RESEARCH ARTICLE

Genotoxicity Evaluation of Ethanolic Leaf Extract of Thai Perilla (*Perilla frutescens* (L.) Britt.) using Micronucleus Assay in V79 Cell Line

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Abstract

The leaves of *Perilla frutescens* (PF) (Nga-Kee-Mon) have been widely used as a functional food and a traditional medicine for various therapeutic applications. This plant possesses many biological activities such as antioxidant, antibacterial, anti-inflammatory, and anticancer properties. However, safety information, especially toxicity studies are very limited. This study aimed to investigate the genotoxic effect of PF leaf extract on fibroblast V79 cell line using micronucleus (MN) test. The cytokinesis-block proliferation index (CBPI), percent cytostasis and MN frequencies were determined in V79 cells treated with the extract (100-250 µg/mL). The results showed that in the test condition without S9 enzyme fraction, the extract at the highest concentration (250 µg/mL) exhibited a higher cytotoxic effect than that with S9, as shown in percent cytostasis of the cells (57.47% and 34.00%, respectively). However, PF extract at all concentrations, with or without S9, did not significantly increase the MN frequency of the cells when compared to the negative control. The results suggested that PF leaf extract had neither direct nor indirect (metabolite form) genotoxic effect on V79 cells, thus enabling its use as a safe food supplement and medicine. Nevertheless, subchronic and chronic toxicity studies in animals should be further performed to provide more information regarding its long-term safety.

Keywords: *Perilla frutescens* leaf, micronucleus, genotoxicity, cytotoxicity, V79 cells

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การศึกษาพิษต่อสารพันธุกรรมของสารสกัดเอทานอลจากใบงาขี้ม้อน (*Perilla frutescens* (L.) Britt.) โดยการทดสอบไมโครนิวเคลียสในเซลล์ V79

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บทคัดย่อ

ใบงาขี้ม้อน (*Perilla frutescens*) ถูกนำมาใช้เป็นอาหารและยาสมุนไพรอย่างแพร่หลาย มีฤทธิ์ทางชีวภาพหลายอย่าง เช่น ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านการอักเสบ และฤทธิ์ต้านมะเร็ง เป็นต้น อย่างไรก็ตาม ข้อมูลความปลอดภัยโดยเฉพาะการทดสอบพิษของพืชชนิดนี้ยังมีน้อยมาก การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาพิษต่อสารพันธุกรรมของสารสกัดจากใบงาขี้ม้อนในเซลล์ไฟโบรบลาส V79 โดยการทดสอบไมโครนิวเคลียส (MN) โดยประเมินหาค่าดัชนีการยับยั้งการแบ่งตัวของเซลล์ (CBPI) ร้อยละการยับยั้งการแบ่งเซลล์ (cytostasis) และความถี่ของการเกิด MN ในเซลล์ V79 ที่ได้รับสารสกัดจากใบงาขี้ม้อน (100-250 มคก./มล.) ผลการศึกษาพบว่า ในสภาวะที่ไม่เหนี่ยวนำด้วยเอนไซม์ S9 สารสกัดที่ความเข้มข้นสูงสุด (250 มคก./มล.) มีความเป็นพิษต่อเซลล์มากกว่าในสภาวะที่เหนี่ยวนำด้วย S9 โดยมีค่าร้อยละการยับยั้งการแบ่งเซลล์เท่ากับ 57.47 และ 34.00 ตามลำดับ แต่สารสกัดทุกความเข้มข้นทั้งในสภาวะที่มีและไม่มี S9 ไม่เพิ่มความถี่ของการเกิด MN อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุมลบ แสดงให้เห็นว่าสารสกัดจากใบงาขี้ม้อนไม่มีพิษทั้งโดยตรงและโดยอ้อม (รูปเมแทบอไลต์) ต่อสารพันธุกรรมในเซลล์ V79 ทำให้สามารถนำไปใช้เป็นอาหารเสริมหรือยาที่ปลอดภัย อย่างไรก็ตาม ควรทำการทดสอบพิษกึ่งเรื้อรังและพิษเรื้อรังในสัตว์ทดลอง เพื่อให้มีข้อมูลเพิ่มเติมเกี่ยวกับความปลอดภัยในระยะยาวต่อไป

คำสำคัญ: ใบงาขี้ม้อน, ไมโครนิวเคลียส, พิษต่อสารพันธุกรรม, พิษต่อเซลล์, เซลล์ V79

Introduction

Perilla frutescens (L.) Britt. (PF), commonly called Perilla, is a medicinal plant in the Lamiaceae family widely cultivated throughout Asian countries. In Thailand, PF is well known as Nga-Kee-Mon or Nga-Mon.[1] All parts of the plant have been widely used as a traditional medicine and a functional food in Asian communities.[2] The leaves of the PF species are listed as herbal medicines for many therapeutic applications and they are also used as a culinary herb and food colorant.[3] In addition, this plant is becoming more important in dermatologic and cosmetic uses as an ingredient for cosmetics formulations, skin creams and dermatological medicinal preparations.[4,5]

Recent reports have shown that PF leaves contain high quantities of phenolic acids, flavonoids, anthocyanin, volatile or essential oils and triterpene compounds, which provide health benefits.[5] Various biological activities of PF leaves have been reported including antioxidant, antibacterial, anti-inflammatory and anticancer activities. Many studies have demonstrated that the leaf extracts of PF possessed a protective effect on the oxidation of low-density lipoprotein (LDL) cholesterol[6] and promoted cellular resistance to oxidative damage through activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element pathway.[7] The essential oil from PF leaves exhibited antibacterial activity to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli*.[8] The ethanolic leaf extract of PF presented significant anti-inflammatory activity via inhibiting the pro-inflammatory cytokine expression, mitogen-activated protein kinase (MAPK) activation, and nuclear factor-kappa B (NF-κB) nuclear translocation in lipopolysaccharide-induced RAW 264.7 macrophages.[9] Luteolin isolated from the ethanolic extract of PF leaves also exhibited an anti-inflammatory effect in BV-2 microglial cells through inhibition of the expression of iNOS, thus exerting beneficial effects on neuro-inflammatory diseases.[10] Lipophilic triterpene acids and tormentic acid isolated from ethanolic leaf extracts of red perilla and green perilla demonstrated significant anti-inflammatory and antitumor-promoting effects in 12-*O*-tetradecanoylphorbol-13-acetate-treated mice, respectively.[11] Moreover, PF leaf extracts also showed potential anticancer activities in both *in vitro* and *in vivo* systems. The aqueous extract of PF leaves was able to induce the expression of apoptosis-related genes and inhibit human hepatoma HepG2 cell proliferation.[12] The 70% ethanolic extract of PF leaves was reported to inhibit MDA-MB-231 human breast carcinoma cell migration and invasion by decreasing the secretion and activity of extracellular matrix degrading enzymes, and by blocking cancer cell invasiveness.[13] The extract also acted as a chemopreventive agent for colon cancer by inducing apoptosis and inhibiting the proliferation of colonic crypt cells, thereby reducing the aberrant crypt foci formation in 1,2-dimethyl-hydrazine-treated rats.[14] Interestingly, this extract was rich in rosmarinic acid that was capable of protecting the stomach against gastric ulcers by decreasing mucosal ulcer, inflammatory infiltration, and degenerative lining cells in indomethacin-treated rats.[15]

