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Clastogenic and Anticlastogenic Potential of Neem Flower Extract Evaluated by Rat Liver Micronucleus Assay

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Abstract

Clastogenic and anticlastogenic effects of neem flower (*Azadirachta indica* A. Juss) extract were determined using a rat liver micronucleus assay. Methanol extracts of neem flowers (MENF) in 15% Tween 80 at 100 and 500 mg/kg body weight were orally given to male Sprague Dawley rats daily for 6 weeks (2 weeks prior to and 4 weeks during repeated administration of diethylnitrosamine (DEN). All rats were anesthetized and their livers were collected. Hepatocytes were isolated from the livers using a collagenase solution without performing liver perfusion, then stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). Incidence of the micronucleated hepatocytes was evaluated by fluorescence microscopy. Results showed that MENF had no clastogenic effects on rat hepatocytes. On the contrary, MENF at high doses significantly reduced micronucleus formation in the rat livers compared to the control group. We concluded that MENF at 100 and 500 mg/kg body weight had no clastogenic effects. Instead, it possesses anticlastogenic potential in the rat liver, particularly at high doses.

Keywords: *Azadirachta indica*, anticlastogenic, clastogenic, liver micronucleus assay, rat

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บทคัดย่อ

ศักยภาพของสารสกัดดอกสะเดาต่อการเกิด/ยับยั้งการเกิดไมโครนิวเคลียสประเมินโดยใช้เซลล์ตับหนูแรท

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ทำการทดสอบศักยภาพของสารสกัดดอกสะเดา (*Azadirachta indica* A. Juss) ต่อการเกิด/ยับยั้งการเกิดไมโครนิวเคลียสในหนูแรทโดยวิธี Liver micronucleus assay โดยการป้อนสารสกัดดอกสะเดาด้วยเมทานอลซึ่งละลายใน 15% Tween 80 ขนาดความเข้มข้น 150 และ 500 มก./น้ำหนักตัว 1 กก. แก่หนูสายพันธุ์ Sprague Dawley เพศผู้ทุกวันเป็นเวลา 6 สัปดาห์ (โดยให้ 2 สัปดาห์ ก่อนได้รับสารก่อมะเร็ง diethylnitrosamine (DEN) และ 4 สัปดาห์ระหว่างได้รับสารก่อมะเร็ง) หลังจากนั้นทำการวางยาสลบและเก็บตับหนูเพื่อนำมาย่อยเซลล์ด้วยสารละลาย collagenase โดยไม่ต้องทำ perfusion ตับ และย้อมเซลล์ด้วย 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) ทำการนับเซลล์ด้วยกล้องจุลทรรศน์ชนิดฟลูออเรสเซนซ์ จากการทดลองพบว่าสารสกัดดอกสะเดาด้วยเมทานอลไม่ก่อให้เกิดไมโครนิวเคลียสในเซลล์ตับของหนูแรท ในทางตรงข้ามสารสกัดดอกสะเดาในขนาดความเข้มข้นสูงสามารถลดจำนวนไมโครนิวเคลียสได้อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุม สรุปได้ว่าสารสกัดดอกสะเดาด้วยเมทานอลในขนาดความเข้มข้น 150 และ 500 มก./น้ำหนักตัว 1 กก. ไม่ก่อให้เกิดไมโครนิวเคลียส แต่มีศักยภาพในการยับยั้งการเกิดไมโครนิวเคลียสในเซลล์ตับของหนูแรทโดยเฉพาะขนาดความเข้มข้นสูง

คำสำคัญ: ดอกสะเดา ไมโครนิวเคลียส เซลล์ตับ หนูแรท

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Introduction

We have reported that the extracts of neem flowers (*Azadirachta indica* A. Juss) and their young leaves exhibit antimutagenic effects against aflatoxin B₁ (AFB₁) and benz(a)pyrene towards *Salmonella typhimurium* (Rojanapo and Tepsuwan, 1992; Kusamran et al., 1998^a). In addition, neem flowers can inhibit 7, 12-dimethylbenz (a) anthracene (DMBA)-induced micronucleated erythrocyte formation in mice (Kupradinun et al., 1997). It has also been demonstrated that neem flowers can cause a marked increase in glutathione-S-transferase (GST) activity in rat liver, while significantly reducing the activity of certain hepatic P450-dependent mono-oxygenases (Kusamran et al., 1998^b). Recently, we have reported that neem flowers also possess strong chemopreventive potential to inhibit carcinogen-induced liver and mammary gland carcinogenesis in rats (Tepsuwan et al., 2002). Furthermore, petroleum ether, chloroform, ethyl acetate and methanol extracts of the neem flowers show the ability to induce quinone reductase activity in Hepa1c1 cells (Sritanaudomchai et al., 2005). In particular, methanol extract of neem flowers (MENF) has a high LD₅₀ value (> 12 g/kg body weight) with subacute toxicity on certain biochemical parameters at 750 and 1500 mg/kg body weight (Kupradinun et al., 2010).

Many investigators have tried to develop a liver micronucleus assay to assess cytogenetic damage

of chemicals *in vivo* (Braithwaite and Ashby, 1988; Suzuki et al., 2004; 2005), as it is well recognized that liver is the most active organ for chemical and xenobiotic detoxification. Moreover, some hepatocarcinogens such as di-alkyl-nitrosamines, nitro aromatic compounds and azo derivatives give negative results in a bone marrow assay (Angelosanto, 1995). A common method to assay liver micronuclei involves liver perfusion with or without partial hepatectomy, which is time-consuming and requires great technical expertise. In addition to this, partial hepatectomy can also stimulate mitotic activity in hepatocytes. Some investigators used 4-acetylaminofluorene (4AAF), a potent rat-liver mitogen, to activate liver cell proliferation (Braithwaite and Ashby, 1988) or used 4-week-old rats whose hepatocytes still had the capacity to proliferate (Parton and Garriott, 1997). In this study, we adapted the existing rat liver micronucleus assay protocol in order to screen for clastogenic and anticlastogenic effects of MENF without both partial hepatectomy and liver perfusion by repeated administration of DEN in adult rats.

Materials and Methods

Animals: Three weeks old male Sprague Dawley rats, (weighing of 41-52 gram), were purchased from the National Laboratory Animal Center (NLAC), Mahidol University, Thailand. The animals were maintained at

the Laboratory Animal Facility of the National Cancer Institute according to the Institute Care Guidelines, which was approved by the Animal Ethics Committee of the National Cancer Institute. The rats were acclimatized for 5 days in a clean conventional room (temperature of 23±2°C, lighting regimen of 12L/12D, relative humidity of 50±20%) and housed in filter-top plastic cages. The rats were allowed pellet diet (Perfect Companion Co Ltd, Thailand) and filtered water *ad libitum*.

Preparation of neem flower extract (MENF): Neem flowers were obtained from local markets in Bangkok and methanol extract was obtained as previously described by Kupradinun et al., 2010. The yield of dried extract from the freeze-dried neem flowers was approximately 25%.

Chemicals and treatment: Diethylnitrosamine (DEN), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and Tween 80 were purchased from Sigma Chemical Co (St. Louis, MO, USA). Collagenase was the product of Yakult Pharmaceutical Ind. Co. (Tokyo, Japan). Pentobarbital sodium was supplied by Ceva Sante Animale (Libourne, France).

After acclimatization, the rats were randomized and divided into five groups of 4-6 rats/group. Group 1 served as the control group, receiving 15% Tween 80 orally for 2 weeks prior to and during the DEN administration (12.5 mg/kg body weight daily for 4 weeks, ig). Groups 2 and 3 were given MENF daily (dissolved in 15% Tween 80) at 100 and 500 mg/kg body weight by gavage for 2 weeks prior to and during DEN administration. Groups 4 and 5 were fed MENF at 100 and 500 mg/kg body weight for 6 weeks. Body weights of all rats were recorded daily during the experiment.

