

In Vitro Enhancement of Doxorubicin Genotoxic Activities and Interference with Cell Cycle Delay by *Plumbago indica* Root Ethanolic Extract in Human Lymphocytes

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Background: Combinations of modern medicines with herbal medicines are being developed for more effectiveness. Data on the safety and drug-herb interactions are needed to be clarified. Ethanolic extract of *Plumbago indica* root (EEPIR) is medicinally used for cancer treatment in Asian traditional medicine. However, its mechanism of action is still inconclusive. Our previous study demonstrated that EEPIR was genotoxic and induced cell cycle delay in human lymphocytes in vitro.

Objective: To investigate genotoxic potency and interference with cell cycle of EEPIR in combination with doxorubicin (DXR), a standard chemotherapeutic agent, in human lymphocytes by in vitro sister chromatid exchange (SCE) assay.

Material and Method: Human lymphocytes were pretreated with EEPIR at 6.25-100 mcg/ml followed by DXR (0.1 mcg/ml). SCE levels and cell cycle kinetics were evaluated.

Results: EEPIR pretreatments (6.5-50 mcg/ml) significantly enhanced genetic damage induced by DXR ($p < 0.05$). Delaying of the cell cycle was detected and related to EEPIR concentration. EEPIR at 100 mcg/ml, on the contrary, did not enhance DXR-induced genotoxicity but tended to lower genotoxicity compared to DXR treatment alone. It significantly delayed cell cycle the most ($p < 0.05$).

Conclusion: EEPIR pretreatments at proper doses enhanced genotoxic damage induced by DXR in human lymphocytes. Delaying cell cycle by EEPIR could lower that potency. Usage of EEPIR, therefore, should be adjusted for safety. Combination of EEPIR with DXR might be useful for more efficient cancer treatment with less DXR toxicity. Further in vivo study is needed to support this in vitro evidence.

Keywords: *Plumbago indica* root, Doxorubicin, Cell cycle, Sister chromatid exchange, Human lymphocytes, Genotoxicity

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Many standard chemotherapeutic agents present several concerns regarding their toxicities. Combinations of modern medicines with herbal medicines are being developed for more effective treatment and less toxicity. Data on the quality, efficacy, safety, and drug-herb interactions are needed to be monitored in herbal products. Ethanolic extract of

Plumbago indica root (EEPIR) is an anecdotal herbal medicine used for cancer treatments in Asian traditional medicine.

Plumbago (family Plumbaginaceae) is an evergreen shrub, widely grown in Asia and Africa with various colored flowers. There are more than ten species around the world e.g. *P. indica* (red flower), *P. zeylanica* (white flower) and *P. auriculata* (blue flower). They possess a variety of pharmacological activities such as antifertility, anthelmintic and antitumor action^(1,2). In Thai traditional medicine (TTM), ethanolic extract of *P. indica* roots (EEPIR) is used as a carminative agent, to stimulate digestive enzyme secretion and intestinal absorption, and also used to treat venereal diseases,

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urinary tract infections, and parasitic infections. It is combined with other types of herbs to make holistic formulations such as “Pikutbenjakul” and “Trinake”.

Pikutbenjakul contains EEPIR as one-fifth volume of its ingredients. It has been used as an adaptogenic drug for cancer patients especially for breast cancer. Folk doctors would give Pikutbenjakul to breast cancer patients for two-three weeks before other breast cancer treatment. Trinake contains a one-third volume of EEPIR. It is used for anti-hemorrhoid treatments.

EEPIR has also been reported to have anti-bacterial activities^(3,4) antimalarial activity⁽⁵⁾ and cytotoxicity against large lung carcinoma cell line (COR-L23)⁽⁶⁾. Bioactive compounds presented in the root extracts of *P. indica* are known to be naphthoquinones e.g. plumbagin, 3,3'-biplumbagin and elliptinone⁽⁷⁾. However, scientific research on effectiveness and safeness of *P. indica* extract in the form of EEPIR, used in Thai traditional medicine, is still limited.

Doxorubicin (DXR) is an anthracycline antibiotic, commonly used alone or in combination, to treat hematological malignancies and solid tumors such as breast, bladder, lung, and ovarian cancers. Its genotoxic mechanisms of action involve intercalation of DNA and induction of free radical damage leading to DNA double-strand breaks⁽⁸⁾. Currently, DXR is a very effective chemo-therapeutic agent with limitations of cardiotoxicity and myelosuppression⁽⁹⁾.

