RESEARCH ARTICLE

Anticlastogenic Effect of *Eryngium foetidum* L. Assessed by Erythrocyte Micronucleus Assay

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Abstract

The aim of this study was to investigate the anticlastogenicity as well as the clastogenicity of *Eryngium foetidum* leaf (EF) using the *in vivo* mouse peripheral blood erythrocyte micronucleus assay. Eighty ICR male mice were fed AIN-76 diet supplemented with ground freeze-dried EF at 0.0%, 0.8%, 1.6% and 3.2% for 2 weeks prior to the administration of both direct-acting, mitomycin C (MMC), and indirect-acting, 7, 12-dimethylbenz(a) anthracene (DMBA) clastogens. Peripheral blood samples were collected from mice just before administration of clastogen and at 24 and 48 h thereafter for MMC. Blood samples were collected at the same times and after 72 h for DMBA. Then, reticulocytes in blood samples were counted using fluorescent microscopy. The results indicated that EF had no clastogenic effect in mice. All doses of diets supplemented with EF decreased the number of micronucleated peripheral reticulocytes in all the MMC-treated groups in a dose dependent manner, but significant reduction was found only at 1.6% and 3.2% EF in the DMBA-treated groups. It can be concluded that EF has no clastogenicity, but possesses anticlastogenic potential against both direct- and indirect-acting types of clastogen in mice.

Keywords: Anticlastogenic - *Eryngium foetidum* - clastogenic - micronucleus - mice

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Introduction

Spices and herbal vegetables have long been traditionally used in habitual diets in tropical regions including Thailand. They contain not only essential nutrients but also are rich in various phytochemicals such as polyphenols, terpenes, alkaloids and phenolic acids, etc. The purified or crude extracts from some plants have been demonstrated to play a vital role in prevention and treatment of some chronic diseases. Several previous studies reported that Thai vegetables, herbs, and spices such as neem flowers, ivy gourd leaf, sesbania flowers, lemon grass, Asiatic pennywort and Indian mulberry, etc. possess antimutagenic, anticarcinogenic and/or anticlastogenic properties (Rojanapo and Tepsuwan, 1993; Kupradinun et al., 1997: 2008: 2011; Kusamran et al., 1998; Tepsuwan et al., 1999: 2002).

Eryngium foetidum L. (Saw tooth leaf, family Apiaceae, EF) is widely used for its unique pungent aroma as an important seasoning in habitual diets (Seaforth et al., 2005). It has been used as traditional medicine for treatment of colds, fits, convulsions, fainting, headaches and malaria (Mitchell et al., 2006; Roumy et al., 2007). The aerial parts of EF are rich in several nutrients including vitamins and carotene (Munsell et al., 1950; Ramcharan and Culantro, 1999). The leaf contains essential oil predominantly with *E*-2-dodecenal (Lo et al., 1991). Triterpenoids, carbonyls, alcohols and terpenes are also isolated from *E. foetidum* leaves (Pual et al., 2011). Stigmasterol, a predominate phytosterol from the organic extract of the leaf, possesses topical anti-inflammatory activity against acute and chronic inflammation in animal model (Garcia et al., 1999). Recently, an ethanol extract from *E. foetidum* leaf showed suppression of LPS-induced pro-inflammatory mediator gene expression in RAW 264.7 murine macrophage (Mekhora et al., 2012). Another previous study showed that oral consumption of *E. foetidum* leaf decoction by rat inhibited carrageenan-induced paw edema and 12-*o*-tetradecanolyphorbol acetate-induced ear edema (Saenz et al., 1997).

So far, there is an increasing interest in using natural products especially in dietary stuffs to prevent or treat some chronic diseases due to their safety properties and low cost relative to pharmacological agents. The *in vivo* rodent micronucleus assay has been accepted as a short-term assay for evaluation of the clastogenicity of chemical compounds as well as the anticlastogenicity of chemopreventive potential of compounds (Ashby et al., 1988; Heo et al., 1996; Kupradinun et al., 1997: 2008; Hwang et al., 2008; Promkum et al., 2010). Mouse peripheral blood, instead of bone marrow cells, was introduced to use in the micronucleus assay (MacGregor et al., 1980) and recently, acridine orange was used for supravital staining of blood cells (Hayashi et al., 1983).

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These modifications offer many advantages to the conventional bone marrow assay and are widely used to evaluate chemical clastogenicity (Morita et al., 1997).

Although *E. foetidum* leaves have been previously reported to exhibit many biological functions, however its anticlastogenicity and clastogenicity have never been reported. Therefore, we applied this technique to determine the anticlastogenicity of EF in this study. The results from the present study will provide a beneficial biological function of EF to promote Thai cuisines using of *E. foetidum* leaf in a healthy diet.

Materials and Methods

Chemicals

Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co., Ltd., (Tokyo, Japan) and 7,12 dimethylbenz(a)anthracene (DMBA) from Sigma Chemical Co. (St. Louis, MO, USA). Acridine orange (AO) was purchased from E. Merck Co (Darmstadt, Germany). For preparing animals diets, AIN-76 minerals mixture was purchased from MP Biomedicals (Solon, California, USA) and vitamins mixture products of Clea Japan Inc. (Osaka, Japan) and was kindly provided by Prof. Dr. Tadashi Okamoto, Kobe Gakuin University, Japan. Vitamin K1 was provided by DSM Nutritional Products Ltd. (Sitesisseln, Switzerland) and cellulose (SOLKA-FLOC® 200 FCC) from FS&D (St. Louis, MO, USA). Sodium caseinate was the product of Erie Foods International, Inc (Erie, Illinois, USA). Other reagents were mostly of analytical grade and obtained locally.

Vegetable preparation

EF was purchased from 4 central distributor markets which represent the vegetables grown in Northern, Northeastern, and Central areas of Thailand. After removing inedible parts of EF, the edible parts were then washed thoroughly with tap water and rinsed with deionized water twice, drained and air-dried and then cut into small pieces. The EF was freeze-dried and was ground in an electric grinder. Then aliquots of each vegetable were pooled, packed in vacuum bags and stored at -20ºC until use.

Animals, diets and experimental procedure

Eighty ICR male mice, 4 weeks old, weight 25±3 grams, were purchased from the National Laboratory Animal Center (NLAC), Mahidol University, Thailand. Animals were maintained at the Laboratory Animal Facility of the National Cancer Institute according to the Institute Care Guidelines which were approved by the Animal Ethic Committees of the National Cancer Institute and Mahidol University. The mice were acclimatized for 5-7 days in a clean conventional room maintained at 23±2°C with 12 h light/dark cycle, controlled relative humidity at 50±20% and housed in filtered top plastic cages. The mice were given AIN-76 diet (basal diet) and water *ad libitum*. The amount of ground freeze-dried EF given to animals was based on the human serving size per person at the 97.5 percentile (Food Consumption Data of Thailand, 2006). The serving size of EF is 0.48 g fresh weight/kg body weight. The experimental diets were prepared by mixing freeze-dried EF in basal AIN-76 diet (Bieri et al., 1977) at the doses of 0.8%, 1.6% and 3.2% with slight modification according to Reeves et al. (1993) which is equivalent approximately to 20, 40 and 80 times human consumption.

Clastogenicity tests

The experimental design to study the effect of freeze-dried EF at various doses on the erythrocyte micronucleus formation is shown in Figure 1. After acclimation, male ICR mice were randomized and divided into four groups of 20 mice/group. Group 1 received basal diet (the control group) while the other three groups were assigned as experimental groups receiving diets supplemented with ground freeze-dried EF at 0.8%, 1.6% and 3.2% for 2 weeks (groups 2, 3 and 4, respectively). Their clastogenic effects were evaluated from the number of micronuclei in reticulocytes of mice receiving either the basal diet or diets containing ground freeze-dried EF according to micronucleus assay as previously described (Kupradinun et al., 2008: 2011). Both control and experimental groups were pair-fed and provided water *ad libitum*. Peripheral blood was collected from the facial vein at day 14 and dropped on AO coated slides. The micronucleated cells were scored under a fluorescence microscope. The extent of cytotoxicity was determined from the incidence of micronuclei in reticulocytes. The frequencies of micronucleated peripheral reticulocytes (MNRETs) were scored from the examination of 1,000 reticulocytes. The reticulocytes are restricted to type I, II and III as classified by Vander et al. (1963).

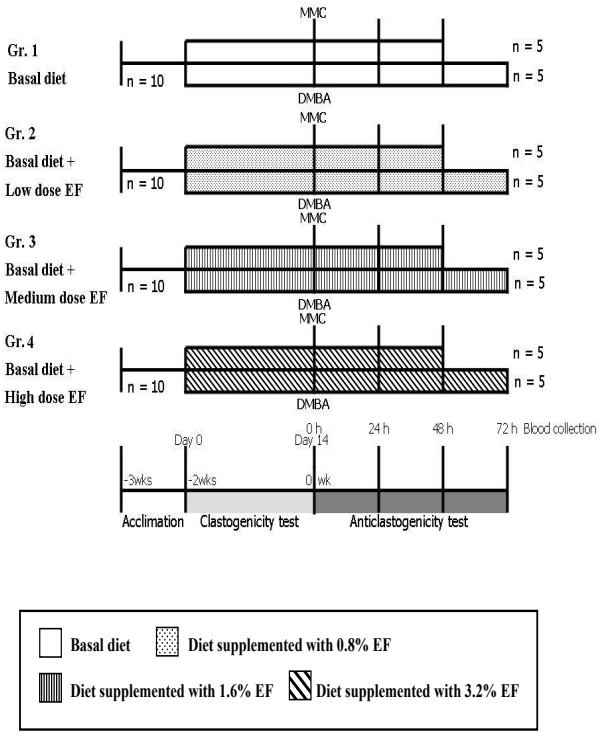

Figure 1. Experimental Design to Study the Clastogenic and Anticlstogenic Effect of *E. foetidum* in mice. MMC: Mitomycin C (1 mg/kg BW, i.p.), DMBA:7,12-dimethylbenz (a) anthracene (40 mg/kg BW, i.g).

Anticlastogenicity tests

On day 14, ten mice of each group were either injected intraperitoneally with MMC (a direct acting clastogen) at 1 mg/kg body weight or gavaged with 40 mg/kg body weight of DMBA (an indirect acting clastogen). All mice continued to receive the basal diet or diets containing ground freeze-dried EF through the entire period of an experiment (Figure 1). Peripheral blood samples were collected at 24, and 48 hours following treatment with MMC and at 24, 48 and 72 hours following treatment with DMBA. The micronucleated cells were scored under fluorescence microscope as described above.

Statistical analysis

The effect of freeze-dried EF on body weight and micronucleus formation was statistically evaluated using the SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). Nonparametric tests with one sample K-S were applied. One-Way ANOVA with *post hoc* multi-comparisons tests was used for indication of any significant difference among groups of animal and comparison of significant differences between groups by Fisher's Least Significant Difference (LSD). *P*-value < 0.05 was considered statistically significant.

Results

Effect of freeze-dried EF on the body weight and food consumption

Body weight and food consumption of mice which fed EF diets were recorded daily during the experiment (Figures 2a-2b). Mean food consumption of mice among the control and experimental groups was 5.1 ± 0.7 g/d and mean body weight was 33.1 ± 2.2 g. The body weight of mice which consumed diets containing 3.2% freeze-dried EF and treated by MMC, was significantly different from those of the control group (Figure 1a) while there was no significant effect on mice body weight among the DMBA treated and the control group (Figure 2b). However, after MMC and DMBA administration, the body weight and food consumed were decreased during day 15-17.

Effect of freeze-dried EF on micronucleus formation

The numbers of MNRETs in each treatment group consuming diets containing freeze-dried EF at 0.8%, 1.6% and 3.2% for 2 weeks were not significantly different compared with the control one (data not shown). The average number of MNRETs in the control group was 4.1±1.9 per 1,000 reticulocytes while in groups 2, 3 and 4 the averages were 3.2±2.3, 3.6±2.0 and 3.7±1.3, respectively. No significant difference ($p>0.05$) was observed between the experimental and the control groups.

Effect of freeze-dried EF on micronucleus formation induced by clastogens

The effect of freeze-dried EF on the micronucleus formation induced by MMC is shown in Figure 3a. The MNRETs were maximized at 48 hours after administration with MMC in the control group. The results indicated that mice consuming EF supplementation had significantly decreased MNRETs in a dose dependent manner compared with the control after MMC administration at 48 hours. Figure 3b shows the number of MNRETs determined at 24, 48 and 72 hours after DMBA treatment. The result indicated that the numbers of MNRETs were continuously increased and reached maximum number at 48 hours and declined at 72 hours. At 24 hours, the MNRETs number after DMBA administration was significantly decreased in all concentrations of EF supplemented in diets compared with those of the control group. Likewise, mice which consumed EF supplements in diets had reduced numbers of MNRETs in a dose dependent manner after DMBA administration at 48 hours and 72 hours but they showed significant differences only in mice which consumed 1.6% and 3.2% EF diets compared with the control group.

Discussion

The present study demonstrated that consumption of EF at 0.8%, 1.6% and 3.2% (approximately equivalent to 1.2, 2.5 and 5.0 g/kg BW of mice) has no effect on the growth rates of mice except at the high dose as the initial body weight was higher than the other groups. These amounts of EF are equivalent to 17, 35 and 71 g (fresh)/kg BW of mice, which represent 33, 73, and 147 times the 97.5th percentile of human consumption (Food Consumption Data of Thailand, 2006). In addition, these consumption amounts have no clastogenic effect as assessed using clastogen induced micronucleated peripheral blood of mice. Our results are consistent with the previous study data indicating that mice which

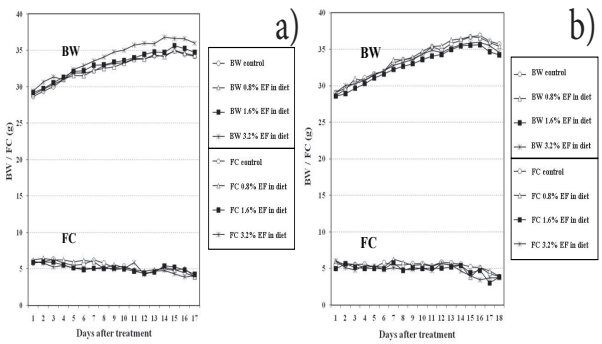

Figure 2. Body Weight (BW) and Food Consumption (FC) of Mice on a) MMC and b) DMBA-Induced Clastogenicity and Anticlastogenicity Tests.

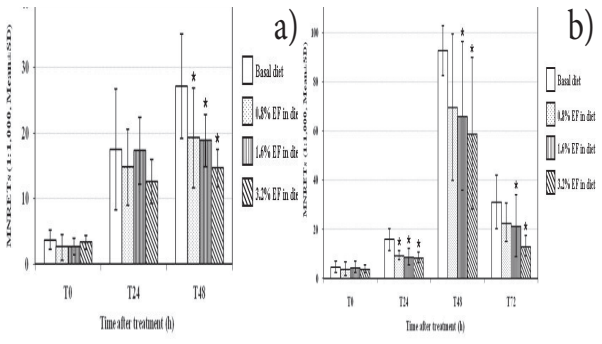

Figure 3. Time-Dependent Effect of *E. foetidum* on MNRETs Induced by a) MMC and b) DMBA. Values are Mean±SD *Significant differences at $p<0.05$.

consumed extract from EF rich in triterpenoids didn't show any toxic effect (Jäger et al., 2009). This result suggested that high consumption of EF does not generate adverse effect on human health.

Regarding the anticlastogenic activity of EF, the numbers of MNRETs were decreased significantly in the groups receiving EF at 1.2 to 5.0 kg BW (0.8%, 1.6% and 3.2% in diets) on both direct and indirect acting clastogens in a dose dependent manner. We can imply that the daily consumption of EF will generate health benefit in term of anticlastogenic effect. Since there were no data on the anticlastogenic study of EF, their anti-clastogenic activity might be due to the various active compounds in the leaves. A lot of terpenic compounds and a variety of phytosterol compounds: α-cholesterol, brassicasterol, campesterol, clerosterol, β-sitosterol, Δ_5-avemasterol, $\Delta_5$24-stigmastadienol, Δ_7-avenasterol, and stigmasterol were reported as the main components (95%) in EF (Saenz et al., 1997). These compounds can act as antioxidants which interact with free radicals caused by toxic chemicals or oxidation reactions and they can suppress NF-κB signaling, the major regulator in the pathogenesis of inflammatory diseases and cancer (Salminen et al., 2008). In addition, phytosterols such as β-sitosterol, campesterol and stigmasterol, were proposed as anticancer dietary components because the structures of plant sterols and cholesterol are similar. Therefore, they have an effect on cell membranes structure, cell membrane-bound enzymes, signal transduction pathways that regulate tumor growth and apoptosis, immune function, etc, and result in increasing potential prevention of carcinogenesis (Awad and Fink, 2000). It is possible that those phytochemical compounds are antioxidant compounds which play an important role in preventing and repairing damages of cells (Floyd, 1990; Bagchi and Puri, 1998; Flora, 2007; Pham-Huy et al., 2008). The antioxidant activity of EF is supported by the study of Chanwitheesuk and coworkers (Chanwitheesuk et al., 2005) who have reported that the contents (mg%) of antioxidant compounds using a β-carotene bleaching method in dried EF were vitamin C (11.4±0.007), vitamin E (0.0069±0.0007), total carotenes (1.92±0.03), total xanthophylls (1.60±0.05), tannins (17.7±0.08), total phenolics (98.4±0.08), and antioxidant index was 5.65±0.46. A recent study provides supportive data on antioxidant and anti-inflammatory properties of EF leaves in LPS-induced murine macrophage cell lines (Mekhora et al., 2012). The same study also analyzed and identified bioactive components in the EF extract which found to have lutein, β-carotene, caffeic acid, chlorogenic acid and kaempferol. The EF extract significantly suppressed LPS-induced pro-inflammatory mediator gene expression including interleukine-6 (IL-6), tumor necrosis factor-alpha (TNF-α), inducible nitric oxide synthase (iNOS) and cyclooxygenease-2 (COX-2) in a dose-dependent manner. Nitric oxide (NO) level and intracellular reactive oxygen species (ROS) content were similarly reduced. These suppressive effects were mediated via an inhibition of LPS-induced phosphorylation of mitogen-activated protein kinases (c-Jun N-terminal kinases and p38) as well as inhibitor κB-α (IκB-α). β-carotene showed a significant effect to protect against benzo(a)pyrene induced chromosome breakage in bone marrow of mice (Raj and Katz, 1985). Kaempferol was reported to protect against the induction of micronuclei by intraperitoneal injection with benzo(a) pyrene in polychromatic erythrocytes of mice (Heo et al., 1992). Since the anticlastogenic activity of EF was shown against both direct acting, MMC, and indirect acting clastogens, DMBA. Thus, we can postulate the mechanism of action of EF may act as blocking agents and/or suppressing agents (Dangubon, 2007; Heo et al., 1992). However, the classification for each type of agents has not been determined.

Taken together, the anticlastogenic activity of EF was confirmed potential health benefit of *E. foetidum* leaf. Regular consumption by adding it as an ingredient in a variety of Thai dishes or as fresh vegetable side dishes should be encouraged. The micronucleus assay could be used as a screening tool for detecting chemopreventive agents.

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