As previously reviewed, although a number of *in vitro* and *in vivo* studies have revealed various potential biological activities of PF leaves, there is very limited information about its safety, especially genotoxicity. The study of DNA damage at the chromosome level is an essential part of genetic toxicology.[16] A chemical agent that

damages genetic materials is referred to a genotoxic agent.[17] Micronucleus (MN) assay has been developed as one of the preferred methods for assessing chromosome damages caused by a variety of genotoxic agents.[16] MN is a tiny nuclear body enclosed by its nuclear membrane and resembles the morphology of its daughter nucleus. It is originated from acentric chromatid/chromosome fragments or whole chromatids/chromosomes that fail to migrate to the poles during nuclear division. The formation of MN can be initiated by defects in the cell repair machinery, DNA damage accumulation and chromosomal aberrations.[18] The technique is based on the principle of detecting MN formation in binucleated cells by using cytochalasin B, a microfilament-assembly inhibitor, to block spindle assembly and prevent cytokinesis that occurs after nuclear division.[19,20] As a result, the assessment of chromosome defects or any aberration induced by a genotoxic agent can be recognized at this stage.[21]

Therefore, to provide more safety information on PF leaves, the purpose of this study was to investigate the genotoxic effect of ethanolic PF leaf extract on lung fibroblast V79 cell line using a standard micronucleus assay.

Materials and Methods

Plant materials and extract preparation

The leaf extract of PF was prepared as previously described by Pintha et al.[13] PF with voucher number QSBG-K2, certified by the Queen Sirikit Botanic Garden Herbarium, Chiang Mai, Thailand, were collected from Wiang-Sa district, Nan province, Thailand. In brief, the dried leaves (100 g) were ground and soaked in 1 L of 70% (v/v) ethanol with continuous shaking (150 rpm) overnight at room temperature. The extraction was performed twice. The supernatant was concentrated using a vacuum rotary evaporator and then freeze-dried and kept at -20°C until use. The extract was dissolved in dimethyl sulfoxide (DMSO) to the concentration of 100 mg/mL as a stock solution before use.

Cell culture

Fibroblast cell line (V79-4) from lung Chinese hamster were obtained from the American Type Culture Collection (ATCC® CCL-93, Manassas, VA, USA). The cells were maintained in 5 mL Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, France) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Germany) and 1% penicillin/streptomycin (Invitrogen, France) in 25 cm^2 tissue-culture flasks and allowed to attach and grow at 37°C in a 5% CO_2 incubator.

Cell viability

Cell viability of V79 cells treated with PF leaf extract was determined using trypan blue exclusion method. The cells (1×10^5 cells/mL) were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin and incubated at 37°C in a 5% CO_2 incubator for 24 h. Subsequently, the cells were exposed to the extracts at various concentrations ranging from 1 to 500 µg/mL for 3 h, then washed with PBS pH 7.4 and incubated in fresh medium for 24 h. The cells were then harvested by trypsinization and immediately mixed with trypan blue solution (with the dilution factor of 2). The number of viable cells and dead cells were counted under a light

microscope and the percentage of cell viability was calculated. 1% DMSO (v/v) was used as a solvent (negative) control.

In vitro MN assay

The V79 cells (5×10^5 cells/mL) (Figure 1A) were grown in DMEM medium containing 10% FBS and 1% penicillin/streptomycin and incubated at 37˚C in a 5% CO_2 incubator for 24 h. Then the cells were treated with PF leaf extracts at concentrations of 100, 150, 200, and 250 µg/mL for 3 h. Next, the cultures were replaced with fresh media and incubated for 3 h when 3 µg/mL of cytochalasin B was added to the media as a mitotic division blocker. After a further 24 h of incubation, the treated cells were harvested by trypsinization and prepared on slides using a cytospin technique. The cells were subsequently fixed with ice cold methanol and incubated on ice for 30 min, following by being dried in air. Then the slides were stained for 30 min with a freshly prepared 5% Giemsa stain solution (1:10 dilution by adding 10 mL of stock solution of Giemsa stain to 80 mL of distilled water and 10 mL of methanol) (Figure 1B). The assays were performed both with and without the metabolic enzyme (S9 fraction) (purchased from Department of Biochemistry, Faculty of Medicine, Chiangmai University). The final concentration of S9 mixed in the treatment medium was 5% (v/v). 1% DMSO (v/v) was used as solvent (negative) control and cyclophosphamide (CP) 20 µg/mL and mitomycin C (MMC) 0.1 µg/mL as the positive controls for assays with S9 (+S9) and without S9 (-S9), respectively.