Our previous study indicated that EEPIR at concentrations of 25-100 mcg/ml were genotoxic and induced cell cycle delay in human lymphocytes in vitro using cytogenetic procedure, sister chromatid exchange (SCE) assay. Cytotoxicity of EEPIR was at 500 mcg/ml and higher⁽¹⁰⁾. Our data informed us that EEPIR could possibly act as a genotoxic and cytotoxic chemotherapeutic agent for anticancer treatment or possibly have advantages when using it in combination with modern chemotherapeutic compound for more effectiveness. However, safeness is a priority in herbal medicinal use. The present study, therefore, investigated the genotoxic and cell cycle modulations induced by EEPIR pre-treatment's at various concentrations followed by DXR, a genotoxic chemotherapeutic agent.

Material and Method

P. indica roots were collected by folk doctors in Bangkok. The samples were then identified by the Herbarium of the Royal Forest Department of Thailand and designated as SKP148160901.

Preparation of the EEPIR

Dried powder of *P. indica* root was percolated with 95% ethanol for three days. The product (EEPIR) was then filtered and dried with reduced pressure, and was kept in a freezer at -20°C. Prior to use, the EEPIR was redissolved in dimethylsulfoxide (DMSO).

A chemical fingerprint of EEPIR, plumbagin, was carried out using high performance liquid chromatography (HPLC) system (Constametric 4100 Bio), with ultraviolet visible detector (Spectromonitor 4100) and automatic injector (Spectra System AS3500). Data were analyzed with TSP PC1000 software. A reversed-phase column, Phenomenex Luna 5m C18 (2) 100A analytical column (250x4.60 mm 5 micron) was used. After this, EEPIR was dissolved in acetonitrile, sonicated for 5 minutes and filtered through a 0.45 m membrane filter before use. One milligram of plumbagin standard, purchased from Sigma-Aldrich, USA was weighed and dissolved in 1 ml acetonitrile.

Chemicals

5'-Bromo-2'-deoxyuridine (BrdU) and Bizbenzimidazole H 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium with HEPES and L-glutamine and fetal bovine serum were from Hyclone, Utah, USA. The Giemsa stain was from Biotech Reagents, Thailand; phytohemagglutinin (PHA) L, penicillin-streptomycin and colcemid were from Seromed, Germany. Doxorubicin (DXR) was from Pfizer (Pharmacia & Upjohn Company, NY, USA).

Sister chromatid exchange assay

Cell cultures

Lymphocyte-enriched buffy coat was cultured in a 5 ml culture medium using standard blood culture conditions⁽¹¹⁾. Prior approval for this experiment was obtained from the Institutional Ethics Committee (MTU-BC-3-CRO48-048/53). Twenty-four hours after the initiation of the culture, lymphocytes were centrifuged to obtain packed cells. The supernatant medium was removed and saved for reuse. The remaining lymphocytes were treated with EEPIR at concentrations of 6.25, 12.5, 25, 50, and 100 mcg/ml dissolved in 0.4% (V/V) DMSO in plain RPMI 1640 culture medium for two hours at 37°C. Following centrifugation at 400 g for 10 minutes, the supernatant medium was discarded. Then, incubation of the treated cells was continued with DXR (0.1 mcg/ml) for two hours at 37°C.

For positive controls, the lymphocytes were simultaneously treated with plain RPMI 1640 culture medium for two hours followed by DXR (0.1 mcg/ml)

for two hours. For negative controls, treatments were made with either plain RPMI 1640 or 0.4% V/V DMSO for two hours, followed by plain RPMI 1640 for two hours.

Cell culture harvest and staining

After treatments, all the lymphocyte cultures were centrifuged to pack the cells together and the supernatant was discarded. Finally, all of the lymphocyte cultures continued to be cultured at 37°C in the dark and mixed with the previously saved medium and Bromodeoxyuridine (BrdU) solution to a final concentration at 5 mcM.

Because of the induction of cell cycle delay, the cells were harvested at 77 hours after initiation. Slides were prepared and stained using the fluorescent plus Giemsa technique, according to the standard protocol^(12,13). Twenty-five cells per dose per experiment showing the second metaphase-staining pattern were scored from the coded slides for frequencies of sister chromatid exchange (SCE). The proliferation index (P.I.) and mitotic index (M.I.) were also evaluated for their cytotoxicity. The mitotic index was determined as number of all mitotic cells/1,000 cells. The proliferation index was determined as $(MI+2MII+3MIII)/100$ cells, where MI is the number of metaphase cells from the first cell cycle (homogeneously-stained chromatids),

MII is the number of metaphase cells from the second cell cycle (heterogeneously-stained chromatids), and MIII is the number of 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

A square root transformation of the SCE data was required to stabilize the variances, according to the procedures of Whorton et al, 1984⁽¹⁴⁾. Frequency of transformed SCE was expressed as a square root of SCE. Dunnett's t-test was performed to analyze the difference between the means of the treated groups and the means of the control groups using the transformed data. Data are shown as mean \pm standard error of mean. Transformed SCE can be expressed as the equation:

$$\text{Transformed SCE (SCE}_T\text{)} = \text{square root SCE}$$

Results

Analysis of the EEPIR samples

The EEPIR used in the present study extracted from Thai *P. indica* roots, yielded approximately 10.6 g/100 g dried *P. indica* roots. The Plumbagin in EEPIR detected by high performance liquid chromatography

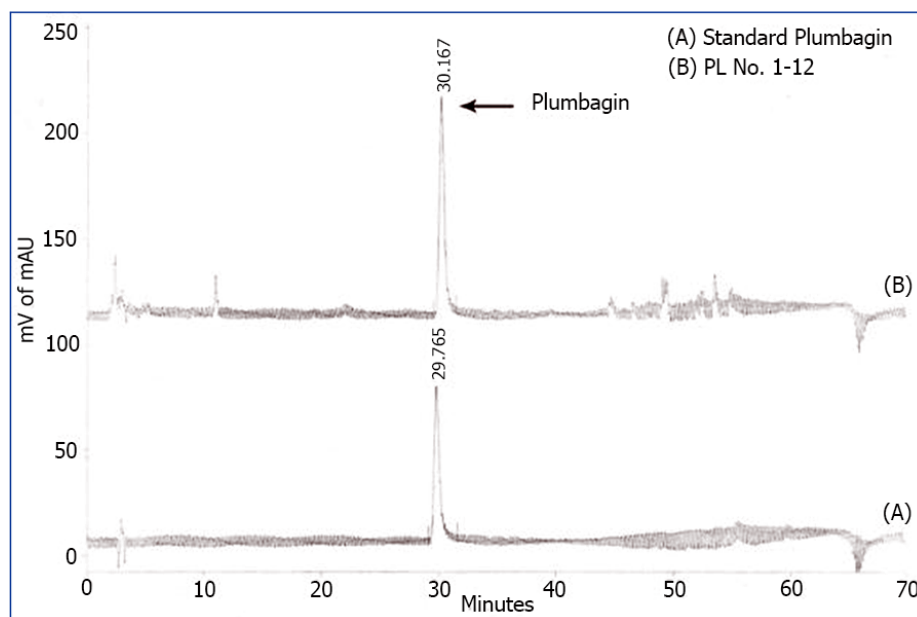


Fig. 1 Fingerprint of EEPIR by high performance liquid chromatography (HPLC). Mobile phase: water (A) -acetonitrile (B) with gradient elution as follows: 0 min: 40% B; 30 min: 50% B; 50 min: 90% B; 60 min: 100% B; Flow rate of 1.0 ml/min; UV detector at 256 nm.

(HPLC) was approximately 0.38 g/100 g EEPIR (Fig. 1).

Genotoxic activities of the EEPIR pretreatments against DXR

EEPIR pretreatments at concentrations of 6.25, 12.5, 25 and 50 mcg/ml followed by DXR treatment (0.1 mcg/ml), significantly increased the SCE level versus that induced by DXR alone ($p < 0.05$) (Fig. 2). These pretreatments raised the SCE level approximately 14% above that of the DXR alone. No dose response was observed. A DMSO (0.4% V/V) pretreatment followed by DXR slightly increased SCE over that induced by

