RESEARCH COMMUNICATION

Evaluation of Genotoxic and Anti-Mutagenic Properties of Cleistanthin A and Cleistanthoside A Tetraacetate

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Abstract

Cleistanthin A (CleinA) and cleistanthoside A (CleisA) isolated from plant Phyllanthus taxodiifolius Beille have previously shown potent anticancer effects. To promote their medicinal benefits, CleisA was modified to cleistanthoside A tetraacetate (CleisTA) and evaluated for genotoxic and anti-mutagenic properties in comparison with CleinA. Both compounds showed no significant mutagenic activity to S. typhimulium bacteria and no cytotoxic effect to normal mammalian cells. The non genotoxic effect of CleinA was further confirmed by un-alteration of cytokinesis-block proliferation index (CBPI) and micronucleus (MN) frequency assays in Chinese hamster lung fibroblast (V79) cells, and of CleisTA was confirmed by un-changes of human peripheral blood lymphocytes (HPBL) chromosomal structure assay. Moreover, the metabolic form of CleinA efficiently demonstrated cytostasis effect to V79 cell and prevented mutagen induced Salmonella TA98 and TA100 reversion, whereas both metabolic and non-metabolic forms of CleisTA reduced HPBL mitotic index (%M.I) in a concentration-dependent relationship. The results support CleinA and CleisTA as the new lead compounds for anti-cancer drug development.

Keywords: Cleistanthin A - cleistanthoside A tetraacetate - Phyllanthus taxodiifolius Beille - genotoxic assay

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Introduction

Plants of the genus Phyllanthus (Euphorbiaceae) are used in traditional medicine in several tropical and subtropical countries. Their known biological activities include anti-bacterial (Mazumder et al., 2006, Meléndez and Capriles, 2006, Oliveira et al., 2007), anti-parasitic (Zirihi et al., 2005, Hout et al., 2006), anti-viral (Venkateswaran et al., 1987, Yang et al., 2005, Balasubramanian et al., 2007), anti-inflammatory (Kassuya et al., 2006) anti-oxidation (Bhattacharjee and Sil, 2007, Chatterjee and Sil, 2006, Kumaran and Karunakaran, 2007), anti-carcinogen and anti-tumor angiogenesis (Rajeshkumar et al., 2002, Sripanidkulchai et al., 2002). Previously, we have found that Phyllanthus taxodiifolius Beille (Euphorbiaceae) extracts from a native Thai plant contained cleistanthin A (CleinA) and cleistanthoside A (CleisA), and both compounds exhibited cytotoxic to several human cancer cell lines (Tuchinda et al., 2006, Pradheepkumar et al., 2000). To potentiate stability of the compounds for future medicinal application, our laboratory has modified Phyllanthus taxodiifolius Beille derived CleisA by acetylation of arylnaphthalide lignin glycosides resulting in CleisA tetraacetate (or CleisTA in this study) (Figure 1). This modification is found enhancing cytotoxic effect of CleisTA (IC50 < 0.04 μg/ml) to human cancer cell lines over that of Clein A (IC50 >20 μg/ml) (Tuchinda et al., 2006). In order to assess the compounds' risk effects, we evaluated and compared the genotoxic effects of CleisTA and CleinA to normal mammalian cells using a set of classical in vitro genotoxic assays, following the OECD guidelines (OECD, 1997a, OECD, 1997b, OECD, 2007) which suggest using the in vitro assays as an alternative assessment to the in vivo assays . The mutagenic system assays include the bacterial gene mutation, micronucleus, and chromosomal aberration assays (Maron and Ames, 1983, Zeiger and Mortelmans, 2000). The anti-mutagenic system assay involves Ames test using Salmonella typhimurium strains TA98, TA100 and compares the results with those of the known mutagenic induction (Ferrer et al., 2001, Ferrer et al., 2002).

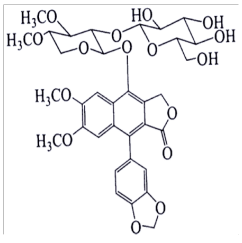

Figure 1. Chemical Structure of Cleistanthin A

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Materials and Methods

Chemicals and supplements

Oxoid nutrient broth No. 2 (Oxoid Ltd., England), Vogel-Bonner minimal glucose agar and Top agar were in-house prepared. Rat liver homogenate contained metabolic enzyme (S9 fraction) was prepared following Matsushima (Matsushima, 1976) at the Department of Biochemistry, Faculty of Medicine, ChiangMai University, Thailand. CleinA and CleisTA were purified from fractions of methanol extracts of the aerial parts of *Phyllanthus taxodiifolius* Beille. CleisA was further modified to CleisTA as previously described (Tuchinda et al., 2006). The compounds were dissolved in dimethysulfoxide (DMSO) to 20 μg/μl concentration and stored at -20˚ C until usage.

Cytotoxicity assay

Human peripheral blood lymphocytes (HPBL) isolated from fresh heparinized venous blood, of ethically obtained healthy volunteers, were incubated with 0.1 ml of 2.5 μg/ml phytohemagglutinin in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) at 37°C in 5% CO2 incubator for 48 h. Viable cells (5×10^4 cells) were distributed in 24 well plates and incubated with various concentrations (5-50μg/ml) of CleisTA for 24 h. Chinese hamster lung fibroblasts (V79) (5×10^4 cells) were grown in 24 well plate at 37˚ C in 5% CO_2 incubator for 48 h and incubated with various concentrations (1-100 μg/ml) of CleinA for 3 h. The control groups were treated with 1% DMSO. The treated-cells were washed, replaced with fresh media and re-incubated for additional 24 h. The concentration of the compound that decease 50% percent cell viability (CC_{50}) were determined by counting of a total number of 200 cells per group using Tryphan blue staining method. Each was performed in triplicate.

Salmonella/Microsome activity (Ames) assay

Salmonella typhimurium histidine auxotrophs tester strains TA98 (hisD3052, rfa, uvrB, pKM101) and TA100 (hisG46, rfa, uvrB, pKM101), originally obtained from Prof. Bruce Ames (University of California, Berkeley, USA), were prepared from frozen stocks and genotypic characterized (Maron and Ames, 1983). The overnight cultured bacteria were incubated (1:1) with various concentrations of the compounds in 0.5 ml phosphate buffer without (-) or with (+) S9 metabolic enzyme activation at 37˚ C, for 20 min (OECD, 1997a, Zeiger and Mortelmans, 2000). After incubation, 2 ml of molten top agar was added, mixed, and poured evenly on minimal glucose agar plates. The plates were air dried and incubated at 37˚ C for 48 h. The spontaneous reversion of each tester strain was examined by colony counting. The experiment was performed in triplicate and repeated twice. Statistical analysis was performed using the independent-sample T test. Significance of the data was considered at $p<0.05$.

Cytokinesis-block proliferation index (CBPI) and Micronucleus (MN) frequency assays

V79 cells (5×10^4 cells) were incubated with various concentrations of CleinA (1, 5, 50 and 100 μg/ml) in the presence (+) or absence (-) of S9 condition at 37˚ C for 3 h. DMSO (0.2%) was used in treatment of a negative control, and mitomycin C (1 μg/ml) and cyclophosphamide (20 μg/ml) were used in treatment of the positive controls. The media were refreshed, and 3μg/ml of cytochalasin B was added at 18 h (for first mitotic division blocker) before the treated-cells were harvested. After 24 h incubation, the treated-cells were stained with Giemsa and examined under the microscope (400x magnification). CBPI was calculated from sum amounts of mononucleated + 2x binucleated, + 3x multinucleated cells in each group divided by total number of the cells. MN was determined under the microscope (1000x magnification) from the number of micronucleus, resulted from acentric fragmented chromosomes during anaphase stage, per 1000 binucleated cells. Percent cytostasis effect was evaluated from cell division inhibition using the formula below:

(%) Cytostasis = 100-100{(CBPIT-1)/(CBPIC-1)}

CBPIT = CBPI from the test compound

CBPIC = CBPI from the vehicle control

All the experiments were performed in triplicate. Data were analyzed by one way ANOVA using SPSS program and Tukey multiple comparison test. Significance of the data was considered at $p<0.05$.

