Original Article

Anticlastogenic Effect of Asiatic Pennywort and Indian Mulberry using Rodent Erythrocyte Micronucleus Assay

Piengchai Kupradinun[1*] Anong Tepsuwan[2] Wannee R. Kusamran[2†]

Abstract

Asiatic pennywort [*Centella asiatica* (L.), Urban, AP] and Indian mulberry (*Morinda citrifolia* Linn., IM), the Asian plants, have been previously demonstrated in our laboratory that they could markedly enhance the activities of some phase II enzymes, but significantly decreased the activities of phase I enzymes, indicating that they may possess cancer chemopreventive potentials. This study was to investigate the anticlastogenic activity of AP and IM against a direct-acting clastogen, mitomycin C (MMC), and indirect-acting clastogens, cyclophosphamide (CYP) and 7, 12-dimethylbenz(a)anthracene (DMBA). Male mice were fed with AP or IM leaves mixing in modified AIN -76 semi-purified diet, or IM fruit juice or fruit powder solution by gavage, for 2 weeks prior to administration of clastogens.

Anticlastogenic effect was determined by using *in vivo* erythrocytes micronucleus assay. Blood samples were collected and counted for reticulocytes with and without a micronucleus using the fluorescent microscope. After comparing with the controls, we found that 25% of AP and IM leaves in diets significant decrease micronucleated peripheral reticulocytes (MNRETs) induced by MMC, CYP and MMC, DMBA, respectively (p<0.05). While IM fruit juice at 10 and 20 ml/kg BW caused a significant decrease in MNRETs induced only by MMC. IM fruit powder at 100 and 500 mg/kg BW decreased MNRETs induced only by MMC in a dose dependent manner, however the significant decrease was found only in the high dose (p<0.05). These results demonstrated that AP leaves and IM (leaves and fruits) were able to inhibit the clastogenic activity of both direct and indirect-acting clastogens in the mouse, particularly IM leaves which showed the highest inhibitory effect.

Keywords: anticlastogenic, Asiatic pennywort, Indian mulberry, micronucleus, MNRETs, mouse

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บทคัดย่อ

ผลการยับยั้งการเกิดไมโครนิวเคลียสของบัวบก และยอในเม็ดเลือดแดงของหนูทดลอง

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บัวบก [*Centella asiatica* (L.), Urban] และยอ (*Morinda citrifolia* Linn.) เป็นพืชประจำท้องถิ่นในเอเชีย งานวิจัยของสถาบันมะเร็งแห่งชาติที่ผ่านมาพบว่า พืชเหล่านี้สามารถกระตุ้นเอนไซม์ในกระบวนการ biotransformation ใน Phase II แต่ยับยั้งเอนไซม์ใน phase I ซึ่งแสดงว่าพืชเหล่านี้มีสารเคมีที่มีศักยภาพในการป้องกันมะเร็ง การศึกษานี้มีจุดประสงค์เพื่อ ตรวจสอบการยับยั้งการเกิดไมโครนิวเคลียสของเม็ดเลือดแดงที่ชักนำด้วยสารก่อมะเร็งที่ออกฤทธิ์โดยตรง (Mitomycin C, MMC) และชนิดที่ออกฤทธิ์ทางอ้อม (Cyclophosphamide, CYP และ 7,12-dimethylbenz(a)anthracene, DMBA) โดยการให้หนูเม้าส์เพศผู้ กินใบบัวบก และยอ (ใบและผล) ที่ผสมในอาหารผงหรือโดยการป้อนทางปาก เป็นเวลา 2 สัปดาห์ก่อนให้สารก่อมะเร็ง ตรวจสอบการยับยั้งการเกิดไมโครนิวเคลียสด้วยวิธี *in vivo* erythrocyte micronucleus assay ผลการศึกษาพบว่าใบบัวบกที่ผสมในอาหารร้อยละ 25 สามารถลดจำนวนเม็ดเลือดแดงที่มีไมโครนิวเคลียส (MNRETs) ที่ชักนำด้วย MMC และ CYP อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม ($p<0.05$) ใบยอในขนาดร้อยละ 25 ในอาหารสามารถลดจำนวน MNRETs ที่ชักนำด้วย MMC และ DMBA ได้อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) น้ำลูกยอในขนาด 10 และ 20 มล./นน.ตัว 1 กก. สามารถลดจำนวน MNRETs ที่ชักนำด้วย MMC เท่านั้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ส่วนผงลูกยอในขนาด 100 และ 500 มก./นน.ตัว 1 กก. สามารถลดจำนวน MNRETs ที่ชักนำด้วย MMC ได้ตามขนาดที่เพิ่มขึ้น อย่างไรก็ตามพบความแตกต่างอย่างมีนัยสำคัญทางสถิติเฉพาะในขนาดสูงเท่านั้น ($p<0.05$) ผลการศึกษานี้แสดงว่าใบบัวบกและยอ (ใบ และผล) มีฤทธิ์ยับยั้งการเกิดไมโครนิวเคลียสที่ชักนำด้วยสารก่อมะเร็งทั้งชนิดที่ออกฤทธิ์โดยตรงและ ชนิดที่ออกฤทธิ์ทางอ้อมในหนูเม้าส์ โดยเฉพาะใบยอมีฤทธิ์ในการยับยั้งสูงที่สุด

