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Anethum graveolens Linn. (dill) extract enhances the mounting frequency and level of testicular tyrosine protein phosphorylation in rats*

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Abstract: Objective: To investigate the effect of *Anethum graveolens* (AG) extracts on the mounting frequency, histology of testis and epididymis, and sperm physiology. Methods: Male rats induced by cold immobilization before treating with vehicle or AG extracts [50, 150, and 450 mg/kg body weight (BW)] via gastric tube for consecutive 1, 7, and 14 d were examined for mounting frequency, testicular phosphorylation level by immunoblotting, sperm concentration, sperm acrosome reaction, and histological structures of testis and epididymis, respectively. Results: AG (50 mg/kg BW) significantly increased the mounting frequency on Days 1 and 7 compared to the control group. Additionally, rat testis treated with 50 mg/kg BW AG showed high levels of phosphorylated proteins as compared with the control group. In histological analyses, AG extract did not affect the sperm concentration, acrosome reaction, and histological structures of testis and epididymis. Conclusions: AG extract enhances the aphrodisiac activity and is not harmful to sperm and male reproductive organs.

Key words: *Anethum graveolens* Linn. (dill), Mounting frequency, Testicular phosphorylation, Sperm acrosome reaction
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1 Introduction

Sexual dysfunction in males results from various stresses and is an important problem in developed and developing countries, including Thailand. In the Western world, a major reason for divorce is the decreased frequency of male mating behaviors (Amato and Previti, 2003). Currently, drugs against erectile dysfunction, including sildenafil citrate (also known as Viagra), are commercially available, but there are some side effects on other vital organ and they are expensive (Mirone *et al*., 2009; Aliferis *et al*., 2012). Therefore, the search for alternative remedies, especially medicinal plants that are effective, non-toxic, and inexpensive in treating sexual dysfunction, is required.

The physical arousal of males is a very complex mechanism via the neuro-gonadal pathway. At the protein posttranslational level, the increments of protein phosphorylation in the male reproductive

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system are found before and during arousal (Wang *et al*., 2007). Basically, protein phosphorylation is essential for sperm and testosterone production in testis (Fardilha *et al*., 2011; Yamashita *et al*., 2011). In fertile males, these phosphorylation events are involved in sperm capacitation and acrosome reaction (Morales *et al*., 2007; Bailey, 2010).

Anethum graveolens (AG) Linn. (also called dill or "Pakchee Lao" in Thais), a vegetable, is commonly used for cooking in Southeast Asia. In addition, AG has been demonstrated to possess significant mucosal protective, antimicrobial, antigastric irritation, and antioxidant activities (Chaurasia and Jain, 1978; Möhle *et al*., 1985; Mahran *et al*., 1992; Faber *et al*., 1997; Yazdanparast and Alavi, 2001; Hosseinzadeh *et al*., 2002; Delaquis *et al*., 2002). Moreover, previous studies have shown that AG could stimulate milk production and change the estrous cycles (Monsefi *et al*., 2006). Indeed, these reports suggest that AG affects the hypothalamo-gonadal axis in females. However, the effects of AG on the male reproductive system have never been documented. Possibly, AG may also affect such axis on the male system that may result in enhancing male sexual behaviors or affect male reproductive organs. The objective of this investigation is to study the effect of AG on male mounting frequency and its toxicity to sperm morphology and function.

2 Materials and methods

2.1 Animals and mounting screening

Forty adult male rats (200–250 g) provided by the Experimental Animal Unit of Medicine Faculty, Khon Kean University, Thailand were determined for mounting frequency using an active estrous female induced with estrogen as a libido inducer. These animals were divided into four groups. The control or vehicle group was fed with distilled water, while the AG-treated groups received the solution of AG extracts [50, 150, and 450 mg/kg body weight (BW)] via gastric tube. The administration was performed for 14 consecutive days. This study was approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand.

