RESEARCH COMMUNICATION

Nutritive Evaluation and Effect of *Moringa oleifera* pod on Clastogenic Potential in the Mouse

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Abstract

***Moringa oleifera* Lam (horseradish tree; tender pod or fruits) has been consumed as a vegetable and utilized as a major ingredient of healthy Thai cuisine. Previous studies have shown that *M. oleifera* pod extracts act as bifunctional inducers along with displaying antioxidant properties and also inhibiting skin papillomagenesis in mice. This study was aimed to determine the nutritive value, and clastogenic and anticlastogenic potentials of *M. oleifera* pod. The nutritive value was determined according to AOAC methods. The clastogenic and anticlastogenic potentials were determined using the *in vivo* erythrocyte micronucleus assay in the mouse. Eighty male mice were fed semi-purified diets containing 1.5%, 3.0% and 6.0% of ground freeze-dried boiled *M. oleifera* pod (bMO) for 2 weeks prior to administration of both direct-acting (mitomycin C, MMC) and indirect-acting (7, 12-dimethylbenz(a)anthracene, DMBA), clastogens. Blood samples were collected at 0, 24, 48 and 72 h, dropped on acridine orange-coated slides, and then counted for reticulocytes both with and without micronuclei by fluorescence microscopy. The nutritive value of 100 g bMO consisted of: moisture content, 8.2 g; protein, 19.2 g; fat, 3.9 g; carbohydrate (dietary fiber included), 60.5 g; dietary fiber, 37.5 g; ash, 8.1 g and energy, 354 kcal. Freeze-dried boiled *M. oleifera* had no clastogenic activity in the mouse while it possessed anticlastogenic activity against both direct and indirect-acting clastogens. Freeze-dried boiled *M. oleifera* pod at 1.5%, 3.0% and 6.0% in the diets decreased the number of micronucleated peripheral reticulocytes (MNRETs) induced by both MMC and DMBA. However, the effect was statistically significant in the dose dependent manner only in the MMC-treated group. In conclusion, the present study demonstrated that bMO has no clastogenicity and possesses anticlastogenic potential against clastogens, and particularly a direct-acting clastogen in the mouse.**

Key words: Anticlastogenic - *Moringa oleifera* - mitomycin C - DMBA - erythrocyte micronucleus assay - nutritive value

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Introduction

The prevalence of non-communicable or chronic diseases including metabolic syndrome associated diseases especially obesity, diabetes mellitus, cardiovascular disease and cancer is remarkably increasing and has become a public health problem all over the world. This is because life-style and consumption behavior has gradually and persistently changed from traditional to Western style, i.e., more high protein, carbohydrate and fat diets consumption but less vegetables and fruits intake, and less exercise. Currently, the rate of mortality from non-communicable or chronic diseases has been increasing in Thailand. The mortality rate from chronic diseases in descending order is cancer, hypertension and cerebrovascular diseases, and heart diseases, respectively (Health Information Unit, Bureau of Policy and Strategy, Thai Ministry of Public Health 2003-2007). Particularly for cancer, nutrition has been proven to play an important role in the prevention processes. Nutrient and non-nutrient content in the habitual diet thus becomes an important issue to reduce the risk of developing chronic diseases (Steinmetz and Potter, 1996). Vegetables, herbs and spices have long been traditionally used in habitual diets in South and South East Asian Countries. Previous studies reported that some Thai vegetables, herbs, and spices have exhibited antimutagenic and/or anticarcinogenic properties. Various edible plants contained in Thai dishes have been reported to have the potential effect on anticlastogenicity induced by clastogens such as neem flowers, ivy gourd leaf, sesbania flowers and lemon grass (Kupradinun et al., 1997; Kupradinun, 2008). *Moringa oleifera* Lam is used as a highly nutritive vegetable in many countries particularly in India, Philippines, Pakistan, Thailand, Hawaii and Africa. Its young leaves, flowers, seeds and tender pods are commonly consumed and there are some medicinal properties (D'Souza and Kulkarni, 1993). The tender pod has been consumed as a major ingredient in Thai dishes. Recently, oral administration of a hydro-alcoholic extract of *M. oleifera* pod was found to increase liver enzymes involved in the reactions of Phase I and Phase II enzymes such as cytochrome b5, cytochrome P450,

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catalase, glutathione-peroxidase, glutathione reductase and glutathione *S*-transferase which are responsible for the detoxification of xenobiotic substances and also inhibit skin papillomagenesis in mice (Bharali et al., 2003).

Interest has been generated in the clastogenicity and anticlastogenicity of *M. oliefera* pod to determine its health benefits. Since there were no reports involving their effect on human health particularly in terms of health benefit effect, studies using animal models for the determination of efficacy dose related health benefit for anticlastogenicity should be elucidated. The *in vivo* rodent erythrocyte micronucleus assay is one form of short-term toxicity testing which has been widely used to assess chemical clastogenicity (Hayashi et al., 1990).

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 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.

Vegetables

Moringa oleifera Lam (horseradish tree) tender pod (MO) was purchased from 2 central distributor markets located in Bangkok and Nakhon Pathom provinces. A central distributor market located in Bangkok represented MO cultivated in Nakhon Sawan, Chachoengsao, and Kanchanaburi provinces and another market, Pathom Mongkol market located in Nakhon Pathom province, represented plants cultivated in Nakhon Pathom. These areas of cultivation represent vegetables which are grown in Northern, Eastern, Central and Western areas of Thailand. Each MO was prepared in traditional fashion: washed with tap water and rinsed with deionized water, drained and air-dried, the peel removed, and the pod cut into small pieces. Then the edible part of MO was boiled for 7 minutes in boiling water (1:1 w/w) and allowed to cool. All boiled vegetables together with boiled water were blended, lyophilized, and ground in an electric grinder. After that, aliquots of each sample were pooled, packed in vacuum bags and stored at -20ºC until use. The yield of bMO from fresh pod was about ten percent.

Nutritive evaluation

The nutritive values of 100g bMO were assayed according to the AOAC methods. Ash content was determined by the gravimetric method while protein content was determined by the Kjeldahl method and fat content was analyzed by acid hydrolysis followed by petroleum ether extraction. Dietary fiber was evaluated by the enzymatic-gravimetric method (AOAC, 2000). Moisture content was determined by the drying method (AOAC, 2005). Carbohydrate and energy were determined by calculation.

Animals

Eighty ICR male mice aged 4 weeks, 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 both the Animal Ethics Committee of the National Cancer Institute and Mahidol University. The animals were acclimatized for 5 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 semi-purified diet (basal diet) and water *ad libitum*.

Preparation of animal diets

The amount of ground bMO in each sample given to animals was based on the human serving size per person at the 97.5 percentile reported from the consumption data of boiled MO in persons aged more than 3 years old (Food Consumption Data of Thailand, 2006). The serving size of boiled MO present in grams (fresh weight) per kilogram body weight per day was 1.98. The experimental diets were prepared by mixing bMO in various concentrations, i.e. 1.5%, 3.0% and 6.0% in basal AIN-76 mouse diet (Bieri et al, 1977), with slight modification according to Reeves et al (1993) which is equivalent approximately to 10, 20 and 40 times human consumption. The composition of basal diet and diets containing each sample is shown in Table 1.

Preparation of Acridine Orange (AO)-coated glass slides

AO-coated slides were prepared according to Hayashi and coworkers (1990). Briefly, AO was dissolved in

Table 1. The Composition of the AIN-76 Semi-Purified Diet and Diets Containing bMO

Ingredients	Amount (g/kg) in diet			
	Basal diet	bMO diet		
		1.5%	3.0%	6.0%
Casein	200	200	200	200
Cornstarch	500	500	500	500
Sucrose	150	150	150	150
Cellulose	50	50	50	50
Corn oil	50	50	50	50
DL-methionine	3	3	3	3
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline bitartrate	2	2	2	2
Vitamin K1	0.002	0.002	0.002	0.002
bMO	0	15	30	60

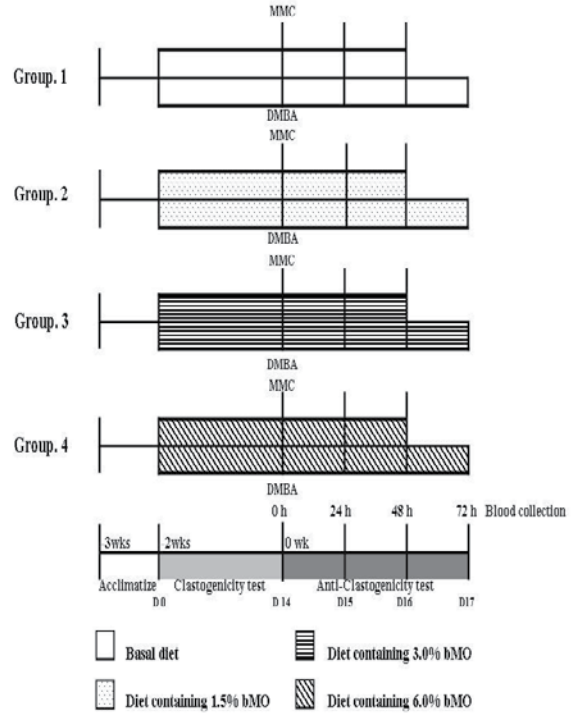

Figure 1. Experimental Design. MMC: Mitomycin C (1mg/kg BW, i.p), DMBA: 7,12- Dimethylbenz (a) anthracene (40mg/kg BW, orally)

distilled water at the concentration of 1 mg/ml. Eight microliters of this solution was dropped on a pre-heated cleaned glass slide and immediately spread by a moving glass rod, air dried and kept in slide boxes in a dry place at room temperature.

Clastogenicity tests

The experimental design to study the effect of bMO at various doses on the erythrocyte micronucleus formation is shown in Figure 1. After 5 days of acclimation, male ICR mice were randomized by weight and divided into four groups of 10 mice each in experiment I. Group 1 received basal diet and was assigned as control group. The other three groups were assigned as experimental groups receiving diets containing bMO at 1.5%, 3.0% and 6.0% for 2 weeks in groups 2, 3 and 4, respectively. The clastogenic effect of bMO was determined by the incidence of micronucleus formation in reticulocytes of mice receiving either the basal diet or diets containing bMO at various concentrations. Both control and experimental groups were therefore pair-fed and provided water *ad libitum*. Peripheral blood was collected from facial vein at day 14 and dropped on AO-coated slides. The micronucleated cells were scored under a fluorescence microscope according to the method of Hayashi et al., 1990. The extent of cytotoxicity was determined from the incidence of micronucleus formation in reticulocytes. For each mouse, 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 of the classification by Vander et al., 1963. The repeat measurement was done in experiment II.

Anticlastogenicity tests

On day 14, ten mice in each group were either injected intraperitoneally with a standard clastogen/carcinogen, MMC (a direct acting clastogen) at 1mg/kg body weight or DMBA (an indirect acting clastogen) administered intragastrically at 40mg/kg body weight. All mice continued to receive the basal diet or diets containing bMO until the end of 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. Peripheral blood smears were prepared at the specified times and the micronucleated cells were scored under fluorescence microscope as described above.

Data and statistical analysis

The effect of bMO 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 were 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

Nutritive evaluation

The nutritive value of 100 g bMO consisted of moisture content 8.23g; protein, 19.2g; fat, 3.9g; carbohydrate (dietary fiber included), 60.5g; dietary fiber, 37.5g; ash, 8.1g; and energy, 354kcal.

Effect of bMO on the body weight and food consumption

In this experiment, the control group was given basal diet and experimental groups were given diets containing 1.5%, 3.0% and 6.0% bMO in pair-fed form. Body weight and food consumption were recorded daily during the experiment. Mean food consumption by each mouse among the control and experimental groups was 4.7±0.6g/d and mean body weight of each mouse was 33.3±2.2g. It was found that there were no significant differences ($p>0.05$) in body weight of mice which received bMO at specified doses in the experiment in which MMC was used as a standard clastogen (data no shown). In DMBA treated groups(Figure 2), the body weights of mice in groups 2, 3 and 4 which were given diets containing bMO at 1.5%, 3.0% and 6.0%, respectively, were higher than that of the control group and a significant increase ($p<0.05$) was found in group 4 in comparison with the control group before and during the treatment period. However, after both carcinogens, MMC and DMBA, administration from day 15-18, the body weight of mice and amount of food consumed were decreased.

