Original Article

Toxicity Testing of Flowers of Neem Tree (*Azadirachta indica* A. Juss)

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Abstract

Flowers of neem (*Azadirachta indica* A. Juss) possess a strong cancer chemopreventive potential in rats as well as antimicronucleus formation in mice. However, toxicity testing has never been evaluated. This study was aimed to determine its acute and subacute toxicities in Wistar rats. Acute toxicity was conducted by feeding methanol extract of neem flowers (MENF) suspending in 20% propylene glycol at 6, 9 and 12 g/kg bw. On the other hand, subacute toxicity testing was carried out by feeding MENF suspending in 0.5% tragacanth at 150, 750 and 1,500 mg/kg bw for 90 consecutive days. Hematology, blood chemistry and histopathology were evaluated at the end of the experiment. The results demonstrated that MENF had LD_{50} value higher than 12 g/kg bw. In subacute toxicity study, MENF caused a significant decrease in the growth of male, but not female rats, but significantly increased the relative liver weights of those receiving MENF at 750 and 1,500 mg/kg bw. Blood chemistry values of most rats were within normal ranges. However, ALP, creatinine and potassium values were significantly higher in female group receiving MENF at 750 mg/kg bw while in male rats, the levels of AST and BUN were lower but that of creatinine was higher than those of the control groups. Histopathological examination of visceral organs showed no significant change. In conclusion, LD_{50} value of MENF in rats was greater than 12 g/kg bw (~ 800 times of human use). Subacute toxicity testing at 750 and 1,500 mg/kg bw (~ 50 and 100 times of human use) showed the effects on some biochemical parameters.

Keywords: *Azadirachta indica*, neem flowers, rat, toxicity

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บทคัดย่อ

การศึกษาความเป็นพิษของดอกสะเดา

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ดอกสะเดา (*Azadirachta indica* A. Juss) มีคุณสมบัติเป็นสารเคมีป้องกันมะเร็งในหนูแร็ท และต้านการเกิดไมโครนิวเคลียสในหนูเม้าส์ อย่างไรก็ตามยังไม่มีการทดสอบถึงความเป็นพิษ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อทดสอบความเป็นพิษทั้งชนิดเฉียบพลันและกึ่งเฉียบพลัน ในหนูแร็ทสายพันธุ์วิสต้าร์ การทดสอบพิษเฉียบพลันทำโดยป้อนสารสกัดด้วยเมทานอลที่ละลายในโพรไพลีนไกลคอลร้อยละ 20 ในขนาด 6, 9 และ 12 ก./กก. ขณะที่การทดสอบพิษกึ่งเฉียบพลันใช้ละลายสารสกัดด้วยทรากาแค็นท์ร้อยละ 0.5 ในขนาด 150, 750 และ 1,500 มก./กก. แล้วป้อนนาน 90 วัน เมื่อสิ้นสุดการทดลองเจาะเลือดเพื่อตรวจวัดค่าทางโลหิตวิทยาและเคมีคลินิก ชันสูตรซากเพื่อตรวจดูลักษณะของอวัยวะต่างๆทางจุลพยาธิวิทยา ผลการทดลองพบว่า ขนาดของสารสกัดดอกสะเดาที่ทำให้หนูตายครึ่งหนึ่ง (LD_{50}) มีค่าสูงกว่า 12 ก./กก. สำหรับการทดสอบพิษกึ่งเฉียบพลันพบว่า น้ำหนักตัวของหนูเพศเมียไม่แตกต่างจากหนูกลุ่มควบคุมทั้ง 2 กลุ่ม (น้ำและทรากาแค็นท์) แต่น้ำหนักตับสัมพัทธ์สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ส่วนหนูเพศผู้มีน้ำหนักตัวน้อยกว่ากลุ่มควบคุม และน้ำหนักตับกลับสูงกว่ากลุ่มควบคุมเช่นเดียวกับหนูเพศเมีย ส่วนค่าเคมีคลินิกอยู่ในระดับปกติยกเว้น ระดับของ ALP, creatinine และโปแตสเซียม มีค่าสูงอย่างมีนัยสำคัญในหนูเพศเมียที่ได้รับสารสกัดในขนาด 750 มก./กก. ขณะที่ในหนูเพศผู้ ระดับของ AST และ BUN มีค่าต่ำ แต่ค่า creatinine สูงกว่าหนูกลุ่มควบคุมทั้ง 2 กลุ่ม ผลทางจุลพยาธิวิทยาของอวัยวะต่างๆพบว่าไม่มีการเปลี่ยนแปลง การศึกษานี้สรุปได้ว่าขนาดของสารสกัดดอกสะเดาที่ทำให้หนูตายครึ่งหนึ่ง มีค่ามากกว่า 12 ก./กก. (~ 800 เท่าของขนาดที่คนบริโภค) และ การทดสอบพิษกึ่งเฉียบพลันในขนาด 750 และ 1,500 มก./กก. (~ 50 และ 100 เท่าของขนาดที่คนบริโภค) มีผลต่อค่าเคมีคลินิกบางตัว