คำสำคัญ: ใบบัวบก ใบยอ ลูกยอ ไมโครนิวเคลียส หนูเม้าส์

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Introduction

It has been estimated that more than two-thirds of human cancers can be prevented through appropriate lifestyle modification (Doll et al., 1981). The risk of some cancers may be reduced by consumption of various kinds of vegetables and fruits (Block et al., 1992). A number of vegetables and fruits contain various kinds of chemicals possessing chemopreventive potentials (Wattenberg, 1985). Chemopreventive agents may function by a variety of mechanisms such as directed at all major stages of carcinogenesis (Wattenberg, 1997); induction of phase II detoxification enzymes; the inhibition of phase I activating enzymes, as well as the inhibition of the mutagenicity/clastogenicity of chemical carcinogens (Talalay, 1992).

We have previously reported that some vegetables, for examples, neem flowers, sesbania flowers, Thai and Chinese bitter gourd fruits, leaves of sweet basil, Siamese cassia, ivy gourd, lemon grass and Indian mulberry, possess antimutagenic activity towards *Salmonella typhimurium* (Rojanapo et al., 1993; Kusamran et al., 1998[a]). Some of these vegetables including neem flowers, Asiatic pennywort leaves, Cassia leaves, Thai bitter gourd fruits, kale and Indian mulberry leaves can also increase the activity of some phase II detoxification enzymes while decreasing the activity of some phase I enzymes (Tepsuwan et al., 1997; Kusamran et al., 1998[b]). In addition, some of them can inhibit the clastogenicity of the clastogens in mice (Kupradinun et al., 1997; Kupradinun 2008) and inhibit some chemically induced carcinogenesis in rats (Kusamran et al., 1998[a]; Tepsuwan et al., 1999; 2002).

Asiatic pennywort (AP) is a tropical plant which is used for health supplement. This plant possesses biological activities such as antioxidation (Jayashree et al., 2003) and chemopreventive property against azoxymethane-induced aberrant crypt foci (ACF) formation in rat colon (Bunpo et al., 2004) and

induction of cell cycle arrest in colon cancer cell line (Caco-2 cells) (Bunpo et al., 2005). Indian mulberry (IM) leaves are commonly used in Thai cuisine while fruit juice is widely consumed as it has been claimed to possess anti-inflammatory and antitumor properties (Hirazumi et al., 1994; Hirazumi and Furusawa, 1999; Wang and Su, 2001; Furusawa et al., 2003).

The *in vivo* rodent micronucleus assay in bone marrow cells has been accepted for evaluation of the clastogenicity of chemical compounds (Matter and Schmid, 1973; Schmid, 1975). In 1980, using mouse peripheral blood instead of bone marrow cells was introduced for use in the micronucleus assay (MacGregor et al., 1980) and acridine orange has been used as supravital staining of blood cells (Hayashi et al., 1983). These modifications offer many advantages to conventional bone marrow assay and widely used to evaluate chemical clastogenicity. This technique has also been shown to be very useful as a short-term assay for evaluating the anticlastogenicity as well as chemopreventive potential of compounds (Heo et al., 1996; Kupradinun et al., 1997; Kupradinun, 2008; Hwan et al., 2008; Promkum et al., 2010).

In this study, we applied this technique to determine the anticlastogenicity of AP and IM which have been demonstrated to possess both antimutagenicity (Kusamran et al., 1998[a]) as well as chemopreventive potentials (Bunpo et al., 2004) against both direct-acting (mitomycin C, MMC) and indirect-acting clastogens (cyclophosphamide, CYP and/or DMBA). Anticlastogenic effect of AP and IM demonstrated in this study would lead to further research on its mechanism or modulating factors related to these effects. Moreover, it will give information whether this technique can be used to predict the chemopreventive potential of chemical compound in vegetables and medicinal plants.

Materials and Methods

Chemicals: MMC, a direct-acting clastogen was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan) while CYP and DMBA (indirect-acting clastogens) were obtained from ASTA Medica AG (Frankfurt am Main, Germany) and Sigma Chemicals (St. Louis, USA), respectively. All vitamins used for the preparation of vitamin mixture were obtained from Sigma Chemicals Co. (St. Louis, USA). Acridine orange (AO) was obtained from E. Merk (Germany). Chemicals used for the preparation of salt mixture were obtained from Fluka Chemicals Co. (Switzerland) and casein (EM HV milk protein) was the product of D.M.V. Co. (The Netherland).

Plant preparations: AP and IM leaves were purchased from local markets in Bangkok. They were washed with tap and distilled water, chopped into small pieces and then lyophilized. Freeze-dried vegetables were blended to powder and kept at -20°C until use. IM fruit juice was purchased from a supermarket in Bangkok while IM fruit powder was obtained from Abhaibhubejhr Hospital, Thailand. IM fruit powder was dissolved in distilled water under 40°-50°C before giving to the animals.

Animals and diets preparation: A total of 270 male ICR mice, 5 weeks old, aged 27±3 g, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were maintained at the Laboratory Animal Facility of the National Cancer Institute according to the Institutional Care Guidelines, which were approved by the Animal Ethic Committee. All animals were housed in shoes box stainless steel cages in an air-conditioned room at 23°±2°C and relative humidity 50±20% with 12 hours light/dark cycle. For each experiment, animals were acclimatized for 5-7 days by giving a modified AIN-76 semi-purified diet (basal diet) according to Bieri et al., 1997 and Reeves et al., 1993 or pellet diet (Perfect Companion Co. Ltd., Thailand) before starting the experiment. The vegetable diets were prepared by substituting the ground freeze-dried AP or IM leaves at 12.5% and 25% in a modified AIN-76 diets as previously described (Kusamran et al., 1998[a]).

Clastogenicity testings

After acclimation, the animals were randomly divided by weight into 3 groups of 8-10 mice each. One group was assigned as control group that continued to receive the basal or pellet diets, while the other two groups were assigned as experimental groups receiving basal diet containing low (12.5%) and high (25%) doses of ground freeze-dried AP/IM or 2 doses (10 and 20 ml/kg BW, approximately equivalent to 5 and 10 times of human consumption) of IM fruit juice and 100 and 500 mg/kg BW of IM fruit powder solutions (approximately equivalent to and 5 times of human consumption) daily by gavage for 2 weeks and continued till the end of the experiment. In groups receiving vegetable diets, both control and experimental groups were pair-fed and water *ad libitum*. For IM fruit juice and powder groups, the mice were given a pellet diet and the control group were given vehicle and distilled water, respectively by gavage. At 2 weeks after feeding the experimental diets or samples, blood samples were collected and subjected to micronucleus assay (Fig. 1) as previously described (Kupradinun, 2008). Briefly, 5-7 µl of mouse peripheral blood were placed on AO-coated glass slides and covered with 22x40 mm cover slip. After few hours, micronucleated peripheral reticulocytes (MNRETs) types I, II, and III as classified by Vander et al. (Vander et al., 1963) were counted under the fluorescent microscope (Olympus Model BH-2, Japan). The frequencies of MNRETs were recorded based on the observation of all 1000 reticulocytes per mouse.