2.2 Treatment and determination of mounting frequency

All animals were stressed by cold immobilization at 4 °C for 30 min to reduce their mounting behaviors before treating with vehicle (polyethylene glycol; PEG diluent) or AG extracts provided by Dr. Panee SRISAARD (Department of Pharmacology, Faculty of Medicine, Chiang Mai University, Thailand). On Days 1, 7, and 14 after AG administration for 30 min, the rats in all groups were examined for the mounting frequency within 1 h after libido stimulation by facing with an active estrous female rat.

2.3 Histopathologic examinations of testis and epididymis

At the end of the experiment, all animals were sacrificed and right testis and epididymis were isolated. The tissues were dissected out and cleaned up to be free from fat pads. They were observed for gross lesions and fixed with 10% formalin in phosphate buffered saline (PBS) (pH 7.4) followed by routine histological processing (5 μm in thickness of the section) and observed under a Nikon ECLIPSE E200 Microscope.

2.4 Western blot analysis

To study the effect of AG extract on the level of testicular protein phosphorylation, the left testis of the mounting-positive group was rapidly kept at 4 °C. Then, the testicular total protein lysate was prepared by homogenization of testis with cocktail protease inhibitors added-RIPA buffer (Cell Signaling Technology Inc., USA). The testicular homogenate was centrifuged at 12000 r/min for 10 min and the supernatant (called testicular total protein lysate) was collected to measure the total protein concentration by NANO drop (NanoDrop ND-1000 Spectrophotometer V3.5 User's Manual, NanoDrop Technologies Inc., USA). Fifty micrograms of total testicular proteins pooled from triplicate samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membrane to detect the intensity of phosphorylation by using the 4G10 primary antibody [1:3000 (v/v), Millipore Corporation, USA]. In immuno Western blotting, bovine serum albumin (BSA; AMRESCO®, USA) and epidermal growth factors

(EGFs; Millipore Corporation, USA) were used as the negative control and positive control, respectively. To detect the phosphorylated proteins, the enhanced chemiluminescence (ECL) substrate was used before visualization under Gel Doct 4 (ImageQuant 400, GH Healthcare, USA).

2.5 Sperm concentration and acrosome reaction analyses

Sperm from the right epididymis and vas deferens were dipped into 1 ml KSOM medium (Millipore). Sperm suspension was diluted (1:20, v/v) with PBS before double counting on hematocytometer under light microscope. In the acrosome reaction assay (Bendahmane *et al.*, 2002; Iamsaard *et al*., 2011), the sperm suspension was centrifuged at 500×*g* for 5 min, and the pellet (composed of sperm cells) was collected. The sperm pellet was fixed with 4% (0.04 g/ml) para form aldehyde-PBS, pH 7.4, at room temperature for 15 min and washed twice by PBS. The sperm suspension was dropped and air-dried on a 0.25% (2.5 g/L) gelatin-coated slide. The dried sperm cells were stained by 0.22% (2.2 g/L) Coomassie blue G-250 (containing 50% CH_3OH, 10% glacial acetic acid, 40% H_2O) for 5 min. The slide was mounted with PBS-glycerol, and observed under a light microscope. Sperm with an intact acrosome showed blue staining at the sperm head, and the acrosomes reacted sperm had no acrosome staining. To determine the percentage of acrosome-reacted sperm, 300 sperm were observed and counted from each slide.

2.6 Statistical analysis

The data were statistically analyzed using the Sigma stat package. One-way analysis of variance (ANOVA) was used to test the hypothesis and the significant difference was considered where *P*-values were <0.05. The data were expressed as mean± standard deviation (SD).

3 Results

3.1 Effects of AG extract on mounting frequency

Male rats that received AG extract had increased mounting frequency (Fig. 1). On Day 1, AG extracts increased the mounting frequency in all doses. The highest mounting frequency was achieved at the dose of 50 mg/kg BW. This effect was reduced on Day 7 but only the 50 mg/kg BW dose induced a significant difference in the mounting frequency. On Day 14, no effect of the extract was shown (Fig. 1).

