Peduto et al., 1996; Dharmani and Jaga, 2005; Konradsen, 2006), especially in suicidal attempts. Over the years synthetic insecticides, owing to their various adverse effects, have been widely replaced by herbal insecticides (e.g., bioinsecticides). The use of bioinsecticides, the natural products, in agriculture is increased worldwide since they are believed to be less toxic than synthetic counterparts. The bioinsceticide Biopes is formulated with the extract of *S. curtisii.* The efficacy for insecticide effect of the plant extract has been reported (Kaltenegger et al., 2003; Kim et al., 2008).

In this study, the safety of the bioinsecticide Biopes was monitored. The acute dermal toxicity study showed that direct exposure to non-diluted Biopes evoked irritation on the skin of all treated animals and caused convulsions and death in two treated guinea pigs. The result indicated the skin penetrating ability of the formulation. However, the diluted solution of Biopes at the concentration used in agriculture [0.05% (w/w) Biopes] could cause the dermal irritation but this effect was weak and recovered within the short period. The results obtained demonstrated that Biopes possesses irritating and toxic effects and might be harmful to the users, especially if they were directly exposed to the non-diluted solution.

In acute oral toxicity study, Biopes showed low toxic effects since LD50 value of Biopes was much higher than those of the chemical insecticides (e.g., oral LD50 of organophosphate parathion = 14 mg/kg) (Gelal et al., 2001). Exposure to even small amounts of organophosphate or carbamate can be fatal and death is usually caused by respiratory failure resulting from bronchospasm, excessive bronchial secretions, paralysis of the diaphragm and intercostal muscles as well as depression of respiratory center in the brain (Peduto et al., 1996; Sungur and Güven, 2001). In this study, lethally high doses of Biopes produced violent clonic convulsions that led to respiratory failure. The underlining mechanism behind this is unknown, but this could possibly be due to disturbance of the central nervous system.

In subchronic toxicity study, the repeated dose of Biopes at the dose of 80 and 140 mg/kg body weight (50 and 100 folds of the concentration used in agriculture) for 90 days caused significant decrease in body weight gain. From histopathological study of visceral organs, this change could be due to the irritating effect on the gastrointestinal tract of the Biopes-treated animals and thereby, consuming less food. Long-term intake of Biopes also caused a slight histopathological change in lungs of rats. A mild degree of lung infiltration accompanied with slight increase of neutrophils in some rats demonstrated lung inflammation which might be due to drug or food aspiration. Regarding to functional status of liver, liver enzyme levels were monitored. Serum enzymes like serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) are very useful biochemical indicators of variety of diseases, especially liver diseases (Wiwanitkit, 2001). In general, abnormal liver enzyme levels may signal liver damage or alteration in bile flow. Elevated serum aminotransferases (SGOT and/or SGPT) suggest injury of hepatocytes (Mahl, 1998). An increase in ALP levels in the serum may reflect physiologic or pathologic changes beyond those of hepatic origin (Mahl, 1998; Wiwanitkit, 2001; Fernandez and Kidney, 2007). The capability of a chemical to cause liver damage often results from the interaction of a series of complex cellular processes that are involved in the intake, biotranformation and elimination of these potentially toxic compounds (Guillouzo, 1998). In this study, Biopes caused a dose-dependent increase in serum ALP level in rats. Although the change was less than 1 fold, and the histopathological study did not show any correlation with the changes of liver enzymes, we postulate the high ALP level in Biopes-treated group predicts the potential of Bipes to cause liver damage if used in very large doses. However, further study such as hepatotoxicity test is still needed to confirm its liver toxic effects.

In this study, some values of hematology (e.g., HGB, HCT, RBC and MCV), blood biochemical indices (e.g., glucose and BUN) and organ weights of Biopes-treated rats were significantly different when compared to those of the control groups. However, these differences could be considered to have no clinical significance because all of these values are still within the normal ranges (Wongcome et al., 2007). This indicated that Biopes might not cause any detectable adverse effect on these parameters.

CONCLUSION

The results indicated that the use of Biopes should be monitored with caution, and avoidance of direct contact with the non-diluted formulation should be encouraged. Moreover, considering the increased in the traditional use of this plant product for insect control in agriculture, additional toxicological evaluations, especially chronic oral toxicity or subchronic dermal toxicity tests are warranted to validate its safety and potential benefits.

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