Anticlastogenicity testings

A. *Anticlastogenicity testing against MMC*

MMC was intraperitoneally injected into mice that have been used previously for clastogenicity test at 1 mg/kg BW just after blood samples were collected. Then blood samples were collected at 24

and 48 hours after MMC injection and micronucleus formation were analyzed (Fig. 1).

B. *Anticlastogenicity testing against CYP and/or DMBA*

Two out of the 3 experimental groups for clastogenicity study were administered with CYP at 50 mg/kg BW (i.p) or DMBA at 40 mg/kg BW (in corn oil, p.o). Blood samples were collected at 24 and 48 hours after clastogens administration and analyzed for reticulocytes as in an experiment A (Fig. 1).

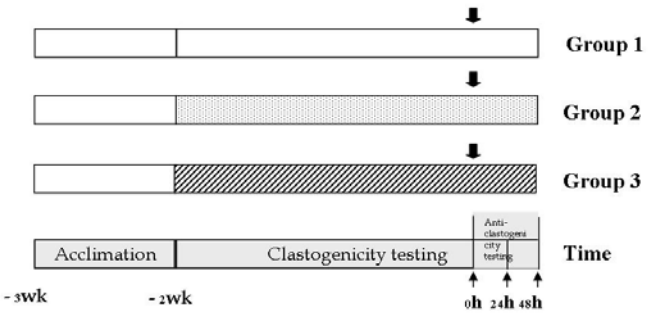

Figure 1 Schematic diagram of experimental design. Open bar was control group (Gr. 1). Dot bar and hatched bar were experimental groups (Grs. 2,3). Arrows: blood collection. Black arrows: clastogens: mitomycin C (MMC); cyclophosphamide (CYP); 7,12-dimethylbenz(a)anthracene (DMBA).

Statistical analysis: Significant difference in the frequencies of MNRETs between the experimental and control groups was analyzed using Kruskal-Wallis H and nonparametric Mann-Whitney U tests at $p<0.05$.

Results

Effect of AP and IM on the body weight and food consumption: Body weight and food consumption were daily recorded for the entire experiment. There were no significant differences in body weight and food consumption between the control and experimental groups (data not shown).

Effect of AP leaves on the micronucleus formation in mice peripheral blood: The effects of AP leaves on the micronucleus formation in male mice treated with a direct-acting clastogen, MMC and indirect-acting clastogens, CYP, and DMBA are shown in Fig. 2. The number of MNRETs in the control and experimental groups were not significantly different. The results showed that the frequency of MNRETs of mice in all groups increased with time-course and reached the maximum number at 48 hours after MMC treatment (Fig. 2A). Therefore, the percent inhibition was calculated to compare the inhibitory effect of these plants against clastogens induced MNRETs at 48 hours after treatment. MNRETs in mice fed with AP leaves decreased at 24 hours and 48 hours in both low and high dose groups, but were significantly different (p=0.049) from those of the control group only a high dose at 48 hours (Fig. 2A). Figure 2B shows MNRETs in mice induced by CYP. MNRETs decreased at 24 and 48 hours in mice fed with AP leaves at both low and high doses. The significant differences (p=0.002) from those of the control were recorded only in high dose group at both time points. While in low dose group, MNRETs significantly decreased only at 24 hours. The inhibitory effects at high and low doses of AP diets were 44% and 10%, respectively. Figure 2C shows MNRETs in mice induced by DMBA. It was found that the feeding of AP leaves resulted in the increase of MNRETs in the low dose group at both time points, which was significantly different (p= 0.045) from those of the control group at 24 hours. However, MNRETs of the high dose group decreased at both time points, but were not significantly different from those of the control group.