Chromosome aberration assay and mitotic index (M.I) measurement

The genotoxic effect of CleisTA was performed by conventional chromosome aberration assay following the OECD guideline (OECD, 1997b). HPBL were incubated with various concentrations of CleisTA (1, 2.5, and 5 μg/ml) at 37C in CO2 incubator for 24 h. DMSO (0.2%) was used in treatment of a negative control, and 1.5 μg/ml mitomycin C or 200 μg/ml cyclophosphamide was used in treatments of the positive controls. In the -S9 condition, 0.25 μg/ml colchicine was added into the treated-cells to arrest metaphase chromosome division at 3 h before harvesting (at 24 h). In the +S9 condition, the treated-cells were incubated with S9-mix in RPMI 1640 medium for 3 h, washed and refreshed with medium supplemented with 20% FBS, then colchicine was added at 3h before harvesting. The harvested cells were analyzed for chromosome structural damage included the aberrations with breaks, exchanges and multiple aberrations by conventional chromosome analysis (Albertini et al., 2000). The number and percentage of structurally aberrant cells were evaluated (Halder et al., 2006). Five independent experiments were performed for each treatment.

To determine the mitosis effect of the compounds, the treated cells were scored by counting the number of metaphases in 2,000 cells per culture for a total of 4,000 cells per group. The percent mitotic index (% M.I) was equal to the number of cells in division x 100, divided by the total number of cells.

Anti-mutagenic activity of the compounds was conducted by modified Ames test assay, using Samonella strains TA98 and TA100 prepared as above, with pre-incubation of the known mutagens; 4-Nitroquinoline-1-oxide (4NQO) (Sigma, USA) in the -S9 condition, and

Benzo (a) pyrene (BAP) (Zeiger and Mortelmans, 2000) in the +S9 condition. Data analysis was based on colony counting and percent inhibition was determined from the formula below:

% Inhibition

$$= \frac{[1 - (\text{test sample R} - \text{spontaneous R})] \times 100}{(\text{mutagen R} - \text{spontaneous R})}$$

Where: test sample R = revertant bacterial colony + positive control; spontaneous R = spontaneous revertant bacterial colony of negative control; mutagen R= Revertant bacterial colony of positive control

Results

The cytotoxic concentration (CC_{50}) of CleinA and CleisTA on the two normal mammalian cells were > 100 μg/ml (data not shown). Treatment of CleinA and CleisTA at the concentration 1-100 μg/plate had no mutagenic effect on S. typhimurium TA98 and TA100 tester stains, both under -S9 and +S9 metabolic enzyme activation conditions. CleinA (concentrations 1-100 μg/ml) effect to V79 cells under -S9 condition also showed no significant difference of CBPI and micronucleus (MN) frequency level when compared to DMSO control (Table 1). Interestingly, under +S9 condition, CleinA at high concentration (50 and 100 μg/ml) significantly decreased CBPI to the same level as cyclophosphamide (20 μg/ml) treated control, but there was no significant increase of MN frequency level (Table 1). In addition, high concentration of CleinA (50 or 100 μg/ml) in the presence of S9 metabolic enzyme activation was found efficient in cytostasis induction of V79 cells (Table 2). CleisTA also showed no genotoxic effect on HPBL. At the concentration 1-5 μg/ml, under the conditions of –S9 and +S9 metabolic activation, CleisTA induced no significant changes in all aspects of chromosome structural aberration when compared to the negative controls (Table 3). However, CleisTA at the high concentrations (2.5-5 μg/ml under –S9 condition, and 5 μg/ml under +S9 condition) trended to decrease % M.I of HPBL treated cells to the same level as the controls (Table 3).

Moreover, CleinA showed anti-mutagenic activities against mutagens induced Salmonella TA98 and TA100 in the Ames assay under +S9 conditions (Table 4). In the absence of enzyme activation, CleinA at the concentrations 25-100 μg/ml induced no significant reversion of bacterial colonies that have been induced mutation with 4NQO. However, in the presence of enzyme activation, CleinA significantly reduced the number of Salmonella TA98 and TA100 revertant bacterial colonies that have been induced mutation with BAP by causing 57-79% and

Table 1. Genotoxic Effect of CleinA on V79 Cells After Treatment Under the Conditions Without S9 (-S9) or With S9 (+S9)

