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The Effects of Cleistanthoside A Tetraacetate Synthesis on Acute Toxicity and Bone Marrow Micronucleus in ICR Mice

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Abstract

Phyllanthus taxodiifolius Beille is used in traditional medicine in tropical and subtropical areas. Although it has been used as a folk medicine for a long time, studies on its safety have been limited. In this study, the bone marrow micronucleus and oral toxicity of Cleistanthoside A tetraacetate, a modified arylnaphthalide lignan of Cleistanthoside A from the *P. taxodiifolius* Beille, were evaluated. Imprinting control region (ICR) female mice were orally administered Cleistanthoside A tetraacetate at doses of 250, 500 and 1,000 mg/kg body weight (BW). Signs of toxicity, bone marrow micronucleus, clinical blood chemistry, and histopathological findings were determined after treatment. No mortality was observed in any of the groups. A significant increase in numbers of bone marrow micronucleus, marked elevation of alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine (CRE) were observed in the mice administered with Cleistanthoside A tetraacetate 1,000 mg/kg BW. These results also correlated with the histopathological scoring of liver and kidney cells in ICR mice. The mice administered with Cleistanthoside A tetraacetate 1,000 mg/kg BW had high toxicity in the liver and kidneys. Long term or chronic toxicity should be further studied for safety.

Keywords: Cleistanthoside A tetraacetate, toxicity, micronucleus, *Phyllanthus* spp.

Introduction

Phyllanthus taxodiifolius Beille, in Thai "Khrai-Hang-Naak", is used in traditional medicine in tropical and subtropical areas [1]. It has been used as an anti-inflammatory, as an anti-diabetic in mice, and to inhibit the growth of cancer cells [2,3]. It has also prevented the growth of epidermal carcinoma in nasopharynx tissue culture [4]. Cleistanthoside A tetraacetate is a derivative modification from arylnaphthalide lignan glycosides extracted from the *P. taxodiifolius* Beille [4]. This structure plays an important role in the growth inhibition of cancer cell types, including oral, colon, breast, and lung cancer cells [5]. It also affects cell cycle arrest and apoptosis with the involvement of p53 in lung cancer cells [6]. Although phytochemicals of medicinal plants have been widely used to treat various ailments [7,8], several substances from natural plants, including Cleistanthoside A tetraacetate, have not been guaranteed in terms of their safety [9,10]. In this study, we investigated the effects of Cleistanthoside A tetraacetate, modified from *P. taxodiifolius* Beille, on bone marrow micronucleus and oral toxicity in Imprinting Control Region (ICR) female mice.

Materials and methods

Cleistanthoside A tetraacetate sample

Cleistanthoside A tetraacetate was obtained from Professor Dr. Patoomratana Tuchinda, Faculty of Science, Mahidol University, Thailand. This phytochemical substance was modified from Cleistanthoside A of *P. taxodiifolius* Beille, described in a previous study [4].

Imprinting control region (ICR) female mice

Approval for this study was received from the Animal Experimentation Ethics Committee of the Faculty of Science, Mahidol University, Thailand (Protocol No. 192). Twenty female ICR mice (25 - 30 g), aged 7 weeks, were used for the bone marrow micronucleus and acute oral toxicity tests. The mice were divided into 4 groups (5 mice/group); group 1 was administered corn oil (control group), and groups 2, 3, and 4 were treated with Cleistanthoside A tetraacetate, at 250, 500 and 1,000 mg/kg body weight (BW), respectively. After dosing, all treated mice were observed for any toxic signs or mortality for 48 h. The mice were sacrificed by an injected overdose of Nembutal.

Bone marrow micronucleus

The micronucleus examination was conducted according to the standard technique [11]. The groups of mice were sacrificed at 24 and 48 h after dosing, by injecting pentobarbital sodium at 50 mg/kg BW. Both femurs were removed, and bone marrows were collected in tubes of 0.5 ml of fetal bovine serum. The solution was then centrifuged for 10 min at 1,000 g. The smears were allowed to air dry prior to fixation and staining with a Giemsa solution. A number of micronuclei were obtained by using light microscopy. The 2000 polychromatic erythrocytes (PCEs) per mouse were examined for micronucleated polychromatic erythrocyte (MnPCE).

Liver and kidney functions

Blood was then drawn from the heart and centrifuged at 3,000 g for 15 min at room temperature. The serum was investigated for liver and kidney functions. The tests of alkaline phosphatase (ALP), blood urea nitrogen (BUN), and creatinine (CRE) were investigated by using an automatic analyzer.