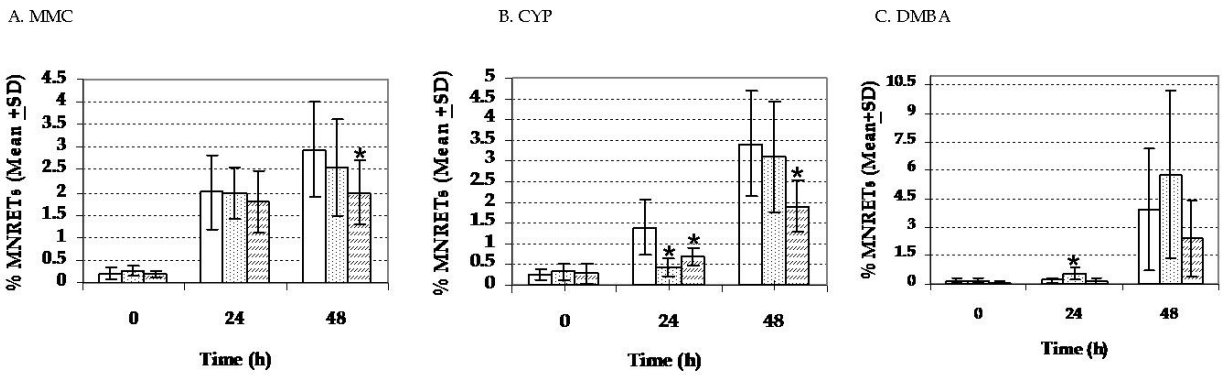

Figure 2 Mean frequencies of MNRETs in mice given AP leaves after clastogens administration. The mean frequencies of MNRETs in the control group received basal diet (open bar) and the experimental groups (dot bar and hatched bar) received ground freeze-dried AP leaves at 12.5% and 25% in the diets, respectively. * Significant differences at $p<0.05$.

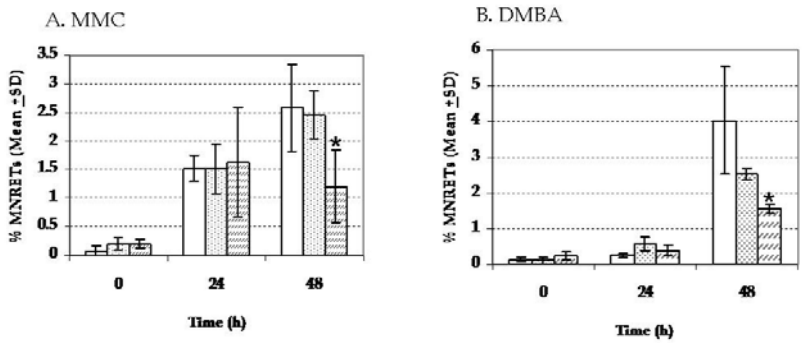

Figure 3 Mean frequencies of MNRETs in mice given IM leaves after clastogens administration. The mean frequencies of MNRETs in the control group received basal diet (open bar) and the experimental groups received ground freeze-dried IM leaves at 12.5% (dot bar) and 25% (hatched bar) in the diets. * Significant differences at $p<0.05$.

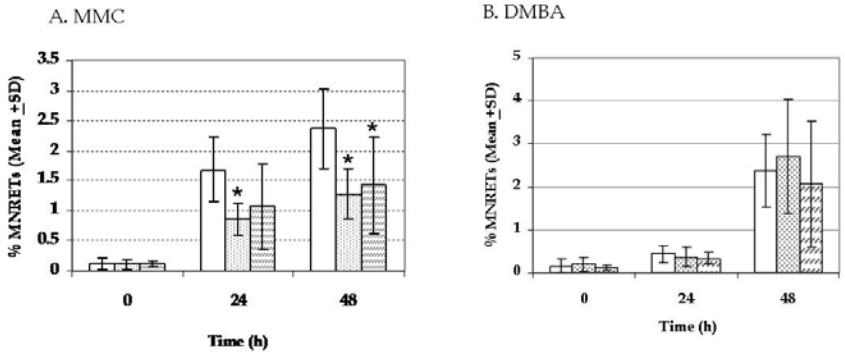

Figure 4 Mean frequencies of MNRETs in mice given with IM fruit juice after clastogens administration. The mean frequencies of MNRETs in the control group which received vehicle (open bar) and the experimental groups received IM fruit juice at 10 (dot bar) and 20 ml/kg BW (hatched bar) by gavage. * Significant differences at $p<0.05$.

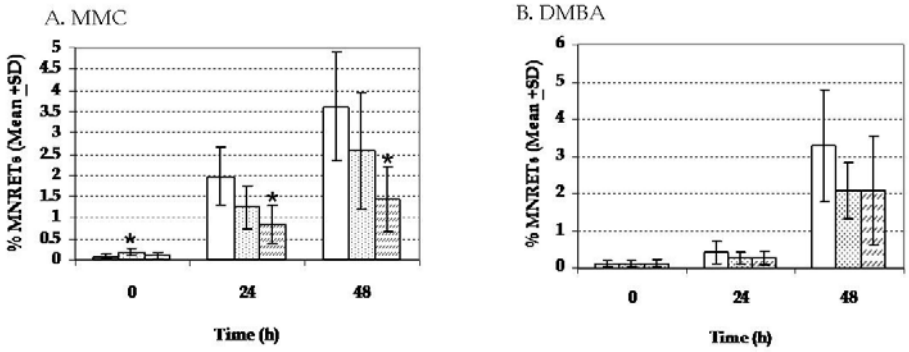

Figure 5 Mean frequencies of MNRETs in mice given with IM fruit powder solutions after clastogens administration. The mean frequencies of MNRETs in the control group which received distilled water (open bar) and the experimental groups received IM fruit powder solutions at 100 (dot bar) and 500 mg/kg BW (hatched bar) by gavage. * Significant differences at $p<0.05$.

Effect of IM leaves and fruits on the micronucleus formation in mice peripheral blood: The number of MNRETs in the control and experimental groups were not significantly different. IM leaves at 12.5% in the diet did not affect MNRETs induced by MMC (Fig 3A). However, IM leaves at 25% in the diet significantly decreased MNRETs ($p=0.05$) at 48 hours with 53% inhibition. IM leaves slightly increased MNRETs induced by DMBA at 24 hours in both low or high dose groups (Fig. 3B). On the contrary, at 48 hours MNRETs decreased in both low and high dose groups and were statistically significant ($p=0.047$) only at the high dose with 53% inhibition.

In mice fed with IM fruit juice, we found that of the dose at 10 and 20 ml/kg BW decreased MNRETs induced by MMC at 24 and 48 hours (Fig. 4A), particularly MNRETs was significantly different from those of the control group at the low dose ($p<0.05$), However, in the high dose group, MNRETs was significantly different ($p=0.018$) only at 48 hours. The inhibitory effects at both low and high doses were quite similar about 40-46%. While in DMBA induced mice in Fig. 4B, MNRETs slightly increased at 48 hours in the low dose group, but slightly decreased in

the high dose group.

Figure 5A illustrates the mean frequency of MNRETs in IM fruit powder treated groups induced by MMC. IM fruit powder shows spontaneous micronucleus formation at low dose (100 mg/kg BW). MNRETs decreased in both low and high dose groups (100 and 500 mg/kg BW) in a dose dependent manner. However, the reduction was statistically significant (p=0.028 and p=0.016), respectively, only in the high dose group at both 24 and 48 hours. The inhibitory effect at high dose was nearly twice higher than at low dose (60% VS 39%). In IM fruit powder treated group induced by DMBA (Fig. 5B), MNRETs either in the low or high dose group slightly decreased at both time points.