Treatment	CBPI		MN frequency	
	-S9	+S9	-S9	+S9
DMSO				
0.2 μg/ml	1.8 ± 0.0	1.8 ± 0.1	7.3 ± 4.4	7.3 ± 2.5
Mitomycin C				
1 μg/ml	1.3 ± 0.1*	NA	42.7 ± 0.3*	NA
Cyclophosphamide				
20 μg/ml	NA	1.3 ±0.1*	NA	62.0 ± 4.2*
Cleistanthin A				
1 μg/ml	1.7 ± 0.1	1.7 ± 0.1	6.7 ± 4.6	7.7 ± 5.0
5 μg/ml	1.6 ± 0.1	1.6 ± 0.2	6.7 ± 1.2	8.0 ± 4.4
50 μg/ml	1.5 ± 0.1	1.3 ± 0.1*	5.3 ± 2.9	8.3 ± 3.5
100 μg/ml	1.6 ± 0.2	1.3 ± 0.2*	5.3 ±1.5	4.8 ±4.2

CBPI = Cytokinesis-block proliferation index assay, indicates that all cells are mononucleated cells (or 100% proliferation inhibition); MN = micronucleus frequency assay; *p<0.05 = significant difference from the control; NA = Not Applied

Table 2. Cytostasis Effect of CleinA on V79 cells

Treatment	% Cytostasis	
	-S9 condition	+S9 condition
Cleistanthin A		
1 μg / ml	10.1	10.7
5 μg / ml	21.5	22.7
50 μg / ml	36.7	58.7
100 μg / ml	30.4	65.3
MitomycinC		
1 μg /ml	58.2	-
Cyclophosphamide		
20 μg/ml	-	56.0

Table 3. Effects of CleisTA on HPBL After Treatment under the Absence of S9 (A) or Presence of S9 (B) Enzyme Activation

Treatment	Chromosome aberrations / cell[a]		Aberrant cells[a] (%)		%M.I[b] ± SD
	+Gaps	-Gaps	+Gaps	-Gaps	
(A) In the absence of exogenous metabolic activation (-S9)					
DMSO (0.2 μg/ml)	0.02±0.02	0.01±0.01	2.20±2.17	1.00±1.00	3.90±0.71
MMC (1.5 μg/ml)	0.63±0.41*	0.44±0.27*	41.3±18.5*	32.7±13.3*	2.21±0.98*
CleisTA 1μg/ml	0.01±0.01	0.01±0.01	1.20±0.83	0.40±0.89	3.39±0.33
CleisTA 2.5 μg/ml	0.04±0.02	0.02±0.01	3.67±2.52	1.67±1.53	2.49±0.59*
CleisTA 5 μg/ml	0.04±0.01	0.01±0.01	4.2±1.30	1.40±0.89	2.03±0.65*
B) In the presence of exogenous metabolic activation (+S9)					
DMSO(0.2 μg/ml)	0.03±0.01	0.01±0.01	3.00±1.00	1.33±0.58	3.19±0.30
CYP (200 μg/ml)	0.36±12.3*	0.23±10.4*	28.0±0.17*	19.3±0.13*	1.35±0.82*
CleisTA 1 μg/ml	0.04±0.02	0.02±0.01	4.00±2.00	1.67±1.53	2.95±0.40
CleisTA 2.5 μg/ml	0.05±0.03	0.02±0.02	3.67±3.06	1.67±2.08	2.72±0.47
CleisTA 5 μg/ml	0.06±0.05	0.02±0.02	6.33±4.93	2.00±2.00	2.41±0.35*

* MMC = Mytomycin C, CYP = Cyclophosphamide, [a]n=5, 100 metaphases scored per group, [b]Mitotic Index was based on 2000 cells per culture, p<0.05 = significant difference from the control

Table 4. Ames Test Results for Anti-mutagenic Effects of CleinA on Salmonella TA98 and TA100 in the Absence of S9 (A) and Presence (B) of S9 Conditions

Treatment	TA98 Revertant colonies / Plate(mean ±SD)	%Inhibition	TA100 Revertant colonies / Plate(mean ±SD)	%Inhibition
	(A) In the absence of exogenous metabolic activation (-S9)			
DMSO	37.5 ± 8.43	-	155 ± 13.2	-
DMSO + 4NQO	131.0 ± 16.4	-	789.3 ± 42.4	-
CleinA 25μg + 4NQO	132.4 ± 16.6	-1.5	900.8 ± 42.8	-17.6
CleinA 50μg + 4NQO	131.6 ± 11.8	-0.6	987.5 ± 54.7	-31.3
CleinA 75μg + 4NQO	134.6 ± 12.8	-3.8	850.0 ± 71.3	-9.6
CleinA 100μg + 4NQO	121.7 ± 14.5	-9.9	848.7 ± 76.3	-9.4
	B) In the presence of exogenous metabolic activation (+S9)			
DMSO	39.3 ± 9.29	-	161.2 ± 6.40	-
DMSO + BAP	468.3 ± 47.3	-	1042.8 ± 108.1	-
CleinA 25μg + BAP	220.1 ± 20.8*	57.9	1001.0 ± 59.06	7.7
CleinA 50μg + BAP	168.3 ± 25.9*	69.9	903.3 ± 69.1*	15.8
CleinA 75μg + BAP	135.7 ± 23.8*	77.5	806.0 ± 131.4*	26.8
CleinA 100μg + BAP	129.5 ± 13.4*	79.0	818.2 ± 104.3*	25.4

