Full Length Research Paper

Non-mutagenic and genotoxic doses of cleistanthoside A tetraacetate isolated from *Phyllanthus taxodiifolius* Beille

Pranom Puchadapirom[1], Achiraya Tammasakchai[1], Sareeya Reungpatthanaphong[1,2], Lakana Himakoun[1], Suraphol Kongtim[1], Patoomratana Tuchinda[3] and Prasit Suwannalert[1]*

[1]Department of Pathobiology, Faculty of Science, Mahidol University, 10400 Bangkok, Thailand.
[2]Department of Pharmaceutical and Natural Products, Thailand Institute of Scientific and Technological Research (TISTR), 12120 Pathum Thani, Thailand.
[3]Department of Chemistry, Faculty of Science, Mahidol University, 10400 Bangkok, Thailand.

Cleistanthoside A tetraacetate isolated from *Phyllanthus taxodiifolius* Beille is claimed to inhibit cancer cell growth, but their toxicity is not reported. Mutagenic and genotoxic effects of cleistanthoside A tetraacetate were investigated in this study. The bacterial reverse mutation (Ames) test using a pre-incubation method was performed in order to determine the mutagenic toxicity of cleistanthoside A tetraacetate at doses ranging from 1, 25, 50 and 100 µg/ plate. *In vitro* and bone marrow micronuclei were used to determine genotoxicity. Cleistanthoside A tetraacetate at the given doses did not show mutagenic toxicity against the tests of bacterial strains of TA98 and TA100 in conditions with and without S9. There was no significance of *in vitro* and bone marrow micronucleated polychromatic erythrocyte at the given doses of isolate compared to the control. Cleistanthoside A tetraacetate isolated from *P. taxodiifolius* Beille at the given doses did not show mutagenic and genotoxic activities both *in vitro* and *in vivo* studies.

Key words: Toxicity, cleistanthoside A tetraacetate, Ames test, micronucleus.

INTRODUCTION

Phytochemical plants and their products have been widely used in preventative health (Okem et al., 2012; Ram et al., 2011). *Phyllanthus taxodiifolius* Beille is commonly used in subtropical countries to treat liver, kidney and bladder diseases (Calixto et al., 1998). Cleistanthoside A was first isolated from the heartwood of *Cleistanthus patulus*. It showed anti-cancer properties against epidermal carcinoma of the nasopharynx in tissue culture (Sastry and Rao, 1983). Cleistanthoside A, isolated from *Phyllanthus* species is beneficial in anti-oxidative stress, anti-inflammation and anti-tumoral activities (Ferrer et al., 2001; Huang et al., 2006).

Cleistanthoside A tetraacetate is a derivative with acetylation modification of the arylnaphthalide lignan glycosides; it has been isolated from a crude extract of *P. taxodiifolius* Beille (Sastry and Rao, 1983; Tuchinda et al., 2006). Cleistanthoside A tetraacetate at 3 to 4 nM is used in cytotoxicity activity in various cancer cells, including oral nasopharyngeal carcinoma, murine lymphocytic leukemia, colon, breast and lung cancer (Tuchinda et al., 2006).

Although medicinal plants are widely used preventa-tively in various diseases (Eddouks et al., 2002; Jouad et al., 2001), many have not been analyzed for their safety (Firenzuoli and Gori, 2007; Wang et al., 2009). The Cleistanthoside A tetraacetate isolated from *P. taxodiifolius* Beille was claimed to inhibit tumor growth in various carcinoma cell lines (Tuchinda et al., 2006), but there is no information about its mutagenicity and genotoxicity. Further studies are needed to establish a

---

*Corresponding author. E-mail: parasit109@yahoo.com or scpsw@mahidol.ac.th. Tel: +66-2-201-5578. Fax: +66-2-354-7158.