คำสำคัญ: ดอกสะเดา พิษเฉียบพลัน พิษกึ่งเฉียบพลัน หนูแร็ท

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Introduction

The flowers of neem tree (*Azadirachta indica* A. Juss, family Meliaceae), Thai variety, is the common vegetable eaten in Thailand especially in the winter. Neem is one of the most widely used as medicinal herbs in the world and is a cornerstone of the Ayurvedic medicine for treatment of skin diseases and dental disorders. It also possesses antimicrobial, antiviral, antifungal, antimalarial, antioxidation and anticarcinogenesis properties (Arakaki et al, 2006; Badam et al., 1987; Dasgupta et al., 2004; Mitra and Patel 1963; SaiRam et al., 1997; 2000; Sithisan et al, 2005). We have reported that the extracts of neem flowers and young leaves exhibited antimutagenicity against indirect mutagens/ carcinogens, namely aflatoxin B_1 (AFB_1) and benz(a)pyrene [B(a)P] towards *Salmonella typhimurium* (Rojanapo and Tepsuwan, 1992; Rojanapo et al., 1998). In addition, neem flowers could inhibit micronucleated erythrocytes formation in mice induced by 7, 12-dimethylbenz(a)anthracene (DMBA) (Kupradinun et al., 1997). It has been clearly demonstrated in our laboratory that neem flowers caused a marked increase in glutathione-*S*-transferase (GST) activity in the rat liver, while resulting in a significant reduction in the activities of some hepatic P450-dependent monooxygenases (Kusamran et al., 1998). Recently, we have reported that neem flowers possessed a strong chemopreventive potential against AFB_1 and DMBA-induced liver and mammary gland carcinogenesis in rats (Tepsuwan et al., 2002). Furthermore, petroleum ether, chloroform, ethyl acetate and methanol extract of neem flowers showed the induction capacity of quinone reductase activity in mouse hepatoma Hepa 1c1c culture cells (Srithanaudomchai, 2005). Therefore, we would like to know whether neem flowers are safe or not if taken for chemopreventive purpose.

Materials and Methods

Preparation of neem flowers extract (MENF): Neem flowers were obtained from local markets in Bangkok. Flowers were removed from the stems, washed with tap and distilled water, and then lyophilized yielding the dry flowers about 10%. Freeze-dried materials were blended to powder and extracted with methanol (20 ml/g) and the suspension left at room temperature overnight. Methanol extract was collected by paper filtration and the extraction was repeated by the same condition. Two methanol fractions were pooled and evaporated by rotary evaporator apparatus (Buchi R-200) at 40^0-45^0C until dryness. The yield of dried extract from freeze-dried neem flowers was about 25%.

Animals and diets: Both male and female Wistar rats, aged 5-6 weeks old, 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 was approved by the Animal Ethic Committee. All animals were housed in the stainless steel cages in a clean conventional room at 23±2^{0}C and humidity 50±20% with a 12-h light/dark cycle. They were provided with a pellet diet (Perfect Companion Group Co. Ltd., Thailand) and water ad libitum.

Treatment of the animals:

Acute toxicity study: Eighty rats (40 males & 40 females), after acclimatization for one week, were randomly divided by weight into 4 groups and were orally administered of 20% propylene glycol in the control and methanol extract of neem flowers (MENF) suspension in 20% propylene glycol at the doses of 6, 9 and 12 g/kg bw which were approximately equivalent to 24, 36 and 48 g/kg bw of freeze-dried neem flowers. The animals were observed for mortality or any sign of abnormality periodically during the first 24 h and twice daily for 14 days thereafter.

Subacute toxicity study: Sixty seven rats of each sex, after acclimatization, were randomly divided by weight into 5 groups of 10-15 rats each. Three groups were assigned as experimental groups which were fed MENF suspension in 0.5% tragacanth daily for 90 consecutive days at the doses of 150, 750 and 1,500 mg/kg bw. These doses were equivalent approximately to 0.6, 3 and 6 g freeze-dried neem flowers. Another two groups were served as control groups receiving either water or 0.5% tragacanth. Body weight was measured weekly and all animals were observed for sign of abnormalities throughout the experiment.

At the end of the study, animals were fasted for several hours, anesthetized with diethylether and sacrificed by drawing blood from posterior vena cava for hematological and biochemical examinations. Hematological analysis was performed using an automatatic hematological analyser (Cell dyne 3500, Abbott). Hematological parameters measured were white blood cell (WBC), % of neutrophil, lymphocyte, monocyte, eosinophil and basophil, red blood cell (RBC), hemoglobin, % of hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoblobin (MCH), mean corpuscular hemoblobin concentration (MCHC), red cell distribution width (RDW) and platelet. Biochemical analysis of serum samples was performed using an automatic chemistry analyzer (Hitachi model 917). Biochemical parameters measured were aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, blood urea nitrogen (BUN), creatinine, uric acid, albumin, sodium, potassium, chloride and carbondioxide. The internal organs were visually observed for any sign of gross lesion, removed, weighed and then fixed with 10% buffered formalin prior to performing histopathological examination by conventional methods.

Statistical Analysis: The data were analyzed using one way ANOVA and Krusskal -Wallis H test at $p<0.05$.

Results

Acute toxicity study: MENF at the doses of 6, 9 and 12 g/kg bw which were equivalent approximately to 400, 600 and 800 times of human use caused no sign and symptom of toxicity in both male and female rats, indicating the LD_{50} value being greater than 12 g/kg bw.

Subacute toxicity study:

Effect of MENF on body weight and relative organ weight: MENF at the doses of 150, 750 and 1,500 mg/kg bw which were equivalent approximately to 10, 50 and 100 times of human use did not have any effect on the growth rate of the female rats, whereas for the male rats, the body weight of all treated groups was significantly lower than that of both control groups (Figure 1). The relative liver weight of the males in high dose group (1,500 mg/kg/day) and of females in groups receiving MENF 750 and 1,500 mg/kg/day was significantly higher than that of the control groups (Table 1).

Effect of MENF on hematological and biochemical parameters: In both male and female rats, there was no difference in most hematological parameters between the controls and treated groups (Table 2), with the exception that MCHC value in all treated male rats was significantly lower than that in both water and tragacanth control groups. In the high dose male group, AST and BUN levels were significantly lower than that in the water control group (Table 3). On the other hand however, creatinine level in the high dose group was significantly higher than that in both water and tragacanth control groups while that in the medium dose group was significantly higher than that only in the tragacanth control group. In the female, only the medium dose group, showed higher level of ALP than that in both water and tragacanth control groups while the levels of creatinine and potassium were significantly higher than only that in the tragacanth control group (Table 3). In addition, creatinine level in all treated groups was significantly higher than that in the tragacanth control group and only that in the high dose group was also higher than that in the water control group.

Effect of MENF on histopathology: From gross examination of visceral organs, no significant gross lesion was observed in any organs of both male and female rats. Histopathological examination of visceral organs were performed on the brain, heart, lung, thyroid gland, liver, kidney, spleen, pancreas, stomach, intestine, bladder, including testis and prostate gland in male rats or uterus and ovary in the females. There was no remarkable histopathological lesion between the controls and treated groups.

Table 1 Relative organ weight (g/100g bw) of rats treated with MENF for 90 days in subacute toxicity study.