* 4NQO = 4-Nitroquinoline-1-oxide (0.2 μg/plate), BAP = Benzo (a) pyrene (0.25 μg/plate), $p<0.05$ = significant difference from the control

15-25% inhibition, respectively. This effect of CleinA was exhibited in a concentration-dependent manner and Salmonella TA98 was more sensitive than Salmonella TA100 (Table 4).

Discussion

Results from this study suggested that CleinA and CleisTA compounds previously determined potent cytotoxic to cancers have no cytotoxic or genotoxic effect to normal mammalian cells. At the concentration ranges (1- 100 μg/ml) applied, both compounds failed to induce 2-folds or greater reversion of the S. typhimurium bacterial colonies than the number of spontaneous revertants in the control group or to maintain a dose-response relationship for several testing concentrations (Cariello and Piegorsch, 1996, OECD, 1997a). According to OECD guideline (OECD, 2007), genotoxicity of the compounds are determined from CBPI of the treated-cells after adding of cytochalasin B, the inhibitor to actin polymerization that stops the interphase of cell division. In the present CBPI and MN frequency assays, almost all CleinA treatments in V79 cells, under –S9 and +S9 conditions, induced similar CBPI and MN levels as the level in the negative control. Except the metabolic form (+S9 condition) of CleinA at high concentrations (50-100 μg/ml) that showed the same CBPI level and induced % cytostasis of V79 cells to the same level as cyclophosphamide (20 μg/ml) treated control. Therefore, CleinA was considered no genotoxicity to V79 cells and it might possess a similar property as cyclophosphamide drug. Although the in vitro anti-mutagenic assay for CleinA by the modified Ames test showed no direct (under -S9 condition) effect against both strains of S. typhimurium, the pre-incubation assay with BAP, under metabolic enzyme activation (under +S9 condition), showed that CleinA significantly reduced the number of Samonella TA98 revertant bacterial colonies more than 30% over the control. The results suggested that CleinA has a positive anti-mutagenic effect (Zeiger and Mortelmans, 2000).

CleisTA has shown more potent cytotoxicity to cancer cells and the lower concentrations than CleinA were used to assess normal cell's genotoxicity in this study. At the concentration 1-5 μg/ml, CleisTA showed no significant chromosomal damage to human blood cells after treatments with metabolic (+S9, for 3 h) and non-metabolic (-S9, for 24 h) forms suggesting the low risk effects of the compound with structural similarity to CleinA. In this study, both metabolic and non-metabolic forms of CleisTA reduced mitosis (%M.I.) level in HPBL to the similar levels as mitomycin C and cyclophosphamide induction. Therefore, CleisTA as well as CleinA are interesting lead compounds containing drug properties that should be worth for further development as anti-cancer drugs.

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References

Albertini RJ, Anderson D, Douglas GR, et al (2000). IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. *Mutat Res/Reviews Mutat Res*, **463**, 111-72.

Balasubramanian G., Sarathi M, Kumar SR, Hameed AS (2007). Screening the antiviral activity of Indian medicinal plants against white spot syndrome virus in shrimp. *Aquaculture*, **263**, 15-9.

