Original Articles

In vitro Cytotoxic and Genotoxic Effects of Ethanolic Extract of Pikutbenjakul on Human Lymphocytes

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Abstract

Pikutbenjakul (PBE) is an adaptogen widely used among patients with cancer. This study aimed to assess *in vitro* genotoxic and cytotoxic effects of ethanolic extract of PBE on human lymphocytes using sister chromatid exchange (SCE) assay. PBE was prepared by macerating its herbal constituents with 95% ethanol for three days, filtered and dried. PBE (dissolved in DMSO) at 50, 100, 200, 400, and 500 μg/ml was used to treat human lymphocytes for three hours. SCE was then scored and analyzed. Plain RPMI and 0.4% (V/V) DMSO were used as the negative controls whereas doxorubicin (0.1 μg/ml) was used as the positive control. Results indicated that PBE ≥ 500 μg/ml was cytotoxic as very few mitotic cells were found. PBE at 100, 200 and 400 μg/ml could significantly increase SCE level ($p<0.05$). Their mitotic index (MI) and proliferation index (PI) were not significantly different from those of the controls. Results clearly showed that PBE was genotoxic and cytotoxic against human lymphocytes at concentrations of ≥ 100 μg/ml and ≥ 500 μg/ml respectively. These toxic effects are possibly due to plumbagin, one of the major compounds in PBE. The usage of PBE was safe at the concentration of ≤ 50 μg/ml. However, the additional *in vivo* studies are needed to clarify the proper dosage for the safety of use in cancer patients.

Key words: Pikutbenjakul, Sister chromatid exchange, Doxorubicin, Genotoxicity, Cytotoxicity

Introduction

Pikutbenjakul (PBE) is one of the most widely used Thai traditional herbs as an adaptogen to enhance normal physiological function, especially for cancer patients. It comprises five important herbs including *Piper chaba* fruit, *Piper sarmentosum* root, *Piper interruptum* stem, *Plumbago indica* root and *Zingiber officinale* rhizome. Each herb has been widely used in Oriental traditional medicine. For example, *P. chaba* (called Di-pli in Thai) is used as an anti-diarrhea and expectorant. *P. sarmentosum* (called Cha-phlu in Thai) is used to stimulate intestinal movement and also used as an expectorant. *P. interruptum* (called Sa-kan in Thai) is used as an anti-flatulant and element tonic. *P. indica* (called Chettamun-Phloeng-Daeng in Thai) is used to treat hemorrhoids and also used as a carminative agent. *Z. officinale* (called Khing in Thai) is used as an antiemetic, antispasmodic, expectorant and also carminative agent.

In recent years, there are a number of scientific studies supporting the use of the aforementioned herbs. The aqueous acetone extract from the fruit of *P.chaba* was found to have protective effects on ethanol- and indomethacin-induced gastric lesions in rats[1]. The ethanolic extract of this fruit had potent cytotoxic activities against the cholangiocarcinoma CL-6 cell line while its methanolic extract had a hepatoprotection over lipopolysaccharide

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(LPS)-induced liver injury in mice[2,3]. This hepatoprotective effect was proved to be the function of piperine, the principal constituent in *P. chaba*[4]. The ethanolic extract of *P. sarmentosum* root was found to prevent the hydrogen peroxide-induced oxidative cell damages in cultured human umbilical vein endothelial cells (HUVECs), and to have anti-inflammatory, anti-nociceptive and antipyretic activities in mice[5,6]. *P. indica* and its major compound, plumbagin, showed the anti-proliferative and apoptotic activities in human colonic cancer cell lines, HT29 and HCT15[7]. The ethyl acetate fraction of *Z. officinale* extract was found to inhibit the expression of the human telomerase reverse transcriptase (hTERT) and c-Myc, two important molecular targets for cancer, in A549 lung cancer cells[8]. In addition, 6-gingerol, a major component of the *Z. officinale* extract, showed apoptotic potential in mouse skin tumors[9] and inhibited metastasis of MDA-MB-231 human breast cancer cell line[10].

While there are several reports supporting the beneficial effects of each individual herbs, the safety of using the combined these five herbs as in the form of PBE has rarely been explored. Itharat et al. (2008) found that PBE exhibited potent cytotoxic activities against lung cancer cell lines (COR-L23) but less active against normal lung fibroblast cell line (MRC-5)[11]. Major compounds isolated from PBE were piperine, plumbagin and 6-gingerol[12,13]. The latter has been reported as a potent mutagen[14,15] which induces DNA strand breaks and chromosome damage in human hepatoma G2 (HepG2) cells by comet assay[16]. Piperine was found to induce selective neurotoxicity in cultured rat hippocampal neurons by inducing lysosomal and mitochondrial damage[17], and plumbagin had been reported to have genotoxic activity in mouse lymphoma L5178Y cells by comet assay[18]. With the cytotoxic actions of these three substances, the safety level of using PBE as the adaptogen and as a chemotherapeutic drug in traditional medicine is of concern. To date, there are few studies exploring the genotoxic and cytotoxic activities regarding to various doses against human cells. This study thus attempted to assess genotoxic and cytotoxic potentials of the PBE at various concentrations in human lymphocytes *in vitro* by sister chromatid exchange (SCE) assay.

