

RESEARCH ARTICLE

Genotoxicity and Interference with Cell Cycle Activities by an Ethanolic Extract from Thai *Plumbago indica* Roots in Human Lymphocytes *in vitro*

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Abstract

In Thai traditional medicine, *Plumbago indica* or Jetamul-Pleung-Dang in Thai is known to have health benefit especially for anti-inflammatory, antibacterial, and antitumor activities. However, the mechanisms of its action are still uncertain. One of which might be genotoxic effects. In the present study, we investigated the genotoxicity of an ethanolic extract of *Plumbago indica* root (EEPIR) by sister chromatid exchange (SCE) assay in human lymphocytes. Results have shown that all treatments with EEPIR (12.5-100 µg/ml) could induce cell cycle delay as shown by significant increase in the number of metaphase cells in the first cell cycle but neither in the second nor the third cell cycle. Only at concentrations of 25, 50, and 100 µg/ml were SCE levels significantly increased above that of the control ($p < 0.05$). EEPIR at a concentration of 500 µg/ml induced cell death as few mitotic cells were shown. Accordingly, EEPIR (25-100 µg/ml) is genotoxic in human lymphocytes and cytotoxic at concentrations of ≥ 500 µg/ml *in vitro*. Therefore, these activities of the EEPIR could serve its potential therapeutic effects, especially as an anticancer agent. Further study of EEPIR *in vivo* is now needed to support this *in vitro* evidence.

Keywords: *Plumbago indica* - cell cycle - cytotoxicity - genotoxicity - SCE

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Introduction

Plumbago (family plumbaginaceae) is an evergreen shrub with colored flowers widely grown in a tropical climate, especially in Southeast Asia, South Asia and South African. There are many species of *Plumbago* e.g. *Plumbago indica* L., syn. *Plumbago rosea* with red flowers, *Plumbago zeylanica* with white flowers and *Plumbago auriculata* with blue flowers. Their major ingredients are naphthoquinones such as plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), 3, 3'-biplumbagin and elliptinone, 4-hydroxybenzaldehyde (V), trans-cinnamic acid (VI), vanillic acid (VII), lupenone and trilinolein (Nguyen, 2004; Zhang et al., 2007; Kaewbumrung and Panichayupakaranant, 2011).

Plumbago indica is more commonly used in Thai traditional medicine. It has various common names in different countries e.g. jetamul-pleung-dang or Pitpiudang (Thailand), red leadwort or Indian leadwort (India), Akar binasa or mehulatu (Indonesia), Cheraka merah or setaka (Malaysia) and Laurel or Pampasapit (Philippines). Plumbagin, a major ingredient isolated from *plumbago*'s roots are known to have various therapeutic effects, such as antiatherogenic (Sharma et al., 1991), anti-bacterial

(Jetty et al., 2010), anti-helminth (Atjanasuppat et al., 2009), anti-fertility (Kini et al., 1997; Premakumari et al., 1997; Azad et al., 1982), anti-inflammation, and anti-tumor activities (Aziz et al., 2008; Gomathinayagam et al., 2008; Shieh et al., 2010).

In Thai traditional medicine, ethanolic extracts of *Plumbago indica* roots (EEPIR) are commonly used to treat hemorrhoids and also used as carminative to stimulate appetite by increasing digestive enzyme secretion and intestinal absorption. It also commonly used to combine with other types of herbs to make new recipes for various traditional medical treatments. For example, the pikutbenjakul recipe which contains a *Plumbago indica* root extract as one-fifth of its ingredients. Pikutenjakul has been used as an adaptogenic drug for cancer patients especially breast cancer. Trinake recipe contains a one-third *Plumbago indica* root extract. However, safety level of using these extracts as a therapeutic drug in traditional medicine is of concern. Genotoxic and cytotoxic studies to determine safety levels in humans are few. Most of the mutagenicity in the extracts was determined by using bacteria, yeast or mice. However, the results are quite inconclusive. Durga et al. (1992) demonstrated that plumbagin is not a mutagen by using the *S.typhimurium*

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Ames test. Kato et al. (1994) stated that no induction of the SOS response and mutations by plumbagin in various *Escherichia coli* mutants defective in the mutM, mutY or soxRS loci. On the contrary, Prieto-Alama et al. (1993) reported that plumbagin was mutagenic in *Escherichia coli* K-12 mutants defective in superoxide dismutase and in catalase.