Bhattacharjee R, Sil PC (2007). Protein isolate from the herb, *Phyllanthus niruri* L. (Euphorbiaceae), plays hepatoprotective role against carbon tetrachloride induced liver damage via its antioxidant properties. *Food Chem*

Toxicol, **45**, 817-26.
Cariello NF, Piegorsch WW (1996). The Ames test: The two-fold rule revisited. *Mutat Res/Genetic Toxicol Env Mutagen*, **369**, 23-31.
Chatterjee M, Sil PC (2006). Hepatoprotective effect of aqueous extract of *Phyllanthus niruri* on nimesulide-induced oxidative stress in vivo. *Indian J Biochem Biophys*, **43**, 299-305.
Ferrer M, Sánchez-Lamar A, Fuentes JL, et al (2001). Studies on the antimutagenesis of Phyllanthus orbicularis: mechanisms involved against aromatic amines. *Mutat Res/Genetic Toxicol Env Mutagen*, **498**, 99-105.
Ferrer M, Sánchez-Lamar A, Fuentes JL, et al (2002). Antimutagenic mechanisms of *Phyllanthus orbicularis* when hydrogen peroxide is tested using Salmonella assay. *Mutat Res/Genetic Toxicol Env Mutagen*, **517**, 251-4.
Halder B, Pramanick S, Mukhopadhyay S, Giri AK (2006). Anticlastogenic effects of black tea polyphenols theaflavins and thearubigins in human lymphocytes in vitro. *Toxicology in Vitro*, **20**, 608-13.
Hout S, Chea A, Bun SS, et al (2006). Screening of selected indigenous plants of Cambodia for antiplasmodial activity. *J Ethnopharmacol*, **107**, 12-8.
Kassuya CAL, Silvestre A, Menezes-De-Lima JO, et al (2006). Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from Phyllanthus amarus: Evidence for interaction with platelet activating factor receptor. *Eur J Pharmacol,* **546**, 182-8.
Kumaran A, Karunakaran RJ (2007). In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. *LWT - Food Science and Technology*, **40**, 344-52.
Maron DM, Ames BN (1983). Revised methods for the Salmonella mutagenicity test. *Mutation Research/ Environmental Mutagenesis and Related Subjects*, **113**, 173-215.
Matsushima T (1976). A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. *In Vitro Metabolic Activation Mutagenesis Testing*, 85-8.
Mazumder A, Mahato A, Mazumder R (2006). Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens. *Nat Prod Res*, **20**, 323-6.
MeLéndez PA, Capriles VA (2006). Antibacterial properties of tropical plants from Puerto Rico. *Phytomedicine*, **13**, 272-6.
OECD 1997a. Test No. 471: Bacterial Reverse Mutation Test, OECD Publishing.
OECD 1997b. Test No. 473: In vitro Mammalian Chromosome Aberration Test, OECD Publishing.
OECD 2007. Test No. 487: In Vitro Mammalian Cell Micronucleus Test, OECD Publishing.
Oliveira DF, Pereira AC, Figueiredo HCP, et al (2007). Antibacterial activity of plant extracts from Brazilian southeast region. *Fitoterapia*, **78**, 142-5.
Pradheepkumar CP, Panneerselvam N, Shanmugam G (2000). Cleistanthin A causes DNA strand breaks and induces apoptosis in cultured cells. *Mutat Res/Genetic Toxicol Env Mutagen*, **464**, 185-93.
Rajeshkumar NV, Joy KL, Kuttan G, et al (2002). Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. *J Ethnopharmacol*, **81**, 17-22.
Sripanidkulchai B, Tattawasart U, Laupatarakasem P, et al (2002). Antimutagenic and anticarcinogenic effects of *Phyllanthus amarus*. *Phytomedicine*, **9**, 26-32.
Tuchinda P, Kumkao A, Pohmakotr M, et al (2006). Cytotoxic arylnaphthalide lignan glycosides from the aerial parts of Phyllanthus taxodiifolius. *Planta Med*, **72**, 60-2.
Venkateswaran PS, Millman I, Blumberg BS (1987). Effects of an extract from Phyllanthus niruri on hepatitis B and woodchuck hepatitis viruses: in vitro and in vivo studies. *Proc Natl Acad Sci USA*, **84**, 274-8.
Yang CM, Cheng HY, Lin TC, et al (2005). Acetone, ethanol and methanol extracts of Phyllanthus urinaria inhibit HSV-2 infection in vitro. *Antiviral Res*, **67**, 24-30.
Zeiger E, Mortelmans K (2000). The Salmonella (Ames) Test for Mutagenicity. Current Protocols in Toxicology. John Wiley & Sons, Inc.
Zirihi GN, Mambu L, GuéDé-Guina F, et al (2005). In vitro antiplasmodial activity and cytotoxicity of 33 West