Parameters	Male rats (g/100g bw)					Female rats (g/100g bw)				
	Control		MENF (mg/kg)			Control		MENF (mg/kg)		
	Water (n=7)	Tragacanth (n=12)	150 (n=15)	750 (n=14)	1,500 (n=11)	Water (n=9)	Tragacanth (n=12)	150 (n=11)	750 (n=11)	1,500 (N=15)
Brain	0.43 ± 0.06	0.46 ± 0.06	0.46 ± 0.05	0.48 ± 0.05	0.46 ± 0.04	0.65 ± 0.14	0.46 ± 0.06	0.70 ± 0.05	0.66 ± 0.05	0.67 ± 0.05
Heart	0.28 ± 0.02	0.27 ± 0.03	0.28 ± 0.04	0.28 ± 0.03	0.28 ± 0.03	0.31 ± 0.03	0.33 ± 0.10	0.29 ± 0.03	0.31 ± 0.04	0.31 ± 0.03
Lung	0.43 ± 0.06	0.49 ± 0.14	0.43 ± 0.08	0.46 ± 0.07	0.42 ± 0.06	0.50 ± 0.09	0.55 ± 0.12	0.53 ± 0.09	0.52 ± 0.06	0.51 ± 0.09
Stomach	0.71 ± 0.17	0.66 ± 0.29	0.72 ± 0.33	0.66 ± 0.19	0.78 ± 0.31	0.74 ± 0.16	0.75 ± 0.15	0.84 ± 0.17	0.85 ± 0.24	0.85 ± 0.22
Liver	2.81 ± 0.44	2.84 ± 0.16	2.87 ± 0.23	2.94 ± 0.27[b]	3.35± 0.24[a]	2.62 ± 0.24	2.66 ± 0.50	2.57 ± 0.74	3.01 ±0.44[a,b]	3.17 ±0.31[a,b]
Rt. Kidney	0.26 ± 0.04	0.25 ± 0.02	0.26 ± 0.03	0.27 ± 0.03	0.27 ± 0.03	0.31 ± 0.03	0.29 ± 0.03	0.24 ± 0.10	0.31 ± 0.03	0.29 ± 0.05
Lt. Kidney	0.26 ± 0.04	0.25 ± 0.02	0.25 ± 0.02	0.27 ± 0.06	0.27 ± 0.04	0.27 ± 0.03	0.28 ± 0.04	0.26 ± 0.09	0.28 ± 0.05	0.27 ± 0.02
Spleen	0.19 ± 0.04	0.21 ± 0.03	0.20 ± 0.03	0.22 ± 0.04	0.22 ± 0.04	0.32 ± 0.03	0.23 ± 0.05	0.23 ± 0.02	0.24 ± 0.03	0.25 ± 0.04

Values are Mean±SD, [a]Significantly different from water control group (p<0.05), [b]Significantly different from tragacanth control group (p<0.05)