SivaKumar et al. (2005) reported that plumbagin is genotoxic in Swiss albino mice by *in vivo* micronucleus assay. Our previous study indicated that pikutbenjakul was genotoxic and cytotoxic against human lymphocytes at concentrations of $>100 \mu\text{g/ml}$ and $\geq 500 \mu\text{g/ml}$ respectively. We hypothesized that the toxicities are possibly due to plumbagin, one of the major compounds in PBE (Ratanavalachai et al., 2012). Therefore, in this study, the cytotoxic and genotoxic activities of the ethanolic extracts from Thai *Plumbago indica* root were conducted on human lymphocytes directly *in vitro* by a sister chromatid exchange assay. In addition, we also determined the effect on lymphocyte's cell cycle in detail by BrdU-Hoechst fluorescence plus the Giemsa technique.

Materials and Methods

Materials

Plumbago indica roots were collected in Bangkok, Thailand and identified by the herbarium of the Department of Forestry, Bangkok. The herbarium vouchers were specified as SKP148160901.

Preparation of the EEPIR

All dried, ground, herb materials (100g) were percolated with 95% ethanol for 3 days. The product was then filtered and dried under reduced pressure. EEPIR was kept in a freezer (-20°C) and it was redissolved in DMSO before use.

Sister chromatid exchange (SCE) assay

Lymphocyte-enriched buffy coat was cultured in 5 ml culture medium using the standard blood culture condition. These studies were approved by our institutional ethical committee (MTU-BC-3-CRO48-048/53). At 24 h, after initiation of the culture, the lymphocyte were treated with EEPIR at concentrations of 12.5, 25, 50, 100 and $500 \mu\text{g/ml}$ in plain RPMI 1640 culture medium for 3 h at 37°C . After treatment, all cultures were centrifuged and the treated lymphocytes were continued to culture at 37°C in the dark with the previously saved medium. Bromodeoxyuridine (BrdU) (Sigma-Aldrich, U.S.A.) was also added in the culture medium for the final concentration of $5 \mu\text{M}$. Doxorubicin ($0.1 \mu\text{g/ml}$) (Roche, Switzerland) was used as the positive control. Plain RPMI 1640 and 0.2% V/V DMSO were used as the negative control. At 77 h after initiation, the cells were harvested. Slides were prepared and stained with the fluorescent plus Giemsa technique, according to the standard protocol (Perry and Wolf, 1974). Twenty-five cells per dose per experiment showing the second metaphase-staining pattern were scored from the coded slides for the frequencies of SCEs. Proliferation index and mitotic index were also evaluated to clarify the cytotoxicity. Mitotic index was determined as

number of all mitotic cells/1,000 cells. Proliferation index was determined as $(\text{MI}+2\text{MII}+3\text{MIII})/100$ cells where MI (M-One) is the number of the metaphase cells from the first cell cycle (homogeneously-stained chromatids), MII (M-Two) is the number of the metaphase cells from the second cell cycle (heterogeneously-stained chromatids) and MIII (M-Three) is the number of the metaphase cells from the third cell cycle (mixed homogeneously-stained and heterogeneously-stained chromatids). Two to three independent experiments were performed for each concentration of the treated compounds.

Statistical analysis

Raw data obtained from the SCE assays were transformed to stabilize the variances by the procedures of Whorton et al. (1984). Dunnett's t-test was performed to analyze the difference between the mean of the treated groups and of the control groups using the transformed data.

Results

The ethanolic extracts from *Plumbago indica* root (EEPIR) preparation

The yield of the ethanolic extract from Thai *Plumbago indica* roots was 10.6% (W/W). The major component in the EEPIR was plumbagin as determined by HPLC. The percent yield was 0.38% (W/W).