MN was identified according to the following criteria: rounded shape, surrounded by a nuclear membrane and occupying the cytoplasm, no link or overlap the main nucleus, diameter less than ½ of diameter of its main nucleus and similar staining pattern as the main nucleus (Figure 1C).[22] The number of MN formation in binucleated cells in each treatment group was scored under a light microscope (×1000 magnification) and the data was expressed as the number of micronuclei per 1000 binucleated cells.

Cytokinesis-block proliferation index (CBPI) and cytotoxicity evaluation

The impact of the test compound on V79 cell proliferation from the micronucleus assay was also determined by considering its cytotoxicity as expressed by the CBPI and percent cytostasis. According to the previous description in Himakoun et al[23], a minimum of 400 cells per treated group were scored for the incidence of mononucleated, binucleated and tri or more nucleated cells under a light microscope (×400 magnification) (Figure 1D). The CBPI indicates the number of cell cycles per cell during the period of exposure to cytochalasin B that was calculated from the following formula:

CBPI = (mononucleated cells + 2× binucleated cells + 3× tri or more nucleated cells) / total number of the cells scored

$$\text{Cytostasis (\%)} = 100 - 100\,[(CBPI_T - 1)/(CBPI_C - 1)]$$

when T = Test chemical
C = Negative control

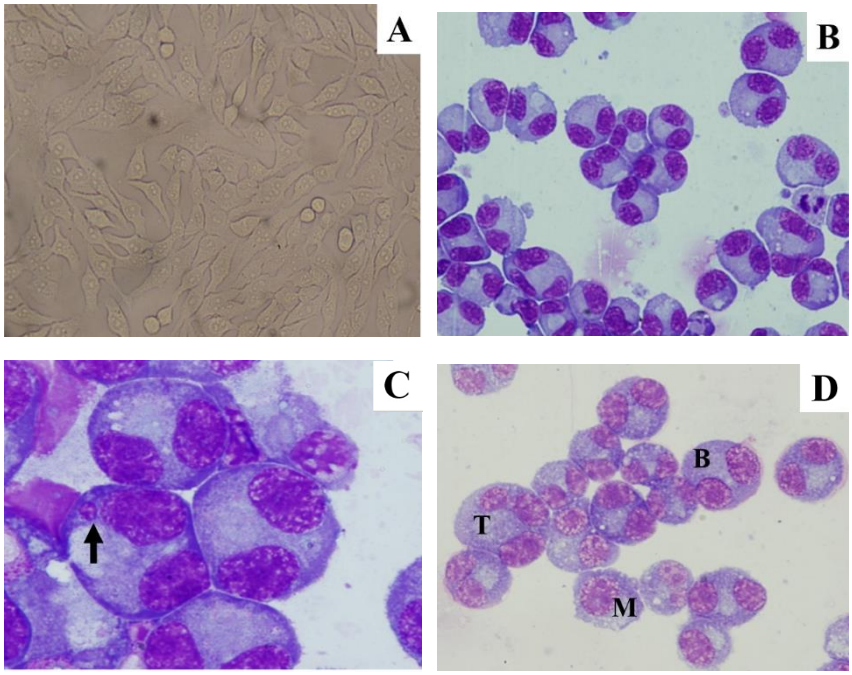

Figure 1. Characteristics of lung fibroblast V79 cells and micronucleus formation. (A) a monolayer of untreated V79 cells (×100); (B) cytochalasin B treated V79 cells after staining with Giemsa (×200); (C) a micronucleus in a binucleated cell (arrow) (×1000); and (D) V79 cells with a mononucleated cell (M), a binucleated cell (B), and a trinucleated cell (T) (×400).

Statistical analysis

All experiments were performed in triplicates and repeated twice. All results were expressed as mean±standard deviation (SD). The differences in the MN frequencies among groups were assessed using one-way analysis of variance (ANOVA). The data were analyzed using SPSS version 16 statistical software program (IBM Corp, Armonk, NY, USA). Statistical significance was considered as $p<0.05$.

Results

Cytotoxicity of PF leaf extract on V79 cells

The cytotoxicity of PF leaf extract on V79 cells treated with cytochalasin B was summarized in Table 1. Under -S9 activation conditions, the CBPI of PF leaf extract at the concentrations of 100, 150, 200 and 250 µg/mL were 1.80±0.06, 1.70±0.04, 1.52±0.09 and 1.37±0.13, respectively. At the concentrations of 200 and 250 µg/mL, the CBPI were significantly lower than that of negative control group (1.87±0.02; $p<0.05$), but comparable to that of the positive (MMC treated) control (1.45±0.08). Also, under +S9 activation condition, the PF leaf extract at the highest concentration (250 µg/mL) showed a significant decrease in CBPI when compared to the negative control (1.66±0.19 *vs.* 1.99±0.01; $p<0.05$). A high concentration of

PF leaf extract (250 µg/mL) under -S9 activation condition was found to be cytotoxic to V79 cells as shown by increased cytostasis (57.47±4.11%), whereas, under +S9 activation condition, all PF extract groups had no significant changes in all aspects of cytostasis. However, PF leaf extracts at all tested concentrations did not induce cytotoxicity on V79 cells, as presented by cell viability of more than 90% in both under -S9 and +S9 activation conditions.

Table 1. Cytotoxic effect of PF leaf extract on V79 cell line under with and without S9 metabolic activation.

Test group	Conc (µg/mL)	CBPI (mean±SD)		%Cytostasis (mean±SD)		%Cell viability (mean±SD)	
		- S9	+ S9	- S9	+ S9	- S9	+ S9
DMSO	-	1.87±0.02	1.99±0.01	0.00±0.00	0.00±0.00	97.74±1.21	97.98±0.43
PF	100	1.80±0.06	1.93±0.02	8.05±4.59	7.00±0.48	98.87±0.95	98.42±0.61
	150	1.70±0.04	1.86±1.10	19.54±9.68	14.00±13.57	97.19±1.87	98.80±0.63
	200	1.52±0.09*	1.78±0.12	40.23±1.68	22.00±13.57	98.40±0.61	97.96±1.16
	250	1.37±0.13*	1.66±0.19*	57.47±4.11[a]	34.00±14.99	95.05±6.70	97.78±1.02
MMC	0.1	1.45±0.08*	ND	48.27±0.50	ND	92.44±5.28	ND
CP	20	ND	1.16±0.01*	ND	84.00±1.19[a]	ND	97.48±1.56

*Significant difference in comparison with negative control (1% DMSO) at $p < 0.05$, [a]Cytostasis increased higher than 50% compared to the negative control, PF: *P. frutescens* leaf extract, Conc: concentrations, CBPI: cytokinesis-block proliferation index, MMC: mitomycin C, CP: cyclophosphamide, -S9: without S9 metabolic activation, +S9: with S9 metabolic activation, ND: not done.

Genotoxic activity of PF leaf extract on V79 cells

PF leaf extract at the range of applied concentrations had no genotoxic effect on V79 cells both under -S9 and +S9 metabolic activation conditions as shown in Figure 2. Without S9 fraction, the numbers of MN per 1000 binucleated cells in PF leaf extract treated groups (100, 150, 200, 250 µg/mL) were 3.33±1.78, 2.67±0.78, 5.33±1.30 and 11.17±6.94, respectively, which showed no significant difference when compared to the negative control group (2.83±0.89 per 1000 cells). However, the MMC treated group showed a strongly increased MN frequency (81.33±7.33 per 1000 cells; $p<0.05$). The trend of MN formation in the cells treated with PF leaf extract under +S9 activation conditions was similar to those without S9. The MN frequencies of the extracts at concentrations of 100, 150, 200, 250 µg/mL were 4.67±1.33, 4.17±1.17, 5.67±1.78 and 5.83±3.78 per 1000 cells, respectively, which was not different from the negative control group (5.33±1.33 per 1000 cells), but was significantly different from the positive control (CP) group (89.83±14.44 per 1000 cells; $p<0.05$).