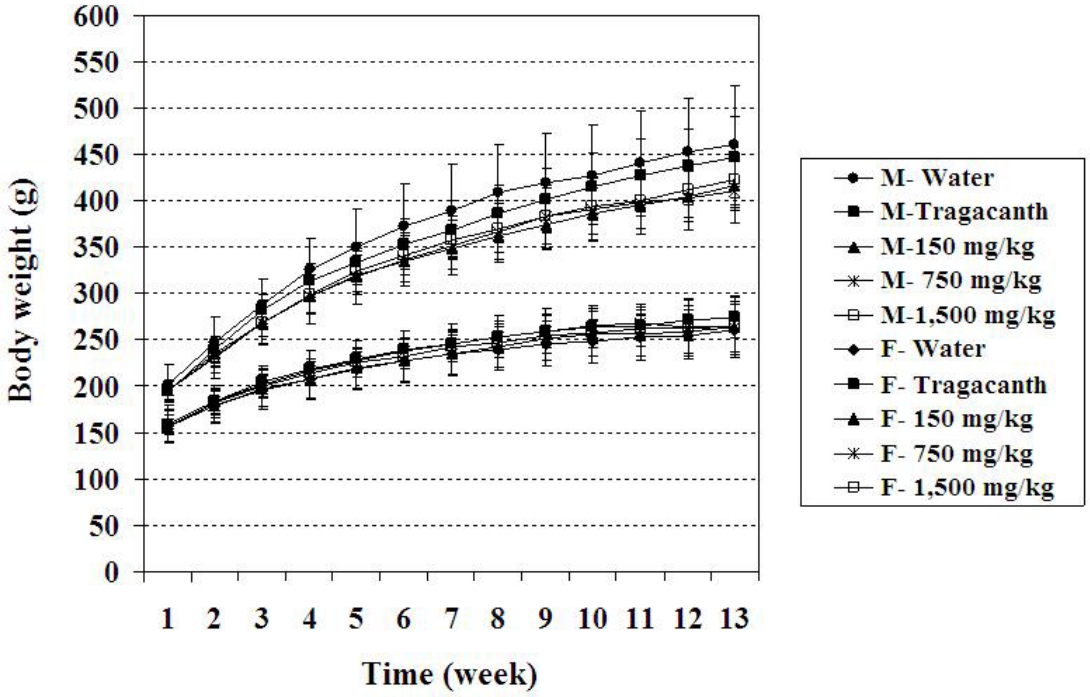

Figure 1 Growth curve of rats treated with MENF for 90 days in subacute toxicity study.

Discussion

For the acute toxicity of MENF in this study, we found that MENF had high LD_{50} value, greater than 12 g/kg bw which was equivalent approximately to 800 times of human use. We calculated the dose usage in animals in this study depending on the maximum serving size in human which is about 35g of fresh vegetable per day per 50 kg human bw. After lyophilization of fresh neem flowers, it resulted 10% freeze-dry material and gave 25% yield of MENF after extraction and evaporation. So the maximum serving size of MENF in human is 17.5 mg/kg bw/day.

There was limitation of the administration of high dose of MENF to the animals because MENF could not be dissolved in water, so we had to dissolve it in propylene glycol or tragacanth instead and the maximum volume of intake in rats was 1 ml/100 g bw (OECD, 2001). In the acute toxicity study, when we would like to use high dose (12 g/kg bw) of the extract sample, it caused the obstruction of the gastric tube. To reduce this problem, we had to give MENF to animals by dividing dose (twice daily) instead of a single high dose.

Our result found in this study is compatible to that of Okapanyi (Okpanyi et al., 1981) who showed that LD_{50} value of ethanol extract of neem bark and leaf in mice was about 13 g/kg. The ethanol extract of neem leaf giving by subcutaneous injection at the dose of 10 g/kg caused no toxic to the mice (Mokkhasmit et al., 1971). The aqueous extract of neem leaves was not toxic to mice up to the dose of 1 g/kg (Subapriya et al, 2005[b]). In contrast, Chattopadhyay reported in 1988 that LD_{50} value of ethanol extract of neem leaf in male rats was 4.57 g/kg.

Azadirachtin is the chemical ingredient which is found in all parts of the tree while nimbolide, β-sitosterol, chlorophylls and flavonoids are major constituents in the flowers (Subramanian and Nair, 1972; Srithanaudomchai et al, 2005). The acute oral toxicity in rats fed azadirachtin in other studies ranged from greater than 3.5 to 5 g/kg (EPA, 1993; Farm Chemicals, 1995; Thomson, 1992).

The result of subacute toxicity study of MENF demonstrated that MENF at the doses used affected the growth rate of only male rats but affected both the weight and some functions of liver of both male and female rats. It also caused an increase in the creatinine level in the high dose groups, indicating that it may affect the kidney function. For hematological and biochemical parameters, it can be seen that some values were fluctuated. It might be that rats have high level of hematocrit and pack red cells, so clot blood can easily occurred and cause error in the determination of components in the serum. On the other hand however, histopathological finding of the visceral organs was within normal limit. There were only hydropic degeneration and fatty change in the liver which were observed only in the high dose groups.

Table 2 Hematological values in rats treated with MENF for 90 days in subacute toxicity study.