Genotoxic activities of the EEPIR determined by sister chromatid exchange assay in human lymphocytes *in vitro*

Treatments of EEPIR at concentrations of 12.5-500 $\mu\text{g/ml}$ for 3 h demonstrated that EEPIR at only concentrations of 25, 50, and $100 \mu\text{g/ml}$ significantly increased SCE levels above that of the negative controls in human lymphocytes *in vitro* (Figure 1). No linear dose response was observed although EEPIR at low concentrations of 12.5, 25, $50 \mu\text{g/ml}$ tended to increase SCE value as the EEPIR concentration increased. However, the genotoxicity of EEPIR at concentrations of $100 \mu\text{g/ml}$ tended to be reduced. Only few mitotic cells were observed at the highest concentration of $500 \mu\text{g/ml}$.

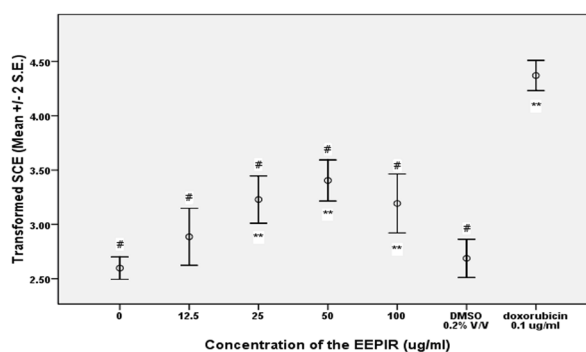


Figure 1. Induction of SCE Levels Represented as Transformed SCE by the EEPIR in Human Lymphocytes *in vitro*. ** $p < 0.05$ significantly different from the negative controls (both plain RPMI and DMSO 0.2% (V/V), # $p < 0.05$ significantly different from the positive control ($0.1 \mu\text{g/ml}$ doxorubicin)

Cytotoxic activities of the EEPIR in human lymphocytes *in vitro*

The EEPIR at the concentration of 500 $\mu\text{g/ml}$ induced potent cytotoxic activities to the cells as only few mitotic cells were found. The mitotic index and proliferation index of the EEPIR treatments at concentrations of 12.5, 25, 50, and 100 $\mu\text{g/ml}$ did not showed statistically significant different from those of the negative control as shown in Table 1. No linear dose response curve was observed.

Influence of the EEPIR on the cell cycle of human lymphocytes *in vitro* as determined by BrdU-Hoechst Fluorescent plus Giemsa technique

In order to clarify the effect of the EEPIR on a lymphocyte's cell cycle, analysis of the number of metaphase cells in each cell cycle were compared. Interestingly, the number of metaphase cells from the first cell cycle are quite high at all concentrations of EEPIR treated (12.5, 25, 50 $\mu\text{g/ml}$). They are all significant higher

Table 1. Mitotic Index and Proliferation Index of the EEPIR

	Mitotic index	Proliferation index
Concentration of EEPIR ($\mu\text{g/ml}$)		
0	10.7 \pm 0.4	2.3 \pm 0.1
12.5	10.4 \pm 4.3	1.8 \pm 1.1
25	10.4 \pm 4.3	1.9 \pm 0.4
50	13.0 \pm 1.8	2.5 \pm 0.4
100	10.6 \pm 1.8	1.6 \pm 0.3
500	toxic	toxic
0.2% V/V DMSO	9.8 \pm 3.9	1.9 \pm 0.9
0.1 $\mu\text{g/ml}$ doxorubicin	7.6 \pm 2.4	1.4 \pm 0.3

*Data are shown as means \pm standard deviations

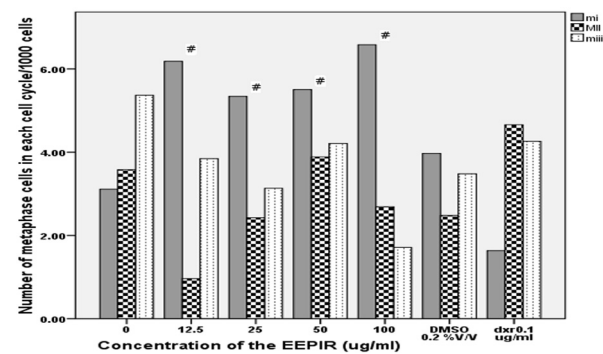


Figure 2. Influence of the EEPIR on the Cell Cycle according to the Number of Metaphase Cells in MI, MII and MIII. # $p < 0.05$ significant different from that of doxorubicin (dxr) treated

than that of the doxorubicin treated as shown in Figure 2 ($p < 0.05$). None of the less, the number of metaphase cells from the second cell cycle tended to increase as the concentration of EEPIR treated increased (12.5, 25, 50 $\mu\text{g/ml}$). Though no statistical significant different was found.