Effect of bMO on micronucleus formation in mouse peripheral reticulocytes

The numbers of MNRETs scored in each treatment

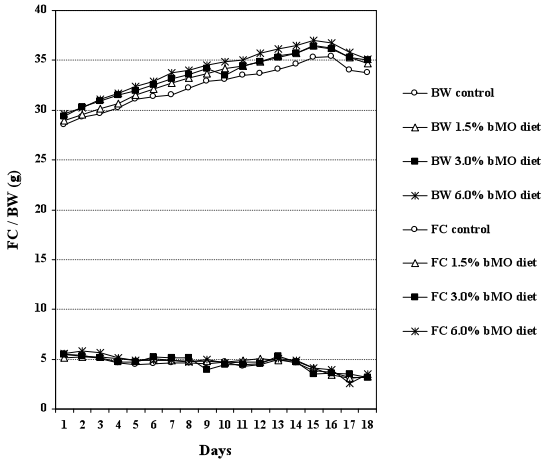

Figure 2. Body Weight (BW) and Food Consumption (FC) of Mice on DMBA-Induced Clastogenicity and Anti-Clastogenicity Tests

group consuming diets containing bMO at 1.5%, 3.0% and 6.0% were not significantly different in comparison with the control group without bMO for 2 weeks. The result from each experiment is shown in Table 2. The average number of MNRETs in the control group was 3.0±1.4 per 1,000 reticulocytes while in groups 2, 3 and 4 were on average 3.6±1.9, 3.1±1.2 and 3.0±1.4, respectively. No significant difference ($p>0.05$) was shown between each experimental groups and the control group.

Effect of bMO on micronucleus formation in mouse peripheral reticulocytes induced by clastogens (MMC and DMBA)

For anticlastogenic testing, the numbers of MNRETs were evaluated at 24 and 48 h. after each control and experimental group was injected intraperitoneally with MMC (Figures 3 and 4). The result demonstrated the highest numbers of MNRETs in the control group at 48 h after mice were treated with MMC. At 24 h, the group of mice which received 1.5% bMO in the diet showed a slight increase in the number of MNRETs while the group of mice which received 3.0% and 6.0% bMO in the diets had a reduction in the number of MNRETs in comparison to the control group. A significant decrease ($p=0.001$) was shown in the group of mice which received diet that contained 3.0% bMO. In addition, at 48 h, the number of MNRETs in all groups of mice consuming diets containing bMO was decreased in a dose dependent manner but significantly different only in groups of mice that received 3.0% and 6.0% bMO in the diets when compared with those of the control group. Figure 4 shows the numbers of MNRETs evaluated at 24, 48 and 72 h after the mice were administered DMBA orally. This resulted continuous increase in the numbers of MNRETs with the highest number at 48 h which declined at 72 h within each group of control or experimental groups. At 24 h after administering DMBA, there was a significant decrease in the number of MNRETs as shown in all concentrations of bMO in the basal diets but no dose response relationship. However, the number of MNRETs was significantly decreased ($p=0.028$) only in the group of mice consuming diet containing 1.5% bMO at 48 h after mice received DMBA.

Table 2. Effect of bMO on the Erythrocyte Micronucleus Formation in Mice. [a]Values are Mean ±sd, MNRETs: Micronucleated peripheral reticulocytes, RETs: reticulocytes.

Groups	The number of MNRETs /1,000 RETs[a]		
	Exp. I	Exp. II	Average
Basal diet (control)	3.6±1.8	2.5±0.6	3.0±1.4
1.5% bMO diet	4.0±2.4	3.2±1.2	3.6±1.9
3.0% bMO diet	3.1±1.1	3.2±1.5	3.1±1.2
6.0% bMO diet	3.6±1.4	2.4±1.1	3.0±1.4

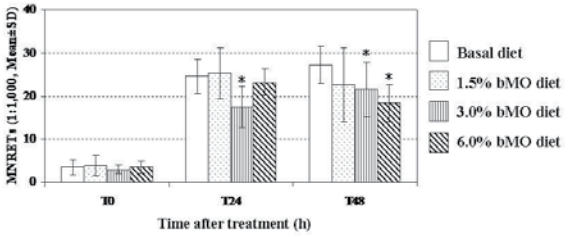

Figure 3. Time-Dependent Effect of bMO on MNRETs Induced by MMC. Values are Mean ±SD. * Significant differences at $p<0.05$