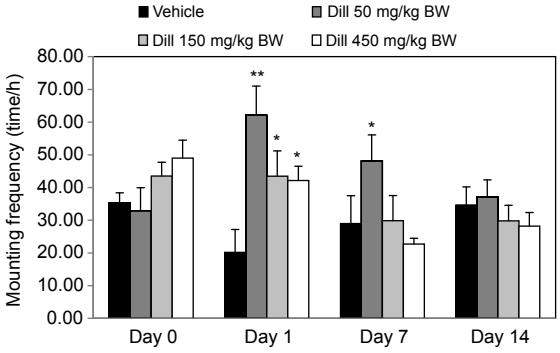

Fig. 1 Effect of AG extract on the mounting frequency
Each bar represents the mean±SD (n=10) in each group. $^{**}P$<0.01, $^{*}P$<0.05, compared to the vehicle-treated controls

3.2 Testicular weight and histology of testis and epididymis

Toxicity effects of AG extract on testes and epididymis were examined by observing both gross and histopathological structures. After treatment for consecutive 14 d, AG did not change the testicular weights (both right and left sides) as compared to the control (Fig. 2). In histological results, the control and AG-treated rats showed mostly testicular epithelium with an orderly arrangement of spermatogenic cells and Sertoli cells (Fig. 3). In addition, no histopathologic alterations (normal epididymal cell arrangement and sperm density) were observed in ductus epididymis of the control or AG-treated rats (Fig. 4).

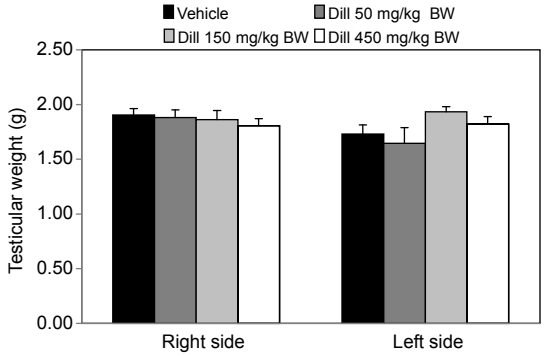

Fig. 2 Testicular weights (right and left sides) after AG extract treatment for 14 d
Data were presented as mean±SD (n=10) in each group. No significant difference between vehicle-treated control group and AG-treated groups was detected

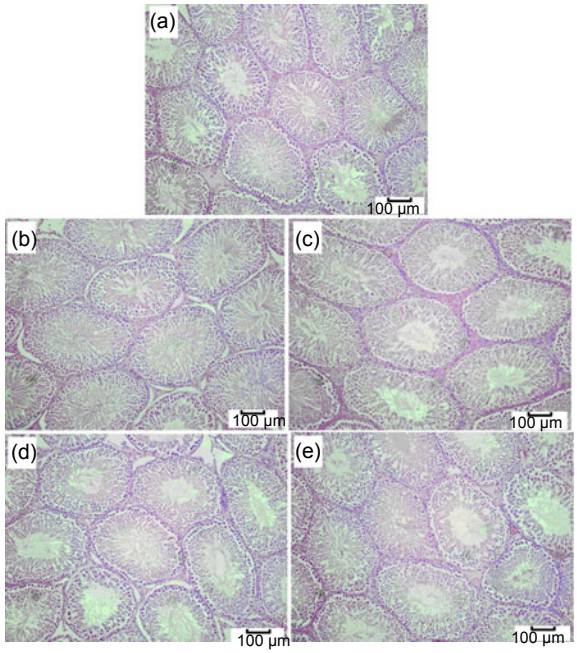

Fig. 3 Photomicrographs of the histology (H&E) of the testes from a representative section
No histological alterations are observed in testes of the control [(a) untreated and (b) vehicle] and AG-treated male rats [(c) 50, (d) 150, and (e) 450 mg/kg BW, respectively]