Discussion

Genotoxic study of the EEPIR in human lymphocytes by SCE assay clearly demonstrated that EEPIR could significantly induce genotoxicity in human cells at concentrations of 25-100 $\mu\text{g/ml}$ above that of the negative control. Nevertheless, the level of genotoxic damage as indicated by SCE level was not very high. EEPIR at lower concentrations of 12.5 $\mu\text{g/ml}$ is neither genotoxic nor cytotoxic and quite safe for human cells. Whereas EEPIR at higher concentrations above 500 $\mu\text{g/ml}$ is potent cytotoxic as few mitotic cells was found.

In addition, the mitotic index and proliferation index of these EEPIR treatments did not show significant differences from those of the negative nor the positive controls. Analysis of the effect on metaphase cells in each cell cycle, clarified by BrdU-Hoechst fluorescent plus Giemsa technique, showed that these EEPIR treatments (12.5-100 $\mu\text{g/ml}$) in human lymphocytes could interrupt metaphase cells to progress further as revealed by the significant increase in the number of metaphase cells left in MI in all concentrations treated. This result is in agreement with a report by Wang (2008) showing that plumbagin could also induce cell cycle arrest in human melanoma A375.S2 cells. In comparison to the potent genotoxic doxorubicin, these EEPIR treatments could better inhibit metaphase cells progression than doxorubicin-treated cells.

According to the mechanism of action of the EEPIR, its genotoxicity might serve well in acting as a chemotherapeutic compound. This result corresponds to the anti-cancer action of plumbagin reported by Xu and Lu (2010). They reported that plumbagin injection in to NB4, promyelocytic leukemia cells xenograft in NOD/SCID mice could reduce tumor volume by 64.5% with no overt manifestation of toxicity which appeared in doxorubicin-treated mice. In addition, induction of cell cycle arrest by EEPIR might be able to allow damaged cells to be repaired before running progressively through cell cycle.

In comparison with the potent genotoxic doxorubicin, this result indicated that the EEPIR might cooperate well with a strong chemotherapeutic agent to potentiate the

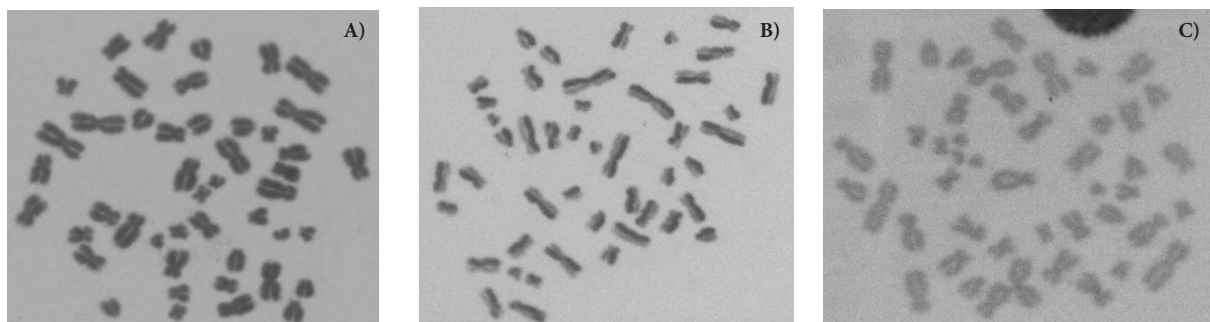


Figure 3. Homogeneous Densely Staining. A) MI, B) MII and C) MIII

anticancer action by potentiating the genotoxicity in the cancer cells while allowing the cell cycle to have time for restoration. However, further study is required to support this evidence.

In conclusion, this study verified that EEPiR at concentrations of 12.5-100 µg/ml are genotoxic while EEPiR at a concentration of ≥500 µg/ml is cytotoxic in human lymphocytes *in vitro*. Therefore, dosage use of *Plumbago indica* root ethanolic extract needs to be of concern in normal healthy humans especially when they are used for tonic treatments. More *in vivo* study is needed to validate its safety dosage in humans.