Hematoxylin and eosin staining

The livers and kidneys of the mice were removed carefully and then fixed in 10 % buffer formalin solution to obtain the pathological changes. The tissues were prepared for a paraffin box by using automatic tissue processing. Paraffin sections were deparaffinized in xylene to remove the paraffin wax. After deparaffinization, the slide was counter stained with hematoxylin and eosin (H&E). A hematoxylin-metal complex acts as a basic dye, staining nucleic acids in the nucleus. Eosin is an acid aniline dye, which stains the basic components within cells, such as cytoplasm and extracellular spaces.

Histopathological examination

The H&E slides of livers and kidneys were obtained for pathological changes. The pathological changes of the livers were defined by 5 scales, depending on the degree of severity. The pathological findings of insignificant degeneration in the centrilobular area (acinar zone III), scatter necrosis in centrilobular area (acinar zone III), necrosis involve centrilobular area (acinar zone I and II), and extensive necrosis in the centrilobular area (acinar zone I, II and III) were defined by the scores of 0, 1+, 2+, 3+ and 4+, respectively. Pathological findings of kidneys were presented in scales of 0 = insignificant; 1+ = cloudy swelling of tubular epithelium; 2+ = pyknotic nuclei, karyorhexis, karyolysis, and 3+ = completed acute tubular necrosis.

Statistical analysis

All data were expressed as a mean ± standard deviation (SD). Statistical analysis among groups was performed employing an SPSS program. The level of statistical significance was set at P-value < 0.05.

Results and discussion

No mortality was observed after administration of Cleistanthoside A tetraacetate at all doses; 250, 500 and 1,000 mg/kg BW. The results indicated that the Cleistanthoside A tetraacetate, a modified arylnaphthalide lignan of Cleistanthoside A from the *P. taxodiifolius* Beille has no signs of toxicity when orally administered.

Table 1 Genotoxicity of the Cleistanthoside A tetraacetate using bone marrow micronucleus test.

Treatment	Dose (mg/kg BW)	Mn/2000 PCEs (mean ± SD)	
		24 h	48 h
Cleistanthoside A tetraacetate	0 (vehicle)	1.25 ± 0.95	1.25 ± 1.08
	250	1.50 ± 1.29	1.25 ± 1.25
	500	1.75 ± 0.95	1.75 ± 1.25
	1,000	2.75 ± 0.50*	3.25 ± 0.95*
Cyclophosphamide (positive control)	60	69.25 ± 16.82**	71.00 ± 13.49**

Mn = micronucleus, PCEs = polychromatic erythrocytes
*, ** were presented statistically significant at $P < 0.05$ and $P < 0.001$, respectively

The bone marrow micronucleus was investigated with a genotoxic test, using a number of MnPCE at 24 and 48 h after the mice were treated. **Table 1** illustrates the number of micronucleus (Mn) per 2000 PCEs in doses of 250, 500 and 1,000 mg/kg BW of cleistanthoside A tetraacetate. There was a slight increase of MnPCE at dose 1,000 mg/kg BW of the treated substance when compared with the vehicle control at 24 and 48 h after the treated ($P = 0.032$ and 0.044, respectively). No significance was found in the other treated doses when compared with the control, while the treated cyclophosphamide showed strongly significant amounts of MnPCE compared with other groups at $P < 0.001$.

The levels of ALP in the control, and treated groups of 250, 500, and 1,000 mg/kg BW, were 132.00 ± 23.28, 344.20 ± 52.86, 446.40 ± 153.73 and 1180.75 ± 497.31 U/l, respectively (**Figure 1a**). The kidney function test of BUN in the group 1,000 mg/kg BW (48.58 ± 16.35 mg/dl) was at a higher level than that of the 500 (21.08 ± 2.15 mg/dl), 250 (19.54 ± 0.50 mg/dl) and control (20.12 ± 0.68 mg/dl) (**Figure 1b**) groups. The CRE level of the highest treated group (0.67 ± 0.26 mg/dl) was also higher than other groups treated with 500 (0.25 ± 0.04 mg/dl), treated with 250 (0.21 ± 0.02 mg/dl) and the control (0.17 ± 0.02 mg/dl) (**Figure 1c**). The treated dose at 1,000 mg/kg BW showed high levels of ALP ($P < 0.001$) and CRE ($P < 0.001$) when compared with other groups. Although no statistic significance of BUN was found in among treated groups, the highest dose at 1,000 mg/kg BW had a tendency to increase with BUN level.