Preparation of hepatocytes: After anesthetization with pentobarbital sodium (50 mg/kg body weight, ip), the rat's liver was removed and weighed at necropsy. Approximately 1 gram of the left lateral lobe was sliced to about 0.5 mm thickness and washed with Hank's balanced salt solution (HBSS). Prepared hepatic tissue was immersed in a collagenase solution (in HBSS, concentration of 100 U/ml, adjusted to pH 7.5), then incubated at 37°C in a shaking water bath at 50 rpm for 1 hour. Halfway through the incubation period, shaking was increased to 180-200 rpm for 1 min. After incubation, the tissue suspension was homogenized by trituration using a pipette. The hepatocyte suspension solution was then filtered through a 70 µm nylon mesh and mixed with an equal

volume of 10% neutral buffered formalin (NBF). The resulting solution was centrifuged at 50xg for 1 min. The pellet was then resuspended in 10% NBF and centrifuged again at 50xg for another 1 min. The final pellet was diluted with an appropriate amount of 10% NBF.

Micronucleus determination: The suspended hepatocytes were stained with DAPI (20 µg/ml) immediately prior to fluorescent cytological examination. Approximately 10-20 µl of stained hepatocytes were dropped on a clean glass slide and mounted with a cover slip. Hepatocytes were analyzed under a fluorescence microscope. Their clastogenic effects were evaluated by number of micronucleated hepatocytes (MNHEPs) of rats receiving either the vehicle control or MENF in accordance with protocols from Braithwaite and Ashby (1980) and Cllet et al. (1989). Frequencies of the MNHEPs were scored from examination of 2000 hepatocytes. MNHEPs were defined as previously described (Braithwaite and Ashby, 1980 and Cllet et al., 1989). Hepatocytes with round or distinct micronuclei stained with the same shade and having diameter of ¼ or less than that of the main nuclei were determined. Number of mitotic cells was also counted in 1000 hepatocytes of each animal to determine mitotic index. Mitotic cells were defined as cells at any stage from prophase to telophase (Suzuki et al., 2004).

Histopathological examination of liver: The remaining lobes of the livers after the collagenase isolation were fixed in 10% NBF. The liver tissues were stained with hematoxylin-eosin in accordance with the standard procedure for the histopathological evaluation.

Statistical analysis: A One-Way ANOVA with post hoc multi-comparison t-test was used to analyze the incidence of MNHEPs. Statistical significance was considered when *p*-value < 0.05.

Results

Effect of MENF on body weight, relative liver weight and histopathology of liver: The mean body weights of rats in the groups treated with DEN and MENF at doses of 100 and 500 mg/kg body weight (Groups 2 and 3) were lower than the control group (Group 1) but no significant difference was found (Table 1). The relative liver weight of rats treated with MENF at

Table 1 Effects of MENF on body and relative liver weights of rats

Groups of Treatment	No. of Rats	Body Weight (g) (Mean±SD)	Relative Liver Weight (%) (Mean±SD)
(1) Control (DEN)	4	314.15±21.40	3.22±0.18
(2) MENF 100mg/kg+DEN	4	290.78±28.23	2.98±0.31
(3) MENF 500mg/kg+DEN	6	286.32±31.54	3.07±0.19
(4) MENF 100 mg/kg	5	314.34±12.75	3.63±0.25
(5) MENF 500 mg/kg	4	342.30±6.39	4.15±0.22*

*Significantly different from the control group (*p* < 0.05)

high dose (Group 5) was significantly higher than that of the control group (*p* < 0.05). In addition, histopathological examination of the liver showed cellular alterations (Fig 1a) and hyperplastic nodules

(Fig 1b) in Groups 1, 2 and 3. However, the alterations found in Groups 2 and 3 were lower than the control group whereas those from the livers in Groups 4 and 5 were within normal limits.

Effect of MENF on micronucleus formation in rat hepatocytes: Table 2 shows the results of liver micronuclei observed in each group. The frequency of MNHEPs in Group 1 (control) was $1.956 \pm 0.764\%$ whereas the mitotic index was $1.056 \pm 0.345\%$. The micronuclei observed in Groups 4 and 5 (MENF at 100 and 500 mg/kg body weight) were $0.050 \pm 0.035\%$, $0.113 \pm 0.048\%$, respectively. MENF at both low and high doses with DEN (Groups 2 and 3) reduced

micronuclei formation in the rat livers ($1.275 \pm 0.348\%$ and $1.017 \pm 0.220\%$, respectively) but a significant difference was only found in Group 3 ($p < 0.05$). Mitotic indices were reduced in all experimental groups but only those from Groups 3 and 4 were significantly lower than that of the control group. The micrographs of micronuclei in rat hepatocytes stained with DAPI can be seen in Figure 2.

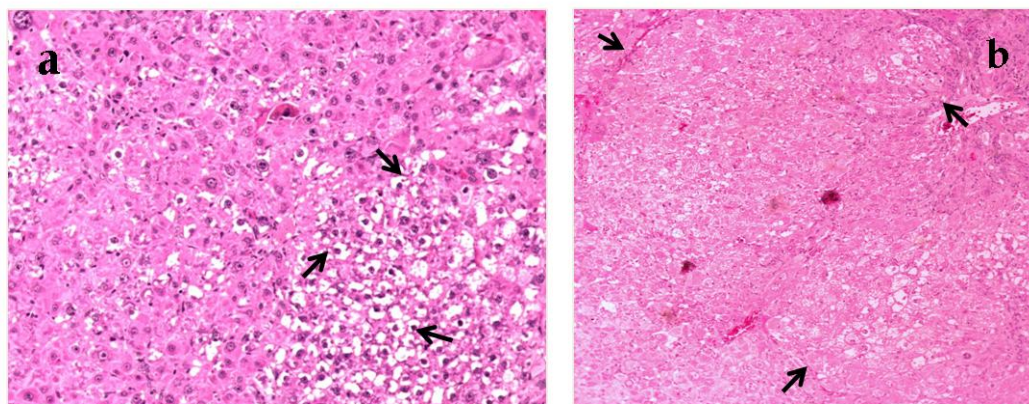


Figure 1 Histopathology findings of rat liver. (a) Cellular alterations; clear cell area are noted. Plant-cell-like hepatocytes (arrows) consist of cells with clear, ground glass containing glycogen. (b) Hyperplastic nodule (arrows); a distinct nodule compresses adjacent parenchyma and may bulge from the liver surface, consisting of enlarged cell with abundant, weakly eosinophilic cytoplasm and large nuclei. (H&E stain, mag (a) x20, (b) x10).

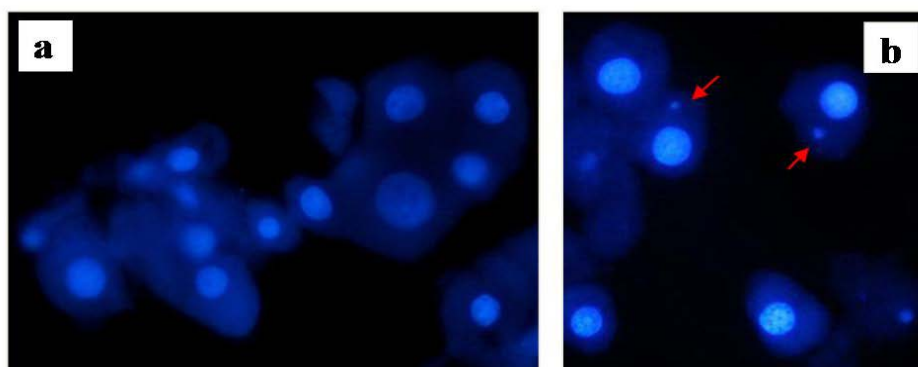


Figure 2 Fluorescent micrograph of micronucleated hepatocytes (MNHEPs) of rat stained with DAPI. (a) Normal hepatocytes have large round nucleus and are stained with blue color (x100). (b) Round micronuclei (arrows) are stained with the same color or lighter than the main nuclei and are 1/4 or less in diameter than the main nuclei (x100).