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References

Atjanasuppat K, Wongkham W, Meepowpan P, et al (2009). *In vitro* screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. *J Ethnopharmacol*, **123**, 475-82.

Azad CAK, Sushanta KC, Azad KAK (1982). Antifertility activity of *Plumbago zeylanica* Linn. root. *Indian J Med Res*, **76**, 99-101.

Aziz MH, Dreckschmidt NE, Verma AK (2008). Plumbagin, a medicinal plant-derived naphthoquinone, is a novel inhibitor of the growth and invasion of hormone-refractory prostate cancer. *Cancer Res*, **68**, 9024-32.

Durga R, Sridhar P, Polasa H (1992). Antimutagenic activity of plumbagin in Ames Salmonella typhimurium test. *Indian J Med Res*, **96**, 143-5.

Gomathinayagam R, Sowmyalakshmi S, Mardhatillah F, et al (2008). Anticancer mechanism of plumbagin, a natural compound, on non-small cell lung cancer cells. *Anticancer Res*, **28**, 785-92.

Jetty A, Subhakar C, Rajagopal D, et al (2010). Antimicrobial activities of neo- and 1-epineo-isoshinanolones from *Plumbago zeylanica* roots. *Pharm Biol*, **48**, 1007-11.

Kaewbumrung S, Panichayupakaranant P (2012). Isolation of three antibacterial naphthoquinones from *Plumbago indica* roots and development of a validated quantitative HPLC analytical method. *Nat Prod Res*, **26**, 2020-3.

Kato T, Watanabe M, Ohta T (1994). Induction of the SOS response and mutations by reactive oxygen-generating compounds in various *Escherichia coli* mutants defective in the mutM, mutY or soxRS loci. *Mutagenesis*, **9**, 245-51.

Kini DP, Pandey S, Shenoy BD, et al (1997). Antitumor and antifertility activities of plumbagin controlled release formulations. *Indian J Exp Biol*, **35**, 374-9.

Nguyen AT, Malonne H, Duez P, et al (2004). Cytotoxic constituents from *Plumbago zeylanica*. *Fitoterapia*, **75**, 500-4.

Perry P, Wolff S (1974). New Giemsa method for the differential staining of sister chromatids. *Nature*, **251**, 156-8.

Premakumari P, Rathinam K, Santhakumari G (1977). Antifertility activity of plumbagin. *Indian J Med Res*, **65**, 829-38.

Prieto-Alamo MJ, Abril N, Pueyo C (1993). Mutagenesis in *Escherichia coli* K-12 mutants defective in superoxide

dismutase or catalase. *Carcinogenesis*, **14**, 237-44.

Ratanavalachai T, Thitiorul S, Tanuchit S, et al (2012). *In vitro* cytotoxic and genotoxic effects of ethanolic extract of *Pikutebnjakul* on human lymphocytes. *Thamma Med J*, **12**, 262-8.

Sharma I, Gusain D, Dixit VP (1991). Hypolipidaemic and antiatherosclerotic effects of plumbagin in rabbits. *Indian J Physiol Pharmacol*, **35**, 10-4.

Shieh JM, Chiang TA, Chang WT, et al (2010). Plumbagin inhibits TPA-induced MMP-2 and u-PA expressions by reducing binding activities of NF-kappaB and AP-1 via ERK signaling pathway in A549 human lung cancer cells. *Mol Cell Biochem*, **335**, 181-93.

SivaKumar V, Prakash R, Murali MR, et al (2005). *In vivo* micronucleus assay and GST activity in assessing genotoxicity of plumbagin in Swiss albino mice. *Drug Chem Toxicol*, **28**, 499-507.

Wang CC, Chiang YM, Sung SC, et al (2008). Plumbagin induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human melanoma A375.S2 cells. *Cancer Lett*, **259**, 82-98.

Xu KH, Lu DP (2010). Plumbagin induces ROS-mediated apoptosis in human promyelocytic leukemia cells *in vivo*. *Leuk Res*, **34**, 658-65.

Zhang QR, Mei ZN, Yang GZ, Xiao YX (2007). Chemical constituents from aerial parts of *Plumbago zeylanica* Linn. *Zhong Yao Cai*, **30**, 558-60.