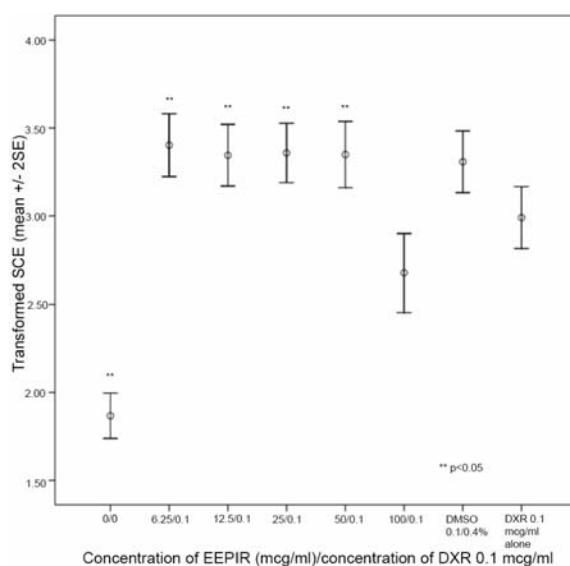
DXR alone, but was not statistically significant. In contrast, EEPIR pretreatment at 100 mcg/ml, tended to decrease SCE levels induced by DXR with no significant difference. The DXR treatment alone had significantly increased the SCE level by 37% above that induced by plain RPMI treatment ($p < 0.05$).

Influence of the EEPIR pretreatments followed by DXR on the cell cycle of human lymphocytes

The mitotic index and the proliferation index of EEPIR pretreatments at all concentrations of 6.25, 12.5, 25, 50 and 100 mcg/ml, as well as those of the negative controls, plain RPMI, and 0.4% V/V DMSO, had no significant difference from that of DXR treatment alone (Table 1). However, when the effects of the EEPIR pretreatments on cell cycle were further analyzed from the number of metaphase cells in each cell cycle, MI, MII, and MIII, there appeared to be a tendency of increasing in the number of metaphase cells in MI relating to the concentrations of EEPIR as shown in Fig. 3. At the highest concentration of 100 mcg/ml EEPIR pretreatment, the number of metaphase cells in MI was the highest compared to those from other treatments. It was significantly higher than the number of metaphase cells in MI from DXR treatment alone ($p < 0.05$).

On the contrary, at the lower concentration of 6.25 and 12.5 mcg/ml EEPIR, the number of metaphase cells in MII tended to be higher than those in MI. This revealed that at these low levels, EEPIR had not yet delayed the cell cycle but had continued to proliferate from MI to MII and to MIII respectively.

For a negative control, plain RPMI, cells were in their normal proliferative phase. Metaphase cells in MII and MIII were higher than that in MI. However, pretreatment with DMSO, a vehicle control, tended to slightly induce cell cycle delay showing the number of



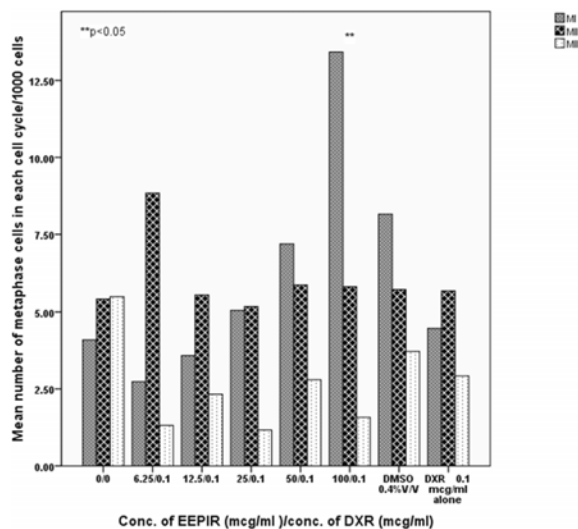
Data are shown as means \pm standard error of mean, $n = 3$
** Significantly different from the positive control, $p < 0.05$

Fig. 2 Sister chromatid exchange levels represented as transformed SCE, induced by EEPIR pretreatments followed by DXR in human lymphocytes in vitro.

Table 1. Mitotic index (M.I.) and proliferation index (P.I.) of the EEPIR pretreatments

Concentration of EEPIR (mcg/ml)/DXR (mcg/ml)	M.I. \pm SE	P.I. \pm SE
0/0	14.9 \pm 2.1	3.1 \pm 0.4
6.25/0.1	13.1 \pm 5.6	2.5 \pm 1.7
12.5/0.1	11.3 \pm 1.6	2.1 \pm 0.3
25/0.1	11.2 \pm 1.7	1.9 \pm 0.3
50/0.1	15.6 \pm 0.9	2.7 \pm 0.2
100/0.1	20.3 \pm 4.8	2.9 \pm 0.9
Negative control: 0.4% V/V DMSO/0.1	19.6 \pm 2.7	3.1 \pm 0.9
Positive control: 0/0.1 (DXR alone)	15.8 \pm 2.5	2.4 \pm 0.9

Data are shown as means \pm standard error of mean, $n = 3$



** Significantly different from the positive control, $p < 0.05$

Fig. 3 The number of metaphase cells in each cell cycle after EEPIR pretreatments at various doses followed by 0.1 mcg/ml DXR.

metaphase cells in MI were slightly higher than those in MII. But they were not significantly different from those of DXR positive control.