Table 1. Mutagenicity of the Cleistanthoside A tetraacetate using Ames test.

| Treatment | Dose (per plate) (µg) | Number of revertant colonies per plate (mean ± SD) | | | |
| | | TA98 | | TA100 | |
		-S9	+S9	-S9	+S9
Cleistanthoside A tetraacetate	1	28.8 ±2.280	32.2 ± 2.950	115.4 ± 10.502	120.0 ± 10.863
	25	29.8 ± 3.701	33.0 ± 3.317	120.2 ± 29.448	109.2 ± 11.520
	50	28.2 ± 2.168	33.2 ± 1.643	121.2 ± 20.657	113.8 ± 5.718
	100	29.4 ± 0.894	31.8 ± 7.225	121.4 ± 8.385	111.6 ± 5.128
100% DMSO	0. 1ml	30.2 ± 4.970	33.4 ± 2.793	120.6 ± 6.269	114.6 ± 11.014
4NQO	0.4	255.4 ± 24.419*	ND	ND	ND
NaN3	0.2	ND	ND	1133.8 ± 75.873*	ND
2-AA	0.5	ND	1649.4 ± 81.806*	ND	1409.6 ± 349.606*

*Statistically significant at $p < 0.001$; ND: not done.

safe dosage of Cleistanthoside A tetraacetate. In this study, the tests of bacterial reverse mutation, *in vitro* micronucleus and bone marrow micronucleus were investigated for mutagenic and genotoxic doses.

MATERIALS AND METHODS

Cleistanthoside A tetraacetate was obtained from the laboratory of Tuchinda P. Cleistanthoside A tetraacetate is a derivative with acetylation modification of Cleistanthoside A that isolated from *P. taxodiifolius* Beille (Tuchinda et al., 2006).

Bacterial reverse mutation (Ames) test

This test was performed using a pre-incubation method (Mortelmans and Zeiger, 2000). The toxicity of Cleistanthoside A tetraacetate isolated from *P. taxodiifolius* Beille was investigated for their capacity to induce reverse mutation by *Salmonella typhimurium* strains of TA98 and TA100 in models with and without S9. Doses ranging from 1, 25, 50 and 100 µg/ plate of Cleistanthoside A tetraacetate were determined for their mutagenic toxicity. 4-Nitroquinoline-1-oxide (4NQO) and sodium azide (NaN3) at concentrations 0.4 and 0.2 µg/plate were used as positive controls in TA98 (-S9) and TA100 (-S9), respectively. A 0.5 µg/plate of 2-aminoanthracene (2-AA) was used as the positive control to TA98 and TA100 with S9, while 0.1 ml of 100% dimethylsulphoxide (DMSO) was used as negative control.

In vitro micronucleus

In vitro micronucleus assay was performed according to the Organization for Economic Co-operation and Development (OECD) guidelines (Seelbach et al., 1993). Hamster lung fibroblast, V79 cells were treated with cleistanthoside A tetraacetate at concentrations of 1, 5, and 50 µg/ml in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) for 18 h at 37°C with 5% CO_2. Cyclophosphamide (20 µg/ml) was used as the positive control, while 0.2% DMSO was used as negative control. Treated cells were collected and stained for binucleated cells. Micronuclei (Mn) per 1000 cells were examined under light microscopy.

Experimental animals

Male ICR mice weighing 25 to 30 g and aged 7 weeks were used for the determination of the genotoxicity. The experimental procedures were approved by the Animal Ethics Committee of the Faculty of Science, Mahidol University, Thailand. Healthy mice were classified into the treated dose according to their concentrations of cleistanthoside A tetraacetate at 0 (corn oil), 250, 500 and 1000 mg/kg BW. In the treated mice, 60 mg/kg of cyclophosphamide was used as a positive genotoxicity, whilst a 100% corn oil vehicle was obtained for negative control.