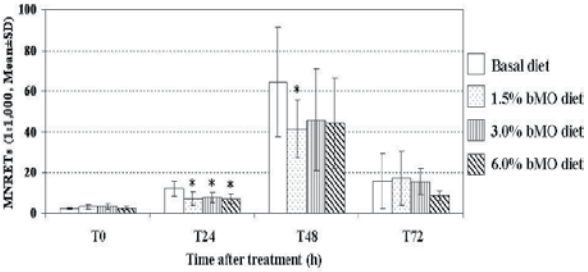

Figure 4. Time-Dependent Effect of bMO on MNRETs Induced by DMBA. Values are Mean ±SD. * Significant differences at $p<0.05$

Discussion

The results from this study show that the growth rates of mice are not different between most treatment and the control groups. The body weight of mice which received 6.0% bMO was increased in comparison with the control group during the treatment period when the experiment was done by using DMBA as a standard clastogen. This is because the mice's body weight in the treatment group, particularly the highest dose of sample, was higher than the control group before the mice received the sample at the start of the treatment period. The weight of the mice increased corresponding to the amount of food consumption. Therefore, pair-feeding was used to avoid this problem. In many reports, almost all parts of *M. oleifera* pod and seeds have been shown to be a great natural source composed of high nutrients, non-nutrients and phytochemical compounds such as protein, vitamins, minerals, β-carotene, amino acid and various phenolics (Farooq et al., 2007). Anti-nutrients such as trypsin and amylase inhibitors were also found in raw *M. oleifera* seed and they can interfere with nutrients digestion and absorption by negatively effecting the metabolic processes in the human body (Oliveira, 1999;

Sharaf, 2009). The anti-nutrient compounds in raw *M. oleifera* seeds were reported at significant amounts such as phytates (30.6g/kg), cyanogenic glucosides (14.02mg/kg), saponins (12.41mg/g) and a small amount of tannins (0.78mg/100g). In this study, we used boiled MO because the boiling process could destroy the anti-nutrient compounds, so body weight of mice was not affected by our sample. Many studies have reported that the anti-nutrient compounds are decreased or detoxified by various processing such as soaking and heat processing (boiling, frying and toasting) (Ugwa and Oranye, 2006; Emiola and Olochobo, 2006). However, after clastogens administration, the body weight and food consumption of mice in all groups decreased due to the effect of clastogens and blood withdrawal.

Previous reports have noted that water extracted from *M. oleifera* seeds has low toxicity (LD_{50}=512.8mg/kg BW), which is considered as gently toxic compared with toxicological human standards (Ferreira et al., 2008). The seed or various parts of this plant have long been used as food and folklore medicine thus edible parts of *M. oleifera* are rather safe for consumption and relevant to consumer health benefit. The studies on genotoxicity of *M. oleifera* seed has shown that sequential extraction with dichloromethane, acetone, methanol and distilled water did not have either effect on coliphages or the enzymes lactic dehydrogenase or invertase. They did not display mutagenic effect on *Salmonella typhimurium* strain TA98 and TA100 both in the presence or absence of metabolic activation by using the Ames *Salmonella* mutagenicity assays (Grabow et al., 1985). The result from the present study shows that bMO does not possess clastogenic activity in mice which received diet containing bMO at 1.5%, 3.0% and 6.0%. These amounts of sample represented 2.1, 4.3, and 8.5 g/kg BW of mice which are equivalent approximately to 8,17 and 33 times typical human consumption. Based on the criterion for a positive result, there was a statistically significant dose-related increase in micronucleated erythrocyte frequency at any time point with at least 1 value significantly exceeding the vehicle control range (Hayashi and Krishna, 2000). This implies that there is no clastogenic activity of bMO as determined by erythrocyte micronucleus assay.