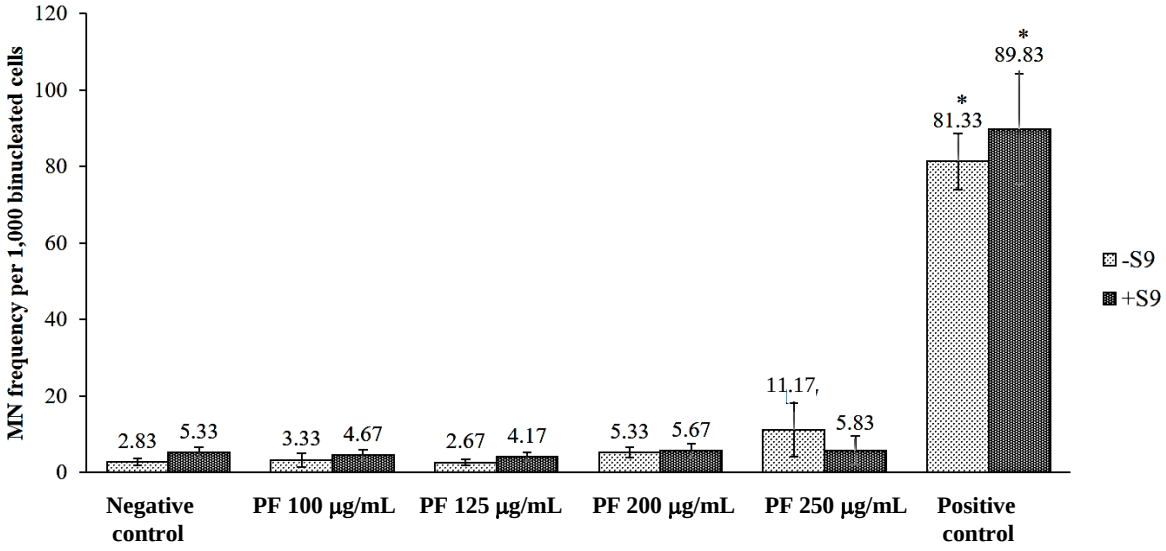

Figure 2. The effect of PF leaf extract on micronucleus formation in V79 cells with and without S9 metabolic activation.

*Significant difference in comparison with solvent (negative) control (1% DMSO) (p<0.05), Positive control groups: MMC 0.1 µg/mL and CP 20 µg/mL for -S9 and +S9 metabolic activating conditions, PF: *P. frutescens* leaf extract.

Discussion

New compounds or herbs intended for use as human food or drug must essentially be screened for toxicity. Leaves of PF have been used as food and for various herbal drugs, but relevant toxicity studies have been very limited.[4,5] MN test is one of standard tests widely used for toxicological screening the potential genotoxic compounds. The MN formation is used as a biomarker for DNA damaging capacity of the test compound. In the present study, possible genotoxicity of 70% ethanolic extract of PF leaves was evaluated using *in vitro* micronucleus assay in V79 cells that is sensitive to mutagens, represents a defined karyotype (diploid or near-diploid) and stable low background frequency of MN.[20]