Bone marrow micronucleus

The micronucleus assay was conducted according to the standard technique (Ha et al., 2011). The groups of mice were killed at 24 and 48 h after dosing, by injecting pentobarbital sodium at 50 mg/kg BW. Both femurs were removed and bone marrow collected in tubes of 0.5 ml of fetal bovine serum. The solution was then centrifuged for 10 min at 1000 rpm/ min. The smears were allowed to air dry prior to fixation and staining with a Giemsa solution. A number of micronucleus was obtained by using light microscopy. The 2000 polychromatic erythrocytes (PCEs) per mouse were examined for micronucleated polychromatic erythrocyte (MnPCE).

Statistical analysis

All results were expressed as mean ± standard deviation (SD). The difference among groups was obtained by one-way ANOVA using the SPSS program. Statistic significance was set at $p<0.05$.

RESULTS

In the reverse mutation test, cleistanthoside A tetraacetate isolated from *P. taxodiifolius* Beille did not show mutagenic activity against the tests of bacterial strains of TA98 and TA100 in conditions with and without S9 at concentrations of 1, 25, 50 and 100 µg/plate (Table 1).

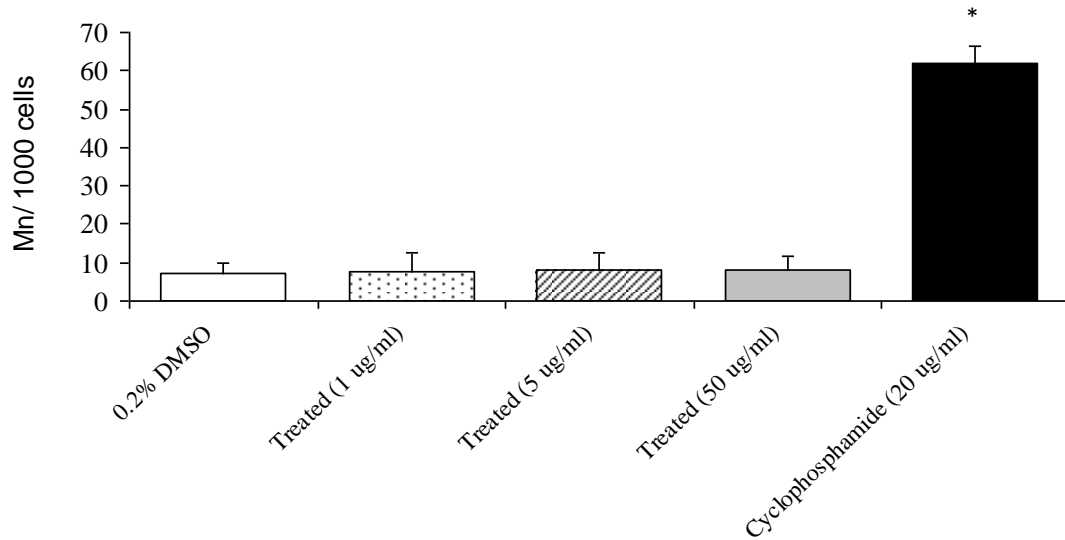Treatment with 0.4 µg/plate 4NQO and 0.2 µg/plate

Figure 1. *In vitro* micronucleus of the Cleistanthoside A tetraacetate in V79 cells. *Statistically significant at *p* < 0.001.

Table 2. Bone marrow micronucleus of cleistanthoside A tetraacetate.

Treatment	Dose (mg/kg BW)	Micronucleus/ 2000 PCE (mean ± SD)	
		24 h	48 h
Cleistanthoside A tetraacetate	0 (Vehicle)	1.640 ± 0.400	1.600 ± 0.894
	250	2.290 ± 1.498	1.600 ± 1.817
	500	2.200 ± 1.304	2.080 ± 1.643
	1000	1.800 ± 1.095	1.900 ± 0.490
Cyclophosphamide	60	29.000 ± 3.937*	29.300 ± 1.761*

*Statistically significant at *p* < 0.001.

NaN3 were used as a positive mutagen to TA98 (-S9) and TA100 (-S9). They had high reverting colony per plate activity when compared with the negative control, 100% DMSO at *p*<0.001 and their treated groups at *p*<0.001. Moreover, treatment with 0.5 µg of 2-AA showed mutagenic activity against bacterial strains of TA98 (+S9) and TA100 (+S9) at *p*<0.001. Cleistanthoside A tetraacetate at concentration 100 µg/ plate showed non-mutagenic activity in test of TA98 and TA100. The result indicated that cleistanthoside A tetraacetate is not mutagenic at concentration of 100 µg/plate in all treatments.

The *in vitro* and bone marrow micronucleus were investigated for genotoxic properties. *In vitro* micronucleus of treated cleistanthoside A tetraacetate at concentrations 1, 5, and 50 µg/ml were 7.67 ± 5.03, 8.00 ± 4.36 and 8.25 ± 3.50 Mn/1000 cells, respectively. No statistical significance of micronucleus was found among groups of all treated doses and 0.2% DMSO (Figure 1). *In vitro* micronucleus of treated cyclophosphamide was statistically significant than that other groups at *p*<0.001. The bone marrow micronucleus was investigated by using a number of micronucleated polychromatic erythrocyte (MnPCE) at 24 and 48 h after the mice were treated. Table 2 illustrates the number of micronucleus (Mn) per 2000 polychromatic erythrocytes (PCEs) in doses of 250, 500 and 1000 mg/ kg of cleistanthoside A tetraacetate. There was no significance of MnPCE at any doses of the isolate when compared with vehicle control (*p*>0.05), while the treated cyclophosphamide showed statistically significant number of MnPCE than other groups at *p*<0.001.

DISCUSSION

Therapeutic substances of phytochemicals extracted from Thai medical plants exhibit positive properties for the treatment of various diseases (Chuanasa et al., 2008; Siriwatanametanon et al., 2010). Cleistanthoside A

tetraacetate is highly effective in preventing cancer cell growth of oral nasopharyngeal carcinoma (KB), murine lymphocytic leukemia (P388), colon cancer (Col-2), breast cancer (MCF) and lung cancer (Lu-1) (Tuchinda et al., 2006). However, there is a lack of research on the mutagenic and genotoxic doses of cleistanthoside A tetracetate. This study was proposed to evaluate the mutagenic and genotoxic doses of the isolate. Treatment with 1 - 100 µg/ plate of cleistanthoside A tetraacetate showed non-mutagenic activity against the test of Ames in conditions with and without S9. Both metabolite (with S9) and non-metabolite (without S9) have been designed to assess the effects of chemicals on mutagenic properties. The human and rat liver S9 factions enhanced mutagenicity of the pro-carcinogens (Hakura et al., 1999). It also increased the quality of mutagenicity in Ames test (Hakura et al., 2001). In conditions of with and without S9 in this study, the result exhibited good safety profile at an effective dose (1 - 100 µg/plate).

In vitro and bone marrow micronucleus in treatment with cleistanthoside A tetraacetate showed no significance when compared with the negative control. The results indicated that cleistanthoside A tetraacetate at high concentration of both V79 cells and mice had no genotoxicity. Cleistanthoside A tetraacetate isolated from *P. taxodiifolius* Beille was claimed to inhibit the growth of cancer cells (Tuchinda et al., 2006). It also demonstrated good safety to genotoxicity. Although the isolate showed non-mutagenic and genotoxic properties in both *in vitro* and *in vivo* models, there is a need to study and clinically evaluate pharmaceutical properties of cleistanthoside A tetraacetate derived from *P. taxodiifolius* Beille in order to establish its suitability for anticancer treatment.

Conclusion

Cleistanthoside A tetraacetate of *P. taxodiifolius* Beille has non-mutagenic activity at concentration of 100 µg/plate of Ames test. It also showed non-mutagenic in both *in vitro* and bone marrow micronucleus. Further studies are needed to develop it into suitable pharmaceutical properties.