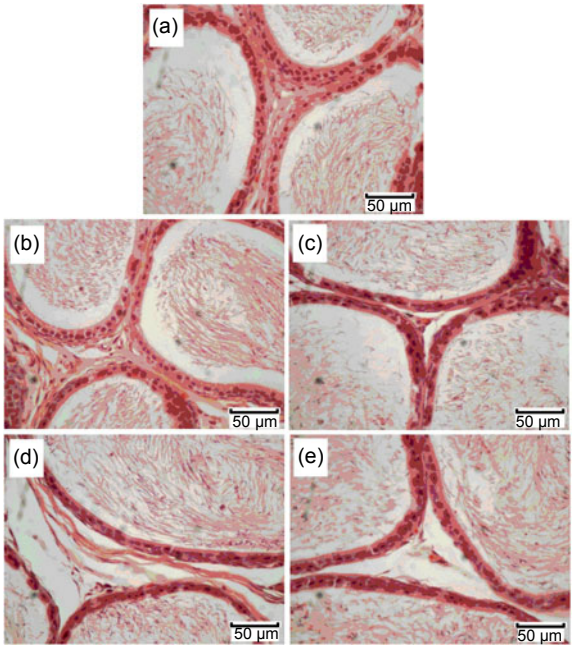

Fig. 4 Photomicrographs of the histology (H&E) of the epididymis from a representative section
No histological alterations are observed in the epididymis of the control [(a) untreated and (b) vehicle] and AG-treated male rats [(c) 50, (d) 150, and (e) 450 mg/kg BW, respectively]

3.3 Effects of AG extract on testicular protein phosphorylation

As shown in Fig. 1, 50 mg/kg BW AG extract significantly increased the mounting frequency on Days 1 and 7. The testes in this group were selected to investigate the level of protein phosphorylation. The testicular lysate of rats treated with 50 mg/kg BW AG extract showed some phosphorylated protein broad bands as compared to those of the control (Fig. 5).

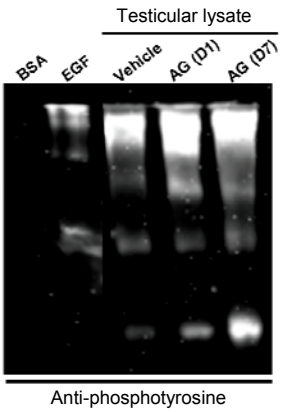

Fig. 5 Immuno Western blot analysis for tyrosine protein phosphorylation levels in testicular lysate (50 μg for each lane) of rats treated with or without AG extract (50 mg/kg BW) on Days 1 and 7 [AG (D1) & AG (D7)]
Bovine serum albumin (BSA) and epidermal growth factor-like growth factor (EGF) were used as negative and positive controls for anti-phosphotyrosine, respectively

3.4 Sperm concentration and acrosome reaction (AR) status

The sperm concentration and AR status were assessed to investigate the direct effect of AG extract on sperm physiology. The results show that rats that received AG extracts are not significantly different in both sperm concentration and AR status (Fig. 6; Table 1) as compared to the control.

4 Discussion

The result in Fig. 1 suggests that low concentration of AG extract could effectively stimulate the mounting frequency with short term treatment. Herein whether a high dose of AG extract and increasing days could not stimulate mounting frequency remains unexplained. It has been suggested that vasodilatation,

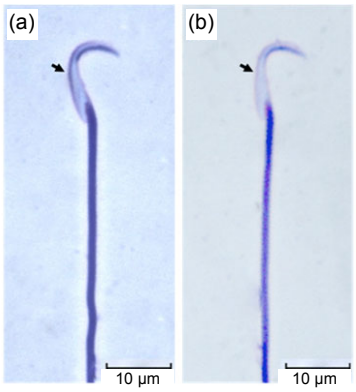

Fig. 6 Photographs showing the representative sperm acrosome status
(a) Acrosome intact sperm stained with Coomassie blue; (b) Acrosome-reacted sperm unstained

Table 1 Epididymal sperm concentration and acrosome reaction of rats treated without or with AG extract on Day 7

Group	Sperm concentration ($\times10^6$ cells/ml)	Acrosome reaction (%)
Control	30±8.4556	4.12±2.4598
Dill (50 mg/kg BW)	31±7.3546	3.17±1.3696
Dill (450 mg/kg BW)	39±9.5539	5.16±1.2697

Sperm concentrations were presented as mean±SD (n=10) in each group

generation of nitric oxide, and/or elevation of androgens and gonadotropins are a major mechanism involved in aphrodisiac activity of many medicinal plants (Yakubu and Akanji, 2011; Prabