Arch Pharm Res Vol 26, No 5, 351-357, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Synthesis of Allylthiopyridazine Derivatives and Inhibition of Aflatoxin B_1-Induced Hepatotoxicity in Rats

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(Received February 4, 2003)

Five kinds of allylthiopyridazine derivatives were synthesized and their chemoprotective activities examined in rats exposed to aflatoxin B_1-toxicant. Rats were pretreated with five allylthiopyridazine derivatives at daily oral doses of 50 mg/kg for 10 consecutive days, and during this period with one or three repeated doses of the potent hepatotoxin, aflatoxin B_1. The hepatoprotective effects of the allylthiopyridazine derivatives against aflatoxin B_1 (1 mg/kg, three times at intervals of 3 days, i.p., or at 3 mg/kg, once at final days, i.p.) administration were showed the significantly normal as compared with control in body and liver weights. Aspartate aminotransferase and alanine aminotransferase levels were markedly elevated after aflatoxin B_1 administration, and pretreatment with allylthiopyridazine derivatives, before aflatoxin B_1 administration, resulted in decreased levels of these enzymes. In addition, the allylthiopyridazine derivatives, K6 (3-methoxy-), K8 (3-chloro-), K16 (3-ethoxy-) and K17 (3-*n*-propoxy), induced elevated hepatic GSH levels. Four kinds of allylthiopyridazine derivatives investigated were effective against aflatoxin B_1-induced hepatotoxicity.

Key words: Allylthiopyridazine derivatives, Aflatoxin B_1-induced hepatotoxicity, Hepatoprotective effect

INTRODUCTION

Aflatoxin B_1 (AFB_1), a mycotoxin produced by the fungi *Aspergillus flavus* and *Aspergillus paracticus*, is known to be a potent hepatotoxin and hepatocellular carcinogen in experimental animals (Towner *et al.*, 2000; Gopalan *et al.*, 1993; Toskulkao *et al.*, 1996). AFB_1 is a category I human carcinogen, and a very potent genotoxic agent. In oriental countries, food contamination with AFB_1 and a high incidence of hepatitis B virus infection are a serious problem (Eaton and Gallagher, 1994; Gyamfi *et al.*, 1996; Shevieila *et al.*, 2001). Recently, it was proposed that AFB_1-induced toxicity is caused by lipid peroxidation and oxidative DNA damage (Souza *et al.*, 1999; Manson *et al.*, 1998; Wang *et al.*, 1999). Exposure AFB_1 in animals is usually followed by pathological changes to the liver, and elevated alanine and aspartate amino transferase levels (Ueng *et al.*, 1995; Metcalfe *et al.*, 1981; Judah *et al.*, 1993 Gorelick *et al.*, 1990).

According to recent reports, the sulfur compounds in garlic inhibit the proliferation of tumor cells and suppress chemically-induced carcinogenesis in liver, thus protecting human organs from toxicants or radiation. They also increase the expressions of mEH (microsomal epoxyhydrolase) and GST (glutathione *S*-transferase), and inhibit the expression of CYP450 2E1. A similar synthetic derivative containing the allylsufur moiety was reported for hepatoprotective effects against chemically-induced toxicity (Ha *et al.*, 1999). Moreover, in terms of molecular structure, it was found that the allylthio group is an important pharmacopore (Jung *et al.*, 2001; Lee *et al.*, 2001). In the preliminary research, chemical compounds containing the allylthio moiety, were synthesized by substituting the allylthio moiety onto a pyridazine nucleus (Kwon, 2002).

Allylthiopyridazine derivatives were designed and synthesized in order to develop hepatoprotective agents, by substituting a halogen or alkoxy at para-position of the allylthio moiety. These allylthiopyridazine derivatives were found to prevent the hepatotoxicity caused by carbon tetrachloride in rats and to induce phase II detoxification enzymes (Jung *et al.*, 2001; Kwon, 2002). In the present investigation, we examined the hepatoprotective effects of allylthiopyridazine derivatives against AFB_1-induced hepatic toxicity.

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MATERIALS AND METHODS

Chemicals

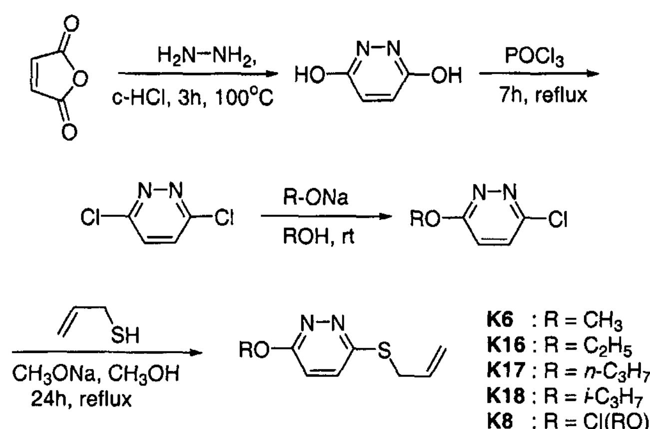
Aflatoxin B_1, Tris acetate, EDTA, DMSO, Tris-base, bovine serum albumin sodium dithionite, 1-chloro-2,4-dinitrobenzene and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO. USA.). The alanine and aspartate aminotransferase kit were purchased from Yeongdong Pharmaceutical Co. (Seoul, Korea) and all other chemicals were purchased from Sigma (St. Louis, MO. USA.). The equipment used included:- high speed centrifuge (DuPont Sorvall Instrument, Model RC5C), ice maker (Welbit Co. USA.), ultracentrifuge (Beckman Co., Lid. L-80), UV-spectrophotometer (Hewlett Packard HP 8452A Diode-Array), osterizer blender (Oster Co., Listed 564A Household type). The analysis of allylthiopyridazine derivatives were confirmed by the NMR (Jeol JMN-LA 400 MHz), IR (Perkin-Elmer FT-IR spectrometer) and melting point (Fischer-Johns melting point apparatus). Commercially available chemicals and solvents were used and purchased from Aldrich, Sigma, or Merck.

General procedure for the synthesis of allylthiopyridazine derivatives

The pyridazine nucleus derivatives were obtained by condensing hydrazine with anhydrides as maleic, 2,3-dimethylmaleic and 3,4,5,6-tetrahydrophthalic anhydride. 3-Alkoxy-6-chloropyridazine were produced from 3,6-dichloropyridazine, sodium and anhydrous alcohols. 3-Chloro-6-thiopyridazine was synthesized from synthetic compound of 3,6-dichloropyridazine and thiourea. 3-Chloro-6-allylthiopyridazine was synthesized from mercaptanes, sodium methoxide and allylbromide 4.33 mL (0.05 mol). 3-chloro-6-thiopyridazine 7.33 g (0.05 mol), after treatment of dissolving sodium 1.15 g (0.05 mol) in anhydrous alcohols (50 mL) at room temperature. The reaction mixture stirred at reflux condition for 3 h. Anhydrous alcohols was evaporated in vacco and the reaction mixture was dissolved in diethyl ether (100 mL). After being stirred 0.2 h, washed with water (50 mL) twice, and dried over anhydrous sodium sulfate. The reaction mixture was filtered and concentrated to give yellowish crude oil residue. The concentrated product was purified by silica gel column chromatography acetate (10:1) as an eluant to give allylthiopyridazine derivatives. The chemical structures of the allylthiopyridazine derivatives are shown in Scheme 1 (Kwon, 2002).

3-Methoxy-6-allylthiopyridazine (K6)

Yield: 3.99 g (53.7%), mp 25-27°C, ^{1}H-NMR ($CDCl_3$) δ 3.95 (d, 2H, SCH_2), 4.08 (s, 3H, OCH_3), 5.25 (dd, 2H, CH_2), 6.00 (m, 1H, CH), 6.80-7.30 (dd, 1H×2, CH), IR (NaCl, cm^{-1}) 3.056 (aromatic), 2.947 (CH), 1.596 (aromatic), (Kwon, 2002).



Scheme 1. Synthesis of allylthiopyridazine derivatives (Kwon, 2002)

3-Chloro-6-allylthiopyridazine (K8)

Yield : 2.03 g (21.8%), mp 68-70°C, ^{1}H-NMR ($CDCl_3$) δ 4.00 (d, 2H, SCH_2), 5.25 (dd, 2H, CH_2), 6.00 (m, 1H, CH), 7.20-7.50 (dd, 1H×2, CH), IR (NaCl, cm^{-1}) 3.096 (aromatic), 1.501 (aromatic), (Kwon, 2002).

Animals

Male Sparague-Dawley rats, 180~220 g, (Daehan Experimental Animal Center, Seoul, Korea) were housed in a controlled environment with food (Samyang Feed) and water available *add libitum*. Rats received daily doses of 5 kinds of allylthiopyridazine derivatives (50 mg/kg body weight, p.o.) for 10 consecutive days, during which time all rats received aflatoxin B_1. It was dissolved (1 mg/kg, i.p.) in a 3 mL/mg saline solution containing dimethylsulfoxide (DMSO, 0.03 mL/kg) three times at intervals of 3 days or (3 mg/kg body weight, i.p.) in a 3 mL/mg saline solution containing DMSO once on the final administration of allylthiopyridazine derivatives. The solution was frozen at -4 °C for storage until use. Control animals were treated with vehicle only (saline solution 3 mL/kg or olive oil, 1 mL/kg body weight, 20% w/v).

Hepatic enzyme assessment

Rats were sacrificed for assay 24 h after the final injection of AFB_1. Blood sample was collected from the abdominal aorta of each rat. The blood was centrifuged at 3,000 rpm for 20 min to separate the serum and store at 4 °C. The activities of alanine and aspartate aminotransferase (ALT and AST) were determined using a kit by the methods reported by Reitman and Frankel (1957). Livers were removed and fixed in 10% buffered neutral formalin solution, then processed by routine techniques with hematoxylin and eosin staining for histopathological assessment.

The portion of rat liver was homogenized in phosphate buffer (pH 7), ground for 1 min, and a 10% liver homo-

genate was acquired. The homogenate was analyzed using a cholesterol kit. Blood serum albumin was analyzed using a UV-spectrophotometer in UV 630 nm using a standard blood serum kit.

Preparation of microsome and cytosol

The rat liver was extracted, sacrificed at 24 h after the final aflatoxin B_1-treatment, washed with a saline solution, sliced, and milled in 2 times volumes of 0.1 M Tris-KCl buffer (0.1 Tris acetate, 0.1 M KCl, 1 mM EDTA, pH 7.4) using an osterizer blender. The liver was ultracentrifuged for 30 min at 10,000×g and 90 min at 105,000×g using a high speed centrifuge at 4 °C, and the supernatant cytosolic fraction was separated. Microsome, a precipitate remained after cytosol of supernatant, was mixed again with 0.1 M sodium pyrophosphate buffer (0.1 M sodium pyrophosphate and 1 mM EDTA), and ultracentrifuged again for 60 min at 105,000×g washed and the microsome was acquired. This microsomal fraction was re-dispersed in 50 mM Tris acetate buffer (50 mM Tris acetate, 20% glycerol, 1 mM EDTA, pH 7.4), divided and stored at -70 °C until used. All operations were executed at below 4, and protein was assayed using the method of Lowry *et al.* (1951).

Measuring glutathione *S*-transferase activity

The activity of glutathione *S*-transferase in the cytosol fraction was determined by the speed of decolorization when 1-chloro-2,4-dinitrobenzene (CDNB) is conjugated with glutathione (GSH) by glutathione *S*-transferase. 25 μg of the cytosol protein in 0.1 M potassium phosphate buffer pH 6.5 was left for 2 min at 25 °C, and 1.0 mM CDNB and 1.0 mM GSH added, absorbance was measured using a UV spectrophotometer for 100 seconds at 10 seconds intervals at 340 nm by the method of Habig *et al.* (1974). Heated and inactivated cytosol was used as a blank, and a molar absorption coefficient was expressed in 9.6 $\mu M^{-1}cm^{-1}$. Protein content was assayed using the Bradford method (1976).

Statistical analysis

The experimental results were summarized as means± S.D. and the statistical significance was performed using by the studentís *t*-test.

RESULTS AND DISCUSSION

The objective of this study was to investigate the hepatoprotective effects of synthetic allylthiopyridazine derivatives. These allylthiopyridazine derivatives were found to inhibit the proliferation of tumor cells, to suppress chemically-induced carcinogenesis in the liver, and to protect human organs from toxic agents or radiation by increasing the expressions of mEH (microsomal epoxyhydrolase) and GST (glutathione *S*-transferase) and inhibiting the expression of CYP450. In addition, it was found that in terms of the molecular structure, that the allylthio group is an important pharmacopore (Jung *et al.*, 2001; Lee *et al.*, 2001).

In a previous report, allylthiopyridazine derivatives prevented the hepatic toxicity caused by AFB_1-toxicant, which requires cytochrome P450-mediated activation to produce carcinogen. The metabolites of AFB_1 implicated in carcinogenesis and mutagenesis include reactive aflatoxin-8,9-oxide, which covalently binds to proteins and nucleic acids. Aflatoxin-8,9-oxide is considered to be detoxified mainly through GST (Gluthathione *S*-transferase)-catalyzed conjugation (Gopalan *et al.*, 1993).

Thus allylthio group as a pharmacologically active group was introduced into pyridazine nucleus and a substituent such as halogen or alkoxy was also introduced into the para-position of the allylthio moeity. 3-Alkoxyñ6-allylthiopyridazine derivatives have been prepared from 3,6-dichloropyridazine, prepared from maleic anhydride. The reaction of 3,6-dichloropyridazine with alcoholic sodium alkoxides affords the corresponding 3-alkoxy-6-chloropyridazines with the selective substitution of the first chlorine atom, which are further converted into the corresponding 3-alkoxy-6- allylthiopyridazines with 2-propenethiol/sodiumethoxide. The nucleophilic displacement of chlorine atom in 3-alkoxy-6-chloropyridazines requires for polonged reaction time at reflux temperature and yields are moderate to low with 22%.

Histopathological observations were characterized by typical signs of hepatic injury, hepatocytes were enlarged with an increased nuclear-cytoplasmic ratio and atypical nuclei were identified, showing irregular contour and coarse chromatin. Intracellular fat vacuole and potential apoptotic bodies are indicated in Fig. 1.

Effect of allylthiopyridazine treatment on body and liver weight changes

The effect of aflatoxin toxicity prevention by the allylthiopyridazine derivatives K6 -with a substituted methoxy group at para-position, K8 -with a substituted chloro group at para-position, and K17, K18 -with substituted normal, isopropoxy group at para-position were examined in terms of their abilities to ameliorate AFB_1-induced hepatotoxicity. Through preliminary experiment, it was certified that the method of continuous oral pretreatment for 10 days with 50 mg/kg of the allylthiopyridazine derivative was appropriate.

A study of inducing acute hepatotoxicity with a single dose (3 mg/kg of intraperitoneal injection) of AFB_1, a group of allylthiopyridazine derivative pretreatment of liver weight showed a preventive effect similar to the most of

normal control group. On the other hand, on attempting to induce a chronic liver damage with three repeated doses (1 mg/kg of intraperitoneal injection) of AFB_1, it was found that rats pretreatment with the allylthiopyridazine showed no significant weight change versus the control group.

Moreover, the ratio of the liver to body weights of rats in the AFB_1-treated and control group was no different (Table I). Pretreatment with the allylthiopyridazine derivatives, K6, K8, K16, K17 and K18, inhibited the body weight reduction induced by AFB_1, but K18 induced an increase compared to the normal control group. It is believed that K18 was toxic as all rats administered this compound died.

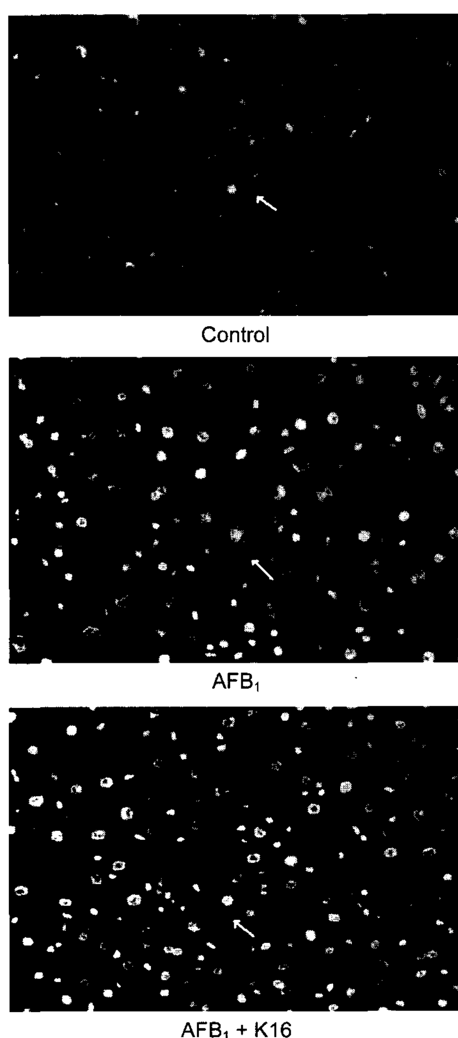


Fig. 1. Histopathological changes in rat liver tissue. Control: Hepatocytes show round nuclei and abundant cytoplasm. AFB_1: hepatocytes are enlarged with increased nuclear-cytoplasmic ratio. Many atypical nuclei are present, showing irregular contours and coarse chromatin. An intracellular fat vacuole is noted and potential apoptotic bodies are indicated. AFB_1+K16: Architecture of the hepatocytic shows a cord-like arrangement. The cytoplasm is more eosinophilic and the nuclei are not increased (H&E stain, ×400).

Effect of allylthiopyridazine treatment on AFB_1-induced hepatotoxicity

The effects of allylthiopyridazine derivatives on AFB_1-induced hepatotoxicity were monitored in rats by assaying blood ALT and AST activities (Table II). Activities of serum enzymes reached values which were indicative of liver damage. There was a statistical difference in ALT and AST activities from AFB_1-treated serum compared to controls ($p<0.01$ for both ALT and AST). The elevation of AST activity in AFB_1-treated serum was significantly higher than that observed for ALT activity. The five of allylthiopyridazine derivative, K6, K8, K16, K17 and K18 group showed a meaningful result compared to AST and ALT activation for acute toxicity with 3 mg/kg of intraperitoneal single doses injection of AFB_1. Results of measuring AST activation level, a acute toxicity with 3 mg/kg of i.p. single doses injection of AFB_1 slightly higher than observed for a chronic liver damage with 1 mg/kg of i.p. three repeated doses injection of AFB_1. Both of results showed an effect of all the allylthiopyridazine derivatives completely decreases in AFB_1 induced elevation of ALT and AST activities, respectively.

To investigate whether the hepatoprotective activity of allylthiopyridazine derivatives against liver injury such as hepatitis with the administration of AFB_1, we measured albumin level in AFB_1-intoxicated rats. The pretreatment of allylthiopyridazine derivatives so slightly decreased albumin level in the AFB_1-treated rats compared with that

Table I. Effect of synthetic allylthiopyridazine derivatives on aflatoxin B_1-induced body and liver weight changes in rats.

Treatment (mg/kg)	Dose (mg/kg)	Body weight (g)	Liver weight (g)	Body/ Liver wt
Untreated	-	231.1±6.52[c]	6.66±0.11	2.8±0.3
AFB_1 (3×1)[a]	-	227.7±2.08	7.22±0.34[d]	2.9±0.1
AFB_1 (3×1)	K6 (50)	219.7±6.55	6.65±0.39	3.0±0.2
AFB_1 (3×1)	K8 (50)	208.1±4.63	6.21±0.51[e]	2.9±0.2
AFB_1 (3×1)	K16 (50)	211.8±3.60	6.42±0.92	3.0±0.1
AFB_1 (3×1)	K17 (50)	227.3±7.54	6.49±0.45	2.8±0.3
AFB_1 (3×1)	K18 (50)	227.9±5.54	6.99±0.61[e]	3.0±0.2
Untreated	-	172.8±3.81	5.59±0.16	3.2±0.1
AFB_1 (1×3)[b]	-	160.6±2.46	6.32±0.16[d]	3.9±0.1[d]
AFB_1 (1×3)	K6 (50)	173.3±5.22	5.45±0.32	3.1±0.2
AFB_1 (1×3)	K8 (50)	162.5±18.7	5.39±0.15	3.3±0.6
AFB_1 (1×3)	K16 (50)	173.6±6.11	5.56±0.38	3.2±0.3
AFB_1 (1×3)	K17 (50)	180.8±25.0	5.79±0.39	3.2±0.8
AFB_1 (1×3)	K18 (50)	177.3±13.8	5.82±0.57	3.3±0.5

[a]Aflatoxin B_1 (3 mg/kg, i.p.) was injected single doses.
[b]Aflatoxin B_1 (1 mg/kg, i.p.) was injected three repeated doses.
[c]The results presented are means±S.D. (n=6).
[d]Significantly different from the control.
[e]Significantly different from the AFB_1-treated alone.

Table II. Effect of synthetic allylthiopyridazine derivatives on hepatic albumin levels and activities of AST, ALT and GST in AFB$_1$-treated rats

Treatment	Dose (mg/kg)	AST (Unit/mL)	ALT (Unit/mL)	Albimin (g/dL)	GST (imol/min /mg protein)
Untreated	–	44.54±3.4[c]	32.4±6.1	3.95±0.12[c]	1.43±0.02
AFB$_1$ (3×1)[a]	–	456.3±20.8[d]	260.3±7.4[d]	4.58±0.29[d]	1.14±0.05
AFB$_1$ (3×1)	K6 (50)	56.3±2	50.2±1.2	3.62±0.26	1.39±0.03[e]
AFB$_1$ (3×1)	K8 (50)	113.2±2.6[e]	102.4±5.8[e]	3.6±0.25	1.35±0.05[e]
AFB$_1$ (3×1)	K16 (50)	132.4±4[e]	140.6±0.8[e]	3.45±0.35	1.22±0.02
AFB$_1$ (3×1)	K17 (50)	100.3±2.1[e]	99.5±7.8[e]	3.77±0.16	1.23±0.01
AFB$_1$ (3×1)	K18 (50)	96.5±1.4	69.4±6.3	3.44±0.76	1.34±0.04
Untreated	–	36.84±10.2	33.82±0.48	3.47±0.43	1.57±0.01
AFB$_1$ (1×3)[b]	–	295.41±47.16[d]	189.61±16.91[d]	4.54±0.16[d]	1.2±0.02[d]
AFB$_1$ (1×3)	K6 (50)	45.15±2.6	32.7±1.4	3.56±0.34	1.42±0.04[e]
AFB$_1$ (1×3)	K8 (50)	44.75±6.1	40.25±3.43	4.12±0.62[e]	1.36±0.06
AFB$_1$ (1×3)	K16 (50)	36.62±2.7	32.48±0.9	3.97±0.27	1.34±0.04
AFB$_1$ (1×3)	K17 (50)	39.22±1.87	49.67±4.25	3.91±0.23	1.34±0.03
AFB$_1$ (1×3)	K18 (50)	73.85±0.66[e]	116.36±12.16[e]	3.92±0.18	1.45±0.05[e]

[a] Aflatoxin B$_1$ (3 mg/kg, i.p.) was injected single doses.
[b] Aflatoxin B$_1$ (1 mg/kg, i.p.) was injected three repeated doses.
[c] The results presented are means±S.D. (n=6).
[d] Significantly different from the control.
[e] Significantly different from the AFB$_1$-treated alone.

of the untreated control group. Meanwhile, pretreatment of allylthiopyridazine derivatives prevented this activity in liver injury that is caused by AFB$_1$.

Self-immunological hepatic diseases are known to result in abnormalities of lipid metabolism (Blanchette-Mackie *et al.*, 1989). Effect of total cholesterol were shown in Fig. 2. We examined measuring cholesterol contents to determine a change of the lipid metabolism of liver with aflatoxin B$_1$-induced toxicity. The pretreatment of K6 showed 119.6 mg/dL, which was the most similar level to the untreated control group. The pretreatment of K18 with 113 mg/dL, showed a slightly low level compared to 117 mg/dL in the untreated control group, but is analyzed to be of normal range. In case of inducing liver disease of chronic aflatoxin B$_1$-induced toxicity (1 mg/kg of i.p. three repeated doses), the pretreatment of K6 with 84.0 mg/dL showed a similar level compared to 65.2 mg/dL in the normal control group. In case of the pretreatment of K18 with 126.7 mg/dL, it was high cholesterol level by accumulating cholesterol in the liver and toxicity in medicine itself was found.

Effect of allylthiopyridazine treatment on hepatic GSH activities

Glutathione *S*-transferase (GST) induction can be considered as a reward cascade for chemical stress caused by electrophilic materials. As an adaptation action responding to bio-texture when toxicity materials for liver exist, it increases GSH inside the particle. Phenolic antioxidant, flavonoid, thiocarbamates, dithiothiones and so on inducing GST, formulate electrophilic material, cause a chemical stress inside the particle, becoming a substrate of GST and incorporated with GSH (Toskulkao *et al.*, 1996). An oxidative stress formulated by an infection of AFB1 will activate phase II antioxidant enzyme is a mechanism stimulating glutathione synthesis so that oxidation-reduction potential inside the particle, which was changed accordingly, can be returned (Jung *et al.*, 2001).

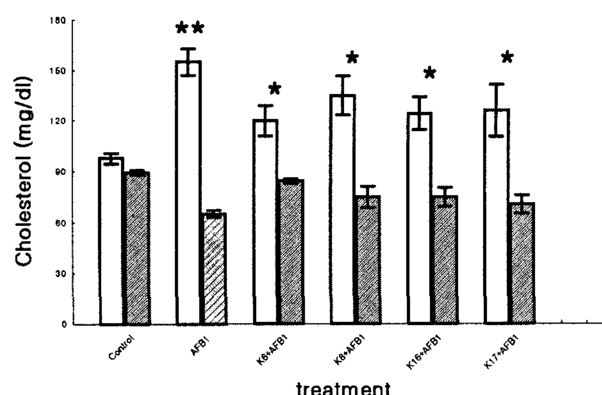


Fig. 2. Effect of synthetic allylthiopyridazine derivatives on hepatic cholesterol contents in aflatoxin B$_1$-treated rats. The results presented are means±S.D. (n=6). **: Significantly different from the control group ($p<0.01$). *: Significantly different from the AFB$_1$-treated alone group ($p<0.05$). □ Acute hepatotoxicity with 3 mg/kg of intraperitoneal single doses injection of AFB$_1$. ▨ Chronic liver damage with 1 mg/kg of intraperitoneal three repeated doses injection of AFB$_1$.

Hepatic GST activities in rat were elevated by 25% an acute toxicity single doses of AFB$_1$ (3 mg/kg of i.p.) slightly lower than observed for a chronic liver damage with 31% three repeated doses of AFB$_1$ (1 mg/kg of i.p). Pretreatment of allylthiopyridazine derivatives with 50 mg/kg p.o. caused significantly elevation in hepatic GST activities, as compared with the treated with AFB$_1$ alone. The results showed an effect of all the allylthiopyridazine derivatives completely in elevating AFB$_1$-induced decreases in hepatic GST activities in rats, respectively (Table II).

From the experimental results above, synthetic the allylthio moiety containing compounds, structurally different of para-position, 3-chloro-6-allylthiopyridazine (K8), 3-ethoxy-6-allylthiopyridazine (K16) and 3-*n*-propoxy-6-allylthiopyridazine (K17) were efficacious protective in hepatotoxicities caused by AFB$_1$. With simple structure 3-methoxy-6-allylthiopyridazine (K6) component as the most prominent leading compound, were proved as a substitute compound anticipated to protect hepatotoxicity by AFB$_1$-induced.

ACKNOWLEDGEMENT

This work was supported by a Promoting Program for the Basis of Women's University Grant (00-N6-05-01-A-02) from KISTEP.

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