Discussion

Our data demonstrated that EEPIR pretreatments at 6.25-50 mcg/ml followed by 0.1 mcg/ml DXR were able to potentiate DXR-induced genotoxicity in human lymphocytes in vitro. This data revealed that EEPIR, although at low concentrations, could potentiate genotoxicity from chemical interactions. Using EEPIR in combination with other compounds for medicinal purpose, therefore, requires scientific testing for biosafety.

Nevertheless, no enhancement of genotoxicity induced by DXR was detected when cells were pretreated with EEPIR at concentration of 100 mcg/ml. More importantly, the treatment was able to delay cell cycle the most, which might be the reason why it could protect cells from increasing genotoxic damage. Delaying cell cycle might allow more time for damaged cells to be repaired. As a consequence, the more cell cycle delay the less the genotoxic damage might occur or vice versa. Our data support this evidence showing that genotoxicity induced by DXR was enhanced when EEPIR pretreatments were treated at lower concentrations (6.25-50 mcg/ml) with lower cell cycle delay.

Currently, DXR is a very effective

chemotherapeutic agent, but has high cardiotoxicity. This is a limitation of the drug. The combination of EEPIR and DXR might be beneficial for increasing efficiency in chemotherapy with genotoxic potential. In addition, the treatment at proper dosage might delay the cell cycle preventing cells from progressing aggressively as well.

Potential of the SCE level induced by a combined treatment between DXR and EEPIR possibly resulted from malfunctioning in DNA topoisomerase II activities. DXR is known to be a potent DNA topoisomerase II inhibitor, which induces DNA double strand breaks and cell cycle arrest in G2⁽⁸⁾. DXR stabilizes the topoisomerase-mediated cleavable complex resulting in DNA damage. Plumbagin and Shikonin, active ingredients in EEPIR were reported to be able to induce DNA topoisomerase II-mediated DNA cleavage⁽¹⁵⁾. Therefore, EEPIR, which is composed of more bioactive compounds other than plumbagin and shikonin, possibly have synergistic effect with DXR to inhibit DNA topoisomerase II enhancing genotoxicity afterwards.

Concerning DMSO as our vehicle control, it was also shown that SCE level induced by the DMSO pretreatment was slightly higher than that induced by DXR treatment alone, though with no statistically significant difference. This combination treatment also slightly affected cell cycle delay by slightly increasing the number of metaphase cells in MI stage. From our previous study, it was demonstrated that DMSO by itself did not increase the SCE level compared to the negative control, plain RPMI⁽¹⁰⁾. Therefore, slight induction of SCE level should be the result from the combination between DMSO and DXR treatments.

DMSO is commonly used to dissolve drugs and chemical compounds that do not dissolve well in water. It has various toxic effects and protective effects. It potentiates the proteinuria and formation of tubular casts in puromycin induced nephrosis in rats⁽¹⁶⁾ and induces widespread apoptosis in the developing central nervous system in mice⁽¹⁷⁾. In contrast, DMSO protected acetaminophen-induced hepatic toxicity in mice⁽¹⁸⁾. It also alters locomotor activity involving behavioral toxicity of larval zebra fish⁽¹⁹⁾. It has also been approved by the US Food and Drug Administration (FDA) for treatment of interstitial cystitis⁽²⁰⁾.

Therefore, our data verified that DMSO at 0.4% V/V tended to enhance genotoxicity induced by DXR. Enhancing genotoxicity by the activities of EEPIR pretreatments in our study partly might result from DMSO. However, EEPIR, by itself, has much more

influence on the results than DMSO does, especially in view of our data showing the relationship between EEPIR concentrations and induction of cell cycle delay. DMSO, at the concentration used in the present study, may function mainly as an effective solvent, enhancing EEPIR to penetrate through cell membrane.

Conclusion

In summary, EEPIR in DMSO pretreatments at lower concentrations, enhanced genotoxicity induced by DXR. Induction of cell cycle delay was related directly to concentrations of EEPIR. EEPIR pretreatment at higher concentrations increased cell cycle delay while decreasing genotoxicity induced by DXR. The data suggested that EEPIR at proper concentrations, in combination with DXR, should be more effective in cancer therapy. On the contrary, usage of EEPIR in normal cells should be used with caution because of its capability of enhancing genotoxicity induced by others e.g. DXR. Scientific testing, for its safeness to normal cells, especially when used in combination with other compounds is required. More in vivo study is needed to confirm this in vitro result.