Materials and Methods

2.1 Materials

PBE was prepared from five dried herb materials which are *P. chaba* fruit, *P. sarmentosum* root, *P. interruptum* stem, *P. indica* root and *Z. officinale* rhizome. All these herbs were collected and identified by the herbarium unit of the Department of Forestry, Ministry of Agriculture, Thailand. The herbarium vouchers were shown in Table 1.

Table 1 Sources of plant materials in Pikutbenjakul

Plants in Pikutbenjakul	Part of plants	Voucher number	Province	Part of Thailand
Piper Chaba Linn.	Fruit	SKP 146160301	Chonburi	East
Piper sarmentosum Roxb	Root	SKP 146161901	Ratchaburi	Middle
Piper interruptum Opiz.	Stem	SKP 146160901	Sakonnakorn	Northeast
Plumbago indica Linn.	Root	SKP 148160901	Bangkok	Middle
Zingiber officinale Roscoe.	Rhizome	SKP 206261561	Pechaboon	Northeast

2.2 Preparation of PBE

All ground herb materials (100 g each) were mixed and percolated with 95% ethanol for three days. Percolated extract was filtered and dried under the reduced pressure. The dried material or PBE was kept at -20°C and dissolved in DMSO before use.

2.3 Sister chromatid exchange (SCE) assay

The lymphocyte-enriched buffy coat was cultured in 5 ml culture medium containing RPMI 1640 (Hyclone, U.S.A.), fetal bovine serum (Hyclone, U.S.A.), autologous plasma, penicillin-streptomycin (Seromed, Germany), phytohemagglutinin (Seromed, Germany) and L-glutamine

(Hyclone, U.S.A.) using standard blood culture conditions as previously described[19]. The present assay had been approved by the institutional ethical committee of Faculty of Medicine, Thammasat University before running the experiment. At 24 hours after initiation of the culture, the lymphocyte cultures were centrifuged for packed cells, and the supernatant medium was removed and saved for reuse after treatment. The remaining lymphocytes were treated with PBE at concentrations of 50, 100, 200, 400, and 500 μg/ml in plain RPMI 1640 culture medium for three hours at 37^{O}C. After treatment, all cultures were centrifuged and the treated lymphocytes were continued to be cultured at 37^{O}C in the dark with the previously saved medium. Bromodeoxyuridine (BrdU) (Sigma-aldrich, U.S.A.) was then added in the culture medium to give the final concentration of 5 μM. Doxorubicin (0.1 μg/ml) (Roche, Switzerland) was used as the positive control. Plain RPMI 1640 and 0.4% (V/V) DMSO were used as the negative controls. At seventy-seven hours after initiation, cells were harvested and prepared the treated and non-treated cells on slides. These prepared slides were stained with the Fluorescent plus Giemsa technique according to the standard protocol[19]. Twenty-five cells per dose per experiment showing the second metaphase-staining pattern were scored from coded slides for the frequencies of SCEs. Proliferation index (PI) and mitotic index (MI) were also evaluated to clarify the cytotoxicity. PI was determined as (MI+2MII+3MIII)/100 cells, and MI was determined as number of mitotic cells/1,000 cells. Three independent experiments were performed for each concentration of the tested compounds.

2.4 Statistical analysis

Raw data obtained from the SCE assay were transformed to stabilize the variance by the procedures of Whorton et al. (1984)[20]. Dunnett's t-test was performed to analyze the difference between the means of the treated and control groups using the transformed data.