Parameters	Male rats					Female rats				
	Control		MENF (mg/kg)			Control		MENF (mg/kg)		
	Water (n=7)	Tragacanth (n=12)	150 (n=15)	750 (n=14)	1,500 (n=11)	Water (n=9)	Tragacanth (n=12)	150 (n=11)	750 (n=11)	1,500 (N=15)
WBC (x10^3/µl)	8.21±4.6	6.90±2.0	8.88 ± 3.5	6.89 ± 2.3	8.18 ± 3.4	5.71±2.2	4.78±1.3	6.80 ± 2.4	5.47 ± 0.9	6.42 ± 2.1
RBC (x10^6/µl)	8.05±2.4	9.22±0.6	9.23±0.4	8.62±2.0	8.78±0.9	8.65±0.4	8.52±0.4	8.44±0.5	8.48±0.5	7.91±0.7
Hemoglobin (g/dL)	14.12±4.1	16.16±0.9	15.82±0.8	14.65±3.5	15.58±0.8	15.73±0.7	15.72±0.4	15.51±0.7	15.43±0.9	14.51±1.6
Hematocrit (%)	62.9±24.4	75.44±4.4	76.78±3.2	70.24±16.7	75.15±4.1	74.46±3.1	74.58±2.1	73.45±4.0	74.03±4.4	69.58±4.9
MCV (fl/red cell)	75.7±13.9	80.4±9.5	83.21±3.3	81.48±2.0	85.9±5.0	86.14±2.5	87.6±2.1	87.07±2.1	87.4±3.5	88.05±4.0
MCH (pg/red cell)	17.67±0.6	17.54±0.5	17.17±0.6	17.011±0.5	17.8±1.1	18.21±0.5	18.24±0.9	18.41±0.5	18.21±0.9	18.5±0.9
MCHC (g/dL)	24.24±5.6	22.22±3.8	20.62±0.5[a]	20.889±0.1[a]	20.7±0.1[a,b]	21.13±0.3	20.84±0.9	21.16±0.2	20.83±0.5	21.02±0.7
RDW (%)	16.26±1.2	16.19±1.1	16.08±1.0	16.1±1.4	17.33 0.7	14.64±0.9	14.625±0.6	14.92±0.6	14.71±1.0	14.87±1.7
Neutrophil (%)	11.20±5.5	17.53±15.8	18.91±15.7	15.62±4.2	18.72±13.8	12.77±7.4	8.61±4.9	10.37±6.4	18.08±9.5	14.50±6.3
Lymphocyte (%)	79.8±4.8	70.46±15.1	71.09±15.5	72.59±4.2	72.0±16.8	77.95±8.4	82.06±6.4	80.06±7.7	70.1±13.7	75.75±7.4
Monocyte (%)	2.98±1.3	2.85±0.8	2.68±1.1	2.72±1.0	3.61±1.5	2.90±1.3	2.92±1.1	2.80±1.4	3.73±2.8	2.14±1.2
Eosinophil (%)	1.56±0.8	2.37±1.6	1.76±0.7	1.48±0.6	1.03±0.7	3.09±4.8	1.36±0.2	1.33±0.4	1.85±0.7	1.77±1.0
Basophil (%)	4.47±2.5	6.80±2.4	5.57±2.4	7.60±2.2	4.65±2.1	4.86±1.7	5.04±2.3	5.43±2.1	6.24±1.9	5.85±2.5
Platelet (x 10^3/µl)	455.69±302.5	634.78±203.2	520.7±252.5	707.78±203.3	443.75±258.6	756.63±74.6	790.88±46.0	696.63±238.9	700.86±145.6	655.78±191.5

Values are Mean±SD, [a]Significantly different from water control group (p<0.05), [b]Significantly different from tragacanth control group (p<0.05)

Table 3 Biochemical values of rats treated with MENF for 90 days in subacute toxicity study.