ACKNOWLEDGEMENT

REFERENCES

Calixto JB, Santos AR, Cechinel FV, Yunes RA (1998). A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential. Med. Res. Rev. 18(4):225-258.

Chuanasa T, Phromjai J, Lipipun V, Likhitwitayawuid K, Suzuki M, Pramyothin P, Hattori M, Shiraki K (2008). Anti-herpes simplex virus (HSV-1) activity of oxyresveratrol derived from Thai medicinal plant: mechanism of action and therapeutic efficacy on cutaneous HSV-1 infection in mice. Antiviral Res. 80(1):62-70.

Eddouks M, Maghrani M, Lemhadri A, Ouahidi ML, Jouad H (2002). Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). J. Ethnopharmacol. 82(2-3):97-103.

Ferrer M, Sanchez-Lamar A, Fuentes JL, Barbe J, Llagostera M (2001). Studies on the antimutagenesis of *Phyllanthus orbicularis*: mechanisms involved against aromatic amines. Mutat. Res. 498(1-2):99-105.

Firenzuoli F, Gori L (2007). Herbal medicine today: clinical and research issues. Evid. Based Complement Alternat. Med. 4(1):37-40.

Ha H, Lee JK, Lee HY, Seo CS, Lee MY, Huh JI, Shin HK (2011). Genotoxicity assessment of a herbal formula, Ojeok-san. J. Ethnopharmacol. 135(2):586-589.

Hakura A, Suzuki S, Satoh T (1999). Advantage of the use of human liver S9 in the Ames test. Mutat. Res. 438(1):29-36.

Hakura A, Suzuki S, Sawada S, Motooka S, Satoh T (2001). An improvement of the Ames test using a modified human liver S9 preparation. J. Pharmacol. Toxicol. Methods 46(3):169-172.

Huang ST, Yang RC, Lee PN, Yang SH, Liao SK, Chen TY, Pang JH (2006). Anti-tumor and anti-angiogenic effects of *Phyllanthus urinaria* in mice bearing Lewis lung carcinoma. Int. Immunopharmacol. 6(6):870-879.

Jouad H, Haloui M, Rhiouani H, El Hilaly J, Eddouks M (2001). Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North centre region of Morocco (Fez-Boulemane). J. Ethnopharmacol. 77(2-3):175-182.

Mortelmans K, Zeiger E (2000). The Ames *Salmonella*/microsome mutagenicity assay. Mutat. Res. 455(1-2):29-60.

Okem A, Finnie JF, Van Staden J (2012). Pharmacological, genotoxic and phytochemical properties of selected South African medicinal plants used in treating stomach-related ailments. J. Ethnopharmacol. 139(3):712-720.

Ram A, Balachandar S, Vijayananth P, Singh VP (2011). Medicinal plants useful for treating chronic obstructive pulmonary disease (COPD): current status and future perspectives. Fitoterapia 82(2):141-151.

Sastry KV, Rao EV (1983). Isolation and structure of cleistanthoside A. Planta Med. 47(4):227-229.

Seelbach A, Fissler B, Madle S (1993). Further evaluation of a modified micronucleus assay with V79 cells for detection of aneugenic effects. Mutat. Res. 303(4):163-169.

Siriwatanametanon N, Fiebich BL, Efferth T, Prieto JM, Heinrich M (2010). Traditionally used Thai medicinal plants: *in vitro* anti-inflammatory, anticancer and antioxidant activities. J. Ethnopharmacol. 130(2):196-207.

Tuchinda P, Kumkao A, Pohmakotr M, Sophasan S, Santisuk T, Reutrakul V (2006). Cytotoxic arylnaphthalide lignan glycosides from the aerial parts of *Phyllanthus taxodiifolius*. Planta Med. 72(1):60-62.

Wang J, Van der Heijden R, Spruit S, Hankermeier T, Chan K, Van der Greef J, Xu G, Wang M (2009). Quality and safety of Chinese herbal medicines guided by a systems biology perspective. J. Ethnopharmacol. 126(1):31-41.