In our preliminary study, the screening for cytotoxic effect of PF leaf extracts (1-500 µg/mL) on V79 cells was performed using a trypan blue exclusion method. The extract at concentrations higher than 250 µg/mL induced more toxic effect, resulting in increasing cell death (>50%) (data not shown). The trypan blue exclusion test of cell viability is one of acute cytotoxicity test recommended for cytotoxicity measurements for *in vitro* micronucleus assay.[24] This method can be used for chemo-sensitivity testing in various mammalian cells both in suspension and monolayer cultures.[25] The method is a good indicator of membrane integrity and is capable of detecting cell kill in nondividing cell populations under a light microscope.[26] Although there is a controversy and limitation that this method cannot distinguish between healthy cells and living cells that are losing cell functions.[27] This may cause an underestimate of cell death when the results of the assay are expressed as percent viability.[28,29] However, a study by Yamamoto et al[30] on the relationship between the

number of viable cells detected using MTT formazan and trypan blue dye exclusion methods, the two most common cytotoxicity tests for *in vitro* micronucleus assay, showed a high correlation (r = 0.9944). This supports the use of trypan blue exclusion method for screening cytotoxicity of the cells for *in vitro* micronucleus assay.

According to the original test guideline by OECD[20], the test concentrations selected should cover a range from that producing 50±5% cytotoxicity, to little or no cytotoxicity. However, only trypan blue exclusion method may not be sufficient as discussed above. Thus, in this study the determinations of CBPI and cytostasis were also performed to establish the appropriate concentration range of the extract for *in vitro* micronucleus assay. The CBPI indicates the average number of cell cycles per cell during the period of exposure to cytochalasin B that should be in the normal range 1.5-2 cycles. A decrease in the CBPI by comparing to the negative control demonstrates cell proliferation inhibition.[31] Non-cytotoxic tested compounds should not have a percent cytostasis of greater than 50%.[32] In this study, as shown in Table 1, the PF leaf extract under the -S9 condition at high concentrations (>200 µg/mL) significantly decreased the CBPI value when compared to negative control (p<0.05). In particular, at the concentration of 250 µg/mL, the CBPI was lower than 1.5 (1.37±0.13) whereas CBPI values of the extracts at the lower concentrations (100 and 150 µg/mL) were in the same level as that of the negative control. Furthermore, the PF leaf extract at 250 µg/mL also increased cytostasis (57.47±4.11%), indicating cytotoxic effect. However, under +S9 activation condition, the PF leaf extract did not affect CBPI and cytostasis at all concentrations. As a result, the concentrations ranging from 100 to 250 µg/mL of PF leaf extracts were chosen for MN evaluation. However, all treatments that achieved the highest concentration of PF leaf extracts in the genotoxic experiment were non-toxic to the V79 cells (percent cell viability >90%).

For genotoxic evaluation, we considered MN formation that occurs during nuclear division after being exposed to the mutagens. Therefore, a compound that induces a significant MN formation can be referred as a genotoxic agent. Exposure to genotoxic agents may have led to cell death, genomic instability, or cancer development.[18] The results of our study, as shown in Figure 2, showed that the MN frequencies of all PF leaf extract treated groups were not significantly different from the negative control in both +S9 and -S9 activation conditions. However, the positive controls (MMC and CP) showed significantly increased MN frequencies (p<0.05). The highest concentration (250 µg/mL) of PF extract in -S9 condition showed the highest increase in MN frequency when compared to other treatment groups, yet not significant. A clear positive result of the test compound can be considered when in any of the experimental conditions examined, at least one of the test concentrations exhibits a statistically significant increase compared to the concurrent negative control.[20] Thus, our finding suggested that the direct form and the metabolic product of ethanolic PF leaf extracts did not cause chromosomal damage as they did not exhibit any genotoxic effect on the V79 cells confirmed by the *in vitro* MN assay. Furthermore, the metabolite form of the extract had trend to reduce the cytotoxic effect on the V79 cells when compared to the direct form of the extract.

Conclusion

The ethanolic extract of PF leaves exhibited neither direct nor indirect genotoxic properties. Thus, the use of PF leaves as a food supplement, oral drug or for external use as skin cream should be safe. However, plant concentration and duration of application (exposure time) are important factors for safety consideration. In addition, subchronic and chronic toxicity tests in animal experiments should be further investigated for long-term safety.

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