Discussion

According to pair feeding of mice in this study, e.g. leaves of Asiatic pennywort and Indian mulberry, including oral administration of Indian mulberry fruits had no effect on the body weight of the mice. AP leaves, IM leaves and fruit juice except IM fruit powder had no effect on the spontaneous formation of MNRETs in the mouse. It means that Indian mulberry fruit powder had both clastogenic and anticlastogenic activities.

The criterion for evaluation of a positive result is a statistically significance with dose-related increase in frequency of micronucleated erythrocyte at any time point with at least 1 value significantly exceeding the vehicle control (Hayashi et al., 1994). In this study all plants significantly reduced MNRETs induced by MMC and CYP but they did not significantly reduce MNRETs induced by DMBA, except IM leaves. It was possible that the different mechanism of action might be used to suppress the micronucleus formation induced by these clastogens (Iyer and Szybaloki, 1964; Erlichman, 1992).

The results of the present study demonstrated that AP leaves and IM (leaves and fruits) had significant effect on the micronucleus formation especially in the high dose. AP leaves at low concentration in the diet increased MNRETs induced by DMBA at both time points. On the contrary, AP leaves at high concentration could reduce MNRETs. It was opposite to the results of Bunpo et al. (2004) which used water extract of AP on AOM-induced ACF formation in rats and found less effective at a high dose. It might be the differences in pharmacokinetic of chemical constituents in this plant such as glycoside, asiatic acid, asiaticoside, medecassic acid and meecassoside (Grimaldi et al., 1990). In addition, our previous study showed that anticarcinogenic potential of AP leaves was uncertain because they caused both induction and reduction of phase I enzymes and also induction of phase II enzymes in rats (Tepsuwan and Kusamran, 1997).

IM leaves had the most potent chemopreventive properties especially at high dose in the diets possess anticlastogenic activity against both direct and indirect-acting clastogens. Its anticlastogenic potential might be modulated via direct acting carcinogenesis related with DNA-cross-linking agents (Iyer and Szybaloki, 1964) and via indirect carcinogenesis, which require metabolic activation to become a highly reactive metabolite and bind to DNA forming labile covalent DNA adducts in order to become mutagenic and/or carcinogenic (Erlichman, 1992). It is correlated with our recent research that IM leaves contain compounds acting as a monofunctional inducer and phase I inhibitor in the rats (Tepsuwan and Kusamran, 1997). In addition, IM leaf extracts have shown antimutagenic activities against an indirect-acting mutagen (Kusamran et al., 1998[a]). IM fruits, both juice and powder at the concentration tested can inhibit the clastogenicity of only a direct-acting clastogen. However, IM fruit powder had both clastogenic activity and more pronounced chemopreventive effect which we should further study. Recent research of IM fruit juice have shown antitumor activity against lung cancer in C57BL/6 mice (Hirazumi et al., 1994). In addition, IM fruit juice has antioxidant activity (Wang and Su, 2001) and cancer preventive effect at the initiation stage of carcinogenesis (Wang et al., 2002). There are possibilities that many phytochemicals found in IM fruit juice as well as vitamins and minerals can suppress tumor growth by stimulating the immune system. However, its chemopreventive properties are not clear at the moment (Potterat et al., 2007).

Conclusion

We concluded that Asiatic pennywort and Indian mulberry contained some anticlastogens, indicating that they might have chemopreventive potential against genotoxicants. Among the 2 vegetables, IM leaves showed the highest chemopreventive potential. The inhibitory effect of these vegetables seemed to correlate quite well with the capacity to enhance phase II enzyme activity and to decrease phase I enzyme activity as well as acting as the blocking agents. Micronucleus assay could be used as screening method for detecting chemopreventive agents.

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