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Potential conflicts of interest

None.

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การเพิ่มความเป็นพิษในระดับจีนของดอกโชรูบิซินและการรบกวนวัฏจักรเซลล์ให้ซาลงโดยสารสกัดเอทานอลจากรากเจตมูลเพลิงแดงในเม็ดเลือดขาวของมนุษย์ในหลอดทดลอง

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ภูมิหลัง: การใช้ตัวยาสมัยใหม่ร่วมกับสมุนไพรทางารแพทย์กำลังอยู่ในระหว่างการพัฒนาเพื่อให้ได้ประสิทธิภาพมากขึ้น ข้อมูลเกี่ยวกับความปลอดภัยและการทำปฏิกิริยาระหว่างยาและสมุนไพรเป็นสิ่งจำเป็นต้องทำให้ชัดเจน สารสกัดเอทานอลจากรากเจตมูลเพลิงแดง (EEPIR) เป็นสมุนไพรที่รักษา มะเร็งในการแพทย์แผนโบราณของเอเชีย อย่างไรก็ตามกลไกการออกฤทธิ์ยังไม่เป็นที่ทราบแน่ชัด ผลการศึกษาเบื้องต้นโดยกลุ่มผู้วิจัยพบว่า EEPIR มีความเป็นพิษระดับจีนและสามารถเหนี่ยวนำให้วัฏจักรเซลล์ซาลงในเม็ดเลือดขาวของมนุษย์ในหลอดทดลอง

วัตถุประสงค์: เพื่อตรวจสอบความรุนแรงในการเป็นพิษระดับจีนจากการใช้ EEPIR ร่วมกับยาดอกโชรูบิซิน (DXR) ที่เป็นเคมีบำบัดมาตรฐาน และการรบกวนวัฏจักรเซลล์ของเม็ดเลือดขาวของมนุษย์ในหลอดทดลองด้วยวิธีซิสเตอร์โครมาติด เอกซเซนจ์แอสเสย์

วัสดุและวิธีการ: ทดสอบ EEPIR ที่ความเข้มข้น 6.25-100 ไมโครกรัม/มิลลิลิตร กับเม็ดเลือดขาวของมนุษย์ตามด้วย DXR ที่ความเข้มข้น 0.1 ไมโครกรัม/มิลลิลิตร ประเมินระดับ SCE และจลนศาสตร์ของวัฏจักรเซลล์

ผลการศึกษา: EEPIR ที่ให้ก่อน DXR ที่ความเข้มข้น 6.5-50 ไมโครกรัม/มิลลิลิตร สามารถกระตุ้นพิษระดับจีนของ DXR ที่ให้ตามมาโดยมีการกระตุ้นพิษเพิ่มขึ้นอย่างมีนัยสำคัญ ($p < 0.05$) ผลยังทำให้วัฏจักรเซลล์ซาลงโดยสัมพันธ์กับความเข้มข้นของ EEPIR ในทางตรงข้าม การให้ EEPIR ก่อน DXR ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร มีแนวโน้มลดความเป็นพิษระดับจีนลงได้ ทั้งยังทำให้วัฏจักรเซลล์ซาลงมากที่สุดอย่างมีนัยสำคัญ ($p < 0.05$)

สรุป: การให้ EEPIR ที่ความเข้มข้นเหมาะสมทำให้เพิ่มความเป็นพิษระดับจีนที่ดูถูกเหนี่ยวนำด้วย DXR ในเม็ดเลือดขาวของมนุษย์ ความสามารถของ EEPIR ในการทำให้วัฏจักรเซลล์ซาลง มีผลลดความเป็นพิษลง ดังนั้นการใช้ EEPIR จำเป็นต้องปรับขนาดความเข้มข้นเพื่ออยู่ในระดับปลอดภัย การใช้ EEPIR ร่วมกับ DXR น่าจะช่วยให้การรักษา มะเร็ง มีประสิทธิภาพเพิ่มขึ้นและลดความเป็นพิษข้างเคียงลง การศึกษาเรื่องนี้ในสิ่งมีชีวิตต่อไปจึงควรได้รับการสนับสนุน เพื่อสนับสนุนข้อมูลที่ได้อจากการศึกษาในหลอดทดลอง