Parameters	Male rats					Female rats				
	Control		MENF (mg/kg)			Control		MENF (mg/kg)		
	Water (n=7)	Tragacanth (n=12)	150 (n=15)	750 (n=14)	1,500 (n=11)	Water (n=9)	Tragacanth (n=12)	150 (n=11)	750 (n=11)	1,500 (N=15)
AST (U/L)	187.7± 85.6	169.9 ± 76.7	174.9 ± 85.4	134.0 ± 44.1	114.6 ±27.4[a]	98.4 ± 16.9	115.67 ± 80.7	78.6 ± 14.5	170.4 ± 139.9	140.3 ± 160.1
ALT (U/L)	93.6± 85.4	97.4 ± 74.9	83.7 ± 58.0	66.6 ± 43.5	62.2 ± 28.7	37.7 ± 13.3	36.25 ± 8.7	29.8 ± 6.5	49.6 ± 28.8	38.9 ± 26.8
ALT (U/L)	83.1 ± 12.6	80.7 ± 10.3	104.1 ± 32.2	80.4 ± 9.0	86.6 ± 13.4	30.0 ± 6.7	39.33 ± 15.4	37.3 ± 7.7	46.1 ± 12.3[a,b]	32.7 ± 8.6
GLU (mg/dl)	135.4 ± 29.8	124.1 ±29.2	153.3 ± 33.9	145.0 ± 27.1	173.2 ± 56.2	146.7 ± 14.5	151.08 ± 14.8	154.6 ± 14.3	139.7 ± 35.0	153.3 ± 43.9
BUN (mg/dl)	26.3 ± 2.9	24.7 ±2.3	26.3 ± 2.8	23.7 ± 3.4	21.4 ± 2.2[a]	22.3 ± 2.4	22.33 ± 2.5	21.6 ± 1.6	25.1 ± 5.3	23.1 ± 4.1
CREA (mg/dl)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1[b]	0.6 ± 0.1[a,b]	0.6 ± 0.1	0.53 ± 0.1	0.6 ± 0.1[b]	0.6 ± 0.1[b]	0.7 ± 0.1[a,b]
UA (mg/dl)	1.3 ± 0.2	1.2 ± 0.4	1.4 ± 0.9	1.2 ± 0.6	1.9 ± 0.9	1.3 ± 0.3	1.21 ± 0.6	1.3 ± 0.5	1.2 ± 0.5	1.6 ± 0.9
Alb (g/dl)	ND	ND	ND	ND	ND	4.4 ± 0.3	4.63 ± 0.2	4.6 ± 0.2	4.4 ± 0.3	4.4 ± 0.3
Na (mmol/l)	148.8 ± 1.0	150.5 ± 1.1	149.4 ± 1.7	149.8 ± 1.5	150.0 ± 1.7	148.4 ± 0.9	148.55 ± 2.0	147.7 ± 1.5	146.8 ± 2.1	147.7 ± 1.7
K (mmol/l)	4.2 ± 0.4	4.2 ± 0.5	4.0 ±0.3	4.0 ± 0.4	4.2 ± 0.6	3.8 ± 0.7	3.5 ± 0.3	3.6 ± 0.3	4.1 ± 0.7[b]	4.1 ± 0.8
Cl (mmol/l)	108.3 ±1.7	108.9 ± 0.7	109.5 ± 1.9	110.6 ± 1.8	110.0 ± 1.2	111.9 ± 1.3	112.64 ± 1.4	112.8 ± 1.5	111.6 ± 2.8	113.1 ± 2.4
CO_2 (mmol/l)	23.0 ± 1.4	24.5 ± 1.0	24.4 ± 1.6	24.0 ± 1.5	20.4 ± 2.8	23.2 ± 1.0	24.27 ± 1.7	23.5 ± 1.0	22.7 ± 3.1	21.9 ± 4.4

Values are Mean±SD, [a]Significantly different from water control group (p<0.05), [b]Significantly different from tragacanth control group (p<0.05)
ND: not determined

Our results might be different from that found by Gbotolorun et al. (2008) who found that alcoholic extract of neem flowers at the dose of 1 g/kg bw for 3 weeks caused significant reduction in the body weight of female rats. This may be due to the difference in the strain of rat used. However, our results might agree with those reported by AgriDyne (1995) that azadirachtin given to rats at 0.5, 2 and 10 g/kg showed no signs of overt systemic toxicity at any dose level after 90 days of feeding except mean body weight was significantly decreased in group fed 10 g/kg in both male and females.

In conclusion, results in the present study revealed that methanol extract of neem flowers had high LD_{50} value, greater than 12 g/kg bw which was about 800 times of human use. In subacute toxicity, methanol extract of neem flowers showed slightly toxicity to rats at the dose greater than 150 mg/kg/day (10 times of human use). These results were inconclusive to suggest whether neem flowers are safe to be used as chemopreventive agent in human. Further studies are needed to be performed before planning the clinical trial.

Acknowledgement

This study was financially supported by a grant from the Department of Medical Services and the National Cancer Institute, Ministry of Public Health, Thailand.

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