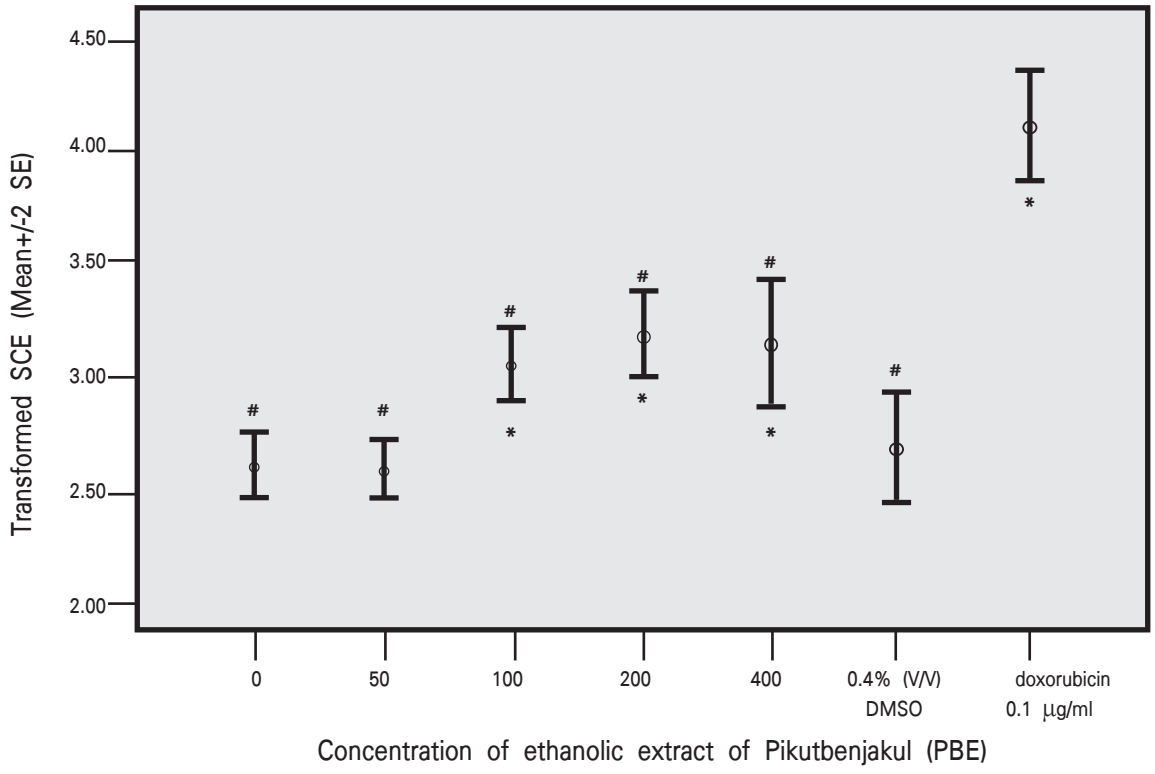
Results

3.1 The PBE Preparation

All five studied Thai plant materials were collected from five provinces of the country. These herbal vouchers, identified by Department of Forestry, Ministry of Agriculture, Thailand, are shown in Table 1. The yield of PBE was 11.1% (w/w) determined from 100g each dried plant material used for Pikutbenjakul preparation.

3.2 Genotoxic activity determined by sister chromatid exchange assay

Figure 1 showed that only the PBE at 50 μg/ml did not significantly induce SCEs above that of the negative controls (both plain RPMI 1640 and 0.4% DMSO treated) while PBE at other concentrations i.e. 100, 200, and 400 μg/ml could significantly increase SCEs ($p<0.05$). PBE at the highest concentration, 500 μg/ml, induced potent cytotoxic to the cells since only few mitotic cells were seen under microscopic observation.



* p<0.05 significantly different from the RPMI1640-treated negative control

\# p<0.05 significantly different from the doxorubicin-treated positive control

Figure 1 Sister chromatid exchanges induced by various concentrations of ethanolic extract of pikutbenjakul (PBE)

3.3 Cytotoxic activity determined by MI and PI values

The MI and PI of PBE treatments at concentrations of 50-400 μg/ml on cultured human lymphocytes tended to increase above those from both negative controls as shown in Table 2, although these values were not significantly different. The MI and PI of 0.4% DMSO treatment were lower than that of RPMI 1640 treatment.

Table 2 The mitotic index (MI) and proliferation index (PI) of PBE treatments

Concentration of PBE (μg/ml)	MI±SE	PI±SE
0	12.9 ± 1.0	2.8 ± 0.3
50	21.3 ± 3.6	4.2 ± 0.5
100	16.1 ± 6.8	3.5 ± 1.7
200	20.1 ± 6.4	3.7 ± 1.3
400	20.0 ± 10.2	3.8 ± 2.8
500	Toxic	Toxic
0.4% (V/V) DMSO	9.8 ± 3.9	1.9 ± 0.9
0.1 μg/ml doxorubicin	5.7 ± 1.7	1.1 ± 0.4

Discussion and Conclusion

The present study demonstrated that treatment of PBE at concentrations of 100-400 μg/ml significantly induced genotoxic effect in cultured human lymphocytes as shown by a significant increase of the frequencies of SCEs ($p<0.05$). This PBE-induced genotoxicity might be due to the effect of plumbagin. Plumbagin has been shown to be moderately mutagenic in exponential phase cells *E. coli* AQ634 cells and to induce DNA damage in mouse lymphoma L5178Y cells[7,21]. However, further investigation of the underlying mechanism of action is needed.

Findings that treatment of PBE at 50 μg/ml did not increase SCE level indicated that PBE at this concentration and below was not genotoxic. Interestingly, PBE at all tested concentrations except at 500 μg/ml caused some stimulating effect on cell proliferation as shown by a slight increase of their MI and PI values, in contrast, they were not significantly different from those of the control. PBE at 500 μg/ml showed potent cytotoxicity.