The pathological findings in the livers and kidneys of treated mice are illustrated in **Figures 2** and **3**, respectively. The treated 250 and 500 mg/kg BW showed slight damage to liver and kidney cells when compared with the control group. However, the group treated with 1,000 mg/kg BW showed severe damage to liver and kidney cells. The histopathological score is shown in **Table 2**. The treated dose 1,000 mg/kg BW showed severe cellular injury; this finding related to the increased amount of MnPCE, liver and kidney function tests at the same treated dose.

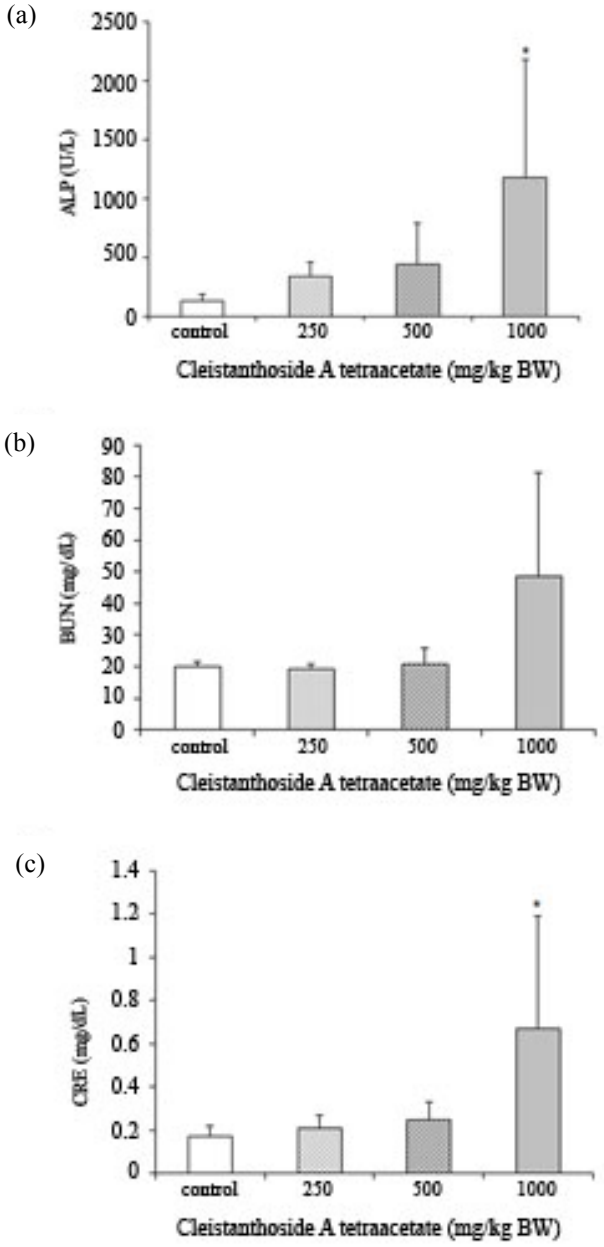

Figure 1 The levels of ALP (a), BUN (b) and CRE (c) in treated groups. Data were expressed as mean ± SD. $P < 0.05$ was considered to be statistically significant.

Phyllanthus taxodiifolius Beille has been widely used as a diuretic drug in Thai traditional herbal medicine [12]. Cleistanthoside A isolated from *Phyllanthus* spp. inhibits cancer growth [13,14]. Although it has been used as folk medicine for a long time, toxicity reports or published data on its safety have been limited. Cleistanthoside A tetraacetate showed slight genotoxicity in bone marrow micronucleus models at the treated dose 1,000 mg/kg BW. The treated dose at 1,000 mg/kg BW also showed toxicity in liver

and kidney cells, in which histopathologic findings corresponded with serum liver and kidney function tests of ALP, BUN, and CRE, respectively. The results indicated that Cleistanthoside A tetraacetate had an acute toxicity to liver and kidney cells at a high treated dose, 1,000 mg/kg BW.

Although phytotherapeutic products contain bioactive ingredients, it may potentially cause adverse effects [15,16]. Acute toxicity is usually defined as adverse changes occurring immediately, or a short time following, a single or short period of exposure to a substance [17,18]. Further study of long term or chronic toxicity is needed to examine and evaluate its safety.

Table 2 Histopathological findings of liver and kidney of ICR mice treated with Cleistanthoside A tetraacetate.