For anti-carcinogenic activity, previous studies reported that isolated bioactive compounds from *M. oliefera* seed such as 4(α-L-rhamnosyloxy)-benzyl isothiocyanate, niazimicin, 3-O-(6'-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol and β-sitosterol-3-O-β-D-glucopyranoside showed inhibitory effect on the Epstein-Barr virus-early antigen (EBV-EA) activation in Raji cells induced by the tumor promoter, TPA *in vitro* assay (Guevara, 1999). For the *in vivo* test, topical application of niazimicin showed inhibitory effect against two-stage carcinogenesis in mouse skin using DMBA as initiator and TPA as tumor promoter. Niazimicin (85 nmol in acetone 0.1 ml) can exhibit 50% delay in the promotion of tumors and decrease the incidence of papilloma bearing mice (Guevara, 1999). Previous studies reported that ascorbic acid (vitamin C) is a main compound in MO, its content was 64.9±0.15 and 53.8±0.20mg/100g dry weight of raw and cooked pod, respectively (Gldamis et al., 2003). The content of vitamin C in 100 g fresh tissues of *M. oliefera* extract was reported in fruits (106.95 mg) and seed (62.11mg) (Brahma et al., 2009). Therefore, vitamin C acts as a free radical scavenging agent which presents its anticlastogenic effect to human peripheral lymphocytes induced by cisplatin (Hilada, 2001). In our study, the anticlastogenic activity in mice has been shown because of the effect of vitamin C content in MO. The numbers of chromosome aberrations were also decreased in Hilada's study (Hilada, 2001). Moreover, Gldamis and coworkers also reported that β-carotene content in 100 g dry weight of MO was found at 2,797.9±81.9mg in raw pod and 2,307±133.2 mg in cooked pod (Gldamis et al., 2003). This compound has anticlastogenic property by protection from the chromosomal breakage in bone marrow of mice from benzo(a) pyrene induction (Raj and Katz, 1986). It provided inhibitory clastogenic activity to human hepatoma cells induced by cyclophosphamide (CP) (Salvadori et al., 1993). In addition, it also showed a significant reduction of micronucleus formation in β-carotene-treated cells, induced by CP (Basu et al., 1987). Interestingly, the number of micronuclei was decreased in dose response with the increasing dose of β-carotene (3, 6, and 12mg/kg BW) in mice pretreated with β-carotene for 5 consecutive days before gamma ray irradiation (Konopacka et al., 1998).

In our study in the anti-clastogenicity test, the bMO in the diets at 1.5%, 3.0% and 6.0% possessed anticlastogenic activity against both direct (MMC) and indirect-acting (DMBA) clastogens. For the indirect acting carcinogenesis process, the numbers of MNRETs decreased in the groups of mice consuming diets containing bMO at 1.5%, 3.0% and 6.0% at both time points at 24 and 48 h after mice received DMBA but significantly decreased ($p=0.028$) only at the lowest dose (1.5% bMO) at 48 h. A significant decrease ($p=0.05$) was seen at 24 h in all doses. However, a dose dependence was not shown in each group of experiments in comparison to the control group. This finding is supported by the study on the modulatory influence of a hydro-alcoholic (80% ethanol: 20% distilled water) extract of MO on hepatic drug metabolizing enzymes and antioxidant status in mice. Their study showed significant increase ($p<0.05$ to $p<0.01$) in the activities of hepatic cytochrome b5, cytochrome P450, catalase, glutathione peroxidase, and glutathione reductase in mice which had received a hydro-alcoholic extract of MO at doses of 0.125g/kg BW mice and 0.25g/kg BW mice for 7 and 14 days (Bharali et al., 2003). In comparison with our study, mice consumed diet containing bMO without solvent extraction at specified doses. Differences in various issues such as types of administration, cooking process and sample extraction can affect mechanism of action. However, there was statistically significant ($p<0.05$) decrease in the number of MNRETs in a dose dependent manner only in MMC-treated group at 48 h after mice received MMC in the experimental group of mice which received bMO at 4.3, and 8.5g/kg BW for 14 days and through the entire period of study.

From the findings of the present study, we can conclude that bMO at dose level 2.1, 4.3 and 8.5g/kg BW in mice

had no clastogenic effect and its anti-clastogenic potential is modulated via the direct acting carcinogenesis process related with DNA cross-linking agents.

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