These data confirmed that PBE at the concentration of ≤50 μg/ml was not toxic to human lymphocytes and thus should be safe to use as the adaptogen. Still, to use as the chemotherapeutic agent, the higher dose that induces genotoxicity is preferred. According to the genotoxic potential with slight enhancing effect on cell proliferation, PBE tends to be useful for both cancer chemotherapy and an adaptogen in enhancing cell physiology. Nonetheless, the mechanisms of action as well as the study *in vivo* to determine the effective dose and safety use in human of PBE should be further investigated. In addition, individual genetic polymorphism, genetic susceptibility and metabolism are also of concern because these factors might interfere with the efficacy of PBE. Unavoidably the stability of the active compounds in PBE has to be clarified. We showed that piperine and plumbagin were unstable and reduced to 85% and 26% in 120 days after being kept in a refrigerator[12].

In conclusion, the genotoxic potency with slight enhancing cell proliferation of PBE is valuable but more studies are needed to ensure the efficacy and safety of PBE as the chemotherapeutic agents and the adaptogen. Mechanism of actions; *in vivo* experiments, stability, specifications for quality controls, and some factors that may influence of the activity of PBE, are areas to be explored in the future.

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บทคัดย่อ

ฤทธิ์ความเป็นพิษในระดับจีนและเซลล์ของสารสกัดเอทานอลจากพิกัดเบญจกูลต่อเม็ดเลือดขาวของมนุษย์ในหลอดทดลอง

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พิกัดเบญจกูลเป็นยาปรับธาตุนิยมใช้กับผู้ป่วยโรคมะเร็ง การศึกษานี้ทดสอบพิษระดับจีนและระดับเซลล์ของสารสกัดเอทานอลของพิกัดเบญจกูล (PBE) ในเม็ดเลือดขาวของมนุษย์ในหลอดทดลองด้วยวิธีซิสเตอร์โครมาติดเอ็กซเช้นจ์ (SCE) แอสเสย์ พิกัดเบญจกูลหมักด้วยร้อยละ ๙๕ เอทานอล เป็นเวลา ๓ วัน กรองและทำให้แห้งได้เป็นสารสกัด PBE ทดสอบ PBE (ละลายใน DMSO) ที่ความเข้มข้น ๕๐, ๑๐๐, ๒๐๐, ๔๐๐ และ ๕๐๐ ไมโครกรัม/มิลลิลิตร กับเม็ดเลือดขาวของมนุษย์เป็นเวลา ๓ ชั่วโมง อ่านและวิเคราะห์ค่า SCE โดยมี RPMI 1640 และร้อยละ ๐.๔ (V/V) DMSO เป็นกลุ่มควบคุมผลลบ และดอกโซรูบิซิน (๐.๑ ไมโครกรัม/มิลลิลิตร) เป็นกลุ่มควบคุมผลบวก ผลการทดลองพบว่า PBE ≥ ๕๐๐ ไมโครกรัม/มิลลิลิตร เป็นพิษระดับเซลล์ โดยพบเซลล์ระยะไมโทซิสน้อยมาก PBE ที่ ๑๐๐, ๒๐๐ และ ๔๐๐ ไมโครกรัม/มิลลิลิตร กระตุ้นระดับ SCE สูงกว่ากลุ่มควบคุมผลลบอย่างมีนัยสำคัญ (ค่าพี < ๐.๐๕) ดัชนีการแบ่งเซลล์ (MI) และดัชนีการเพิ่มจำนวนเซลล์ (PI) ไม่ต่างจากกลุ่มควบคุม ผลการทดลองแสดงว่า PBE เป็นพิษต่อเม็ดเลือดขาวของมนุษย์ในระดับจีน และระดับเซลล์ความเข้มข้นที่ ≥ ๑๐๐ ไมโครกรัม/มิลลิลิตร และ ≥ ๕๐๐ ไมโครกรัม/มิลลิลิตร ตามลำดับ ความเป็นพิษของ PBE น่าจะมาจากสาร plumbagin ที่เป็นองค์ประกอบสำคัญของ PBE ระดับปลอดภัยของ PBE คือ ≤ ๕๐ ไมโครกรัม/มิลลิลิตร อย่างไรก็ตามการศึกษาในสิ่งมีชีวิตในขั้นต่อไปเป็นสิ่งจำเป็น เพื่อบ่งชี้ความเข้มข้นที่ปลอดภัยในผู้ป่วยโรคมะเร็ง

คำสำคัญ: พิกัดเบญจกูล, ซิสเตอร์โครมาติดเอ็กซเช้นจ์, ดอกโซรูบิซิน, พิษระดับจีน, พิษระดับเซลล์