Organ	Histopathological score	Group (mg/kg BW)			
		Control	Treated 250	Treated 500	Treated 1000
Liver	0	5/5	1/5	0/5	0/5
	1+	0/5	3/5	3/5	0/5
	2+	0/5	1/5	1/5	0/5
	3+	0/5	0/5	0/5	2/5
	4+	0/5	0/5	1/5	3/5
Kidney	0	5/5	3/5	1/5	0/5
	1+	0/5	2/5	2/5	0/5
	2+	0/5	0/5	1/5	4/5
	3+	0/5	0/5	1/5	1/5

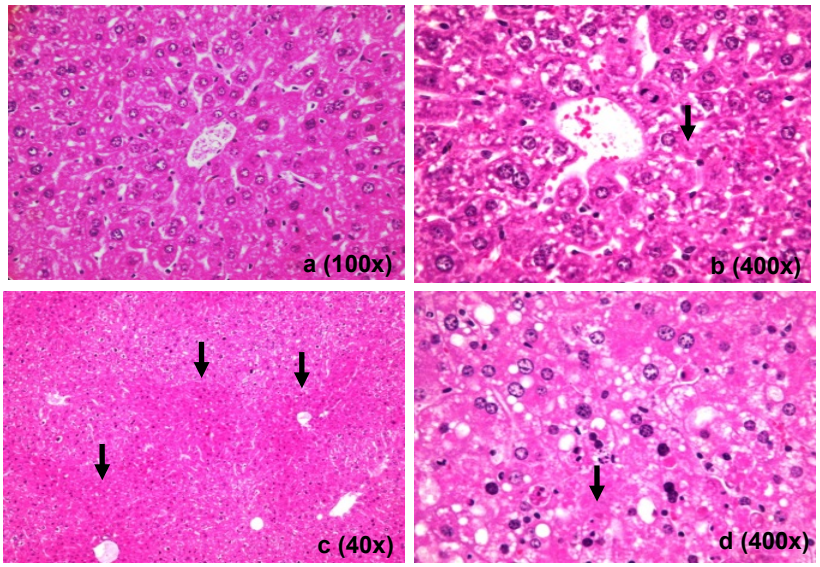

Figure 2 Histopathological findings in liver; unremarkable (a), mild centrilobular degeneration (b), extensive lobular necrosis in low (c) and high (d) power fields. The arrows show areas of necrosis.

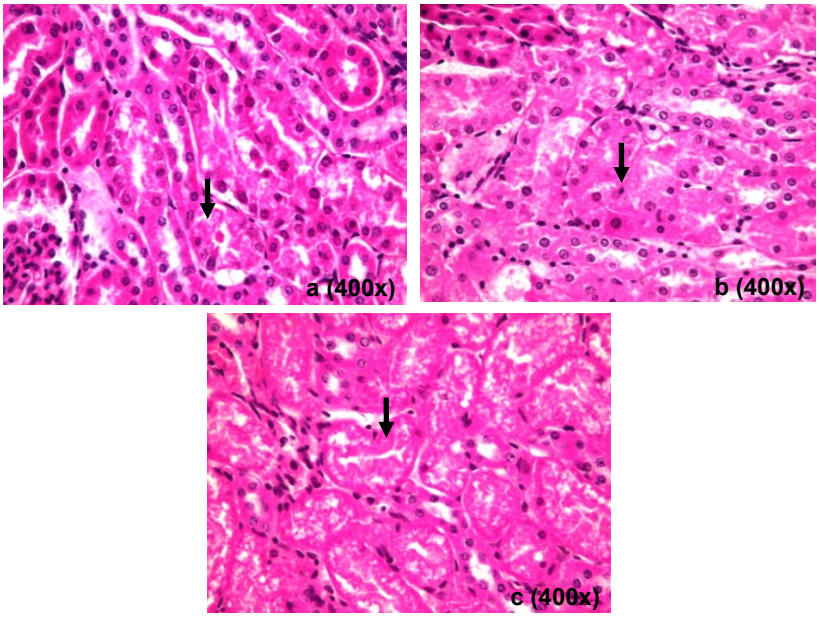

Figure 3 Histopathological findings in kidneys; the arrows show the area of cloudy swelling of tubular epithelium (a), pyknotic and karyorhexis of tubular epithelium cells (b), and acute tubular necrosis (c).

Conclusions

In summary, Cleistanthoside A tetraacetate, a derivative from *P. taxodiifolius* Beille, had toxicity on liver and kidney functions when administered at a high dose of 1,000 mg/kg BW, which correlated with histopathological damage in liver and kidney tissues.

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