Table 2 Mean frequencies of MNHEPs and mitotic indices in rats

Groups of Treatment	MNHEPs (%) (Mean±SD)	Mitotic Cells (%) (Mean±SD)
(1) Control (DEN)	1.956 ± 0.764	1.056 ± 0.345
(2) MENF 100 mg/kg+DEN	1.275 ± 0.348	0.838 ± 0.312
(3) MENF 500 mg/kg+DEN	$1.017 \pm 0.220^*$	$0.450 \pm 0.117^*$
(4) MENF 100 mg/kg	$0.050 \pm 0.035^*$	$0.057 \pm 0.104^*$
(5) MENF 500 mg/kg	$0.113 \pm 0.048^*$	0.800 ± 0.178

Frequencies of MNHEPs were scored from 2000 hepatocytes

DEN: diethylnitrosamine, 12.5 mg/kg body weight for 4 weeks, MENF: methanol extract of neem flower

*Significantly different from the control group ($p < 0.05$)

Discussion

Previous reports strongly indicated that neem flowers contained cancer chemopreventive agents (Kupradinun et al., 1997; Kusamran et al.,

1998^b; Tepsuwan et al., 2002; Sritanaudomchai et al., 2005). It was also demonstrated in our laboratory that dietary intake of neem flowers caused a marked increase in the activity of GST in the rat liver as well as a significant reduction in hepatic P450-dependent

monooxygenases. In addition, neem flower extracts possess antimutagenic activity against AFB₁ and benz(a)pyrene towards *Salmonella typhimurium* (Rojanapo and Tepsuwan, 1992; Kusamran et al., 1998^a). Nakahara et al. (2008) has also shown the antimutagenic activity of MENF against Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole), Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) towards *Salmonella typhimurium* TA 98. In this study, the MENF at 100 and 500 mg/kg body weight had no clastogenic effect in the rat hepatocytes. An anticlastogenic effect was found particularly at high doses. It is possible that the chemical constituents in the flowers such as nimbolide, β -sitosterol, chlorophylls and flavonoids are accountable for the anticlastogenic effects (Subramanian and Nair, 1972; Sritanaudomchai et al., 2005). Moreover, four prenylated flavones possessing anticlastogenic effects were previously isolated from the methanol extract of neem flowers (Nakahara et al., 2003).

The body weight and relative liver weight of the rats treated with DEN and MENF were lower than the control and MENF treated groups. Histopathological changes of the liver such as hyperplastic nodules and cellular alterations were found more and were more severe in the control group (DEN only) than the groups treated with DEN and MENF at both low and high doses. Normal microscopic findings of liver were found in the groups treated with only the MENF at both low and high doses. It was indicated that MENF could promote health benefits in rats, while protecting the liver from carcinogens. The mitotic index observed in this study was comparable to a young rat liver micronucleus assay conducted by Suzuki (2004). The mean incidence of MNHEPs in low and high doses of MENF groups were relatively low when compared to the standard method.

Here, we adapted and simplified the conventional method for assaying liver micronuclei using adult Sprague Dawley rats without performing partial hepatectomy and liver perfusion to assess the clastogenic and anticlastogenic activity of neem flower extract in liver cells. We repeated the administration of DEN for 28 days, which reliably induced the formation of micronucleated hepatocytes. This method requires less technical expertise and demands less time and is less invasive, while providing the assessment of high sensitivity relative to previously described protocols (Braithwaite and Ashby, 1980; Cliet et al., 1989; Suzuki et al., 2004). Therefore, we propose that this protocol is a highly valuable tool for evaluating the clastogenic and anticlastogenic effects of dietary substances with medicinal properties and other ingested chemical compounds.

In conclusion, the methanol extract of neem flowers at 100 and 500 mg/kg body weight had no clastogenicity. On the contrary, it possesses anticlastogenic properties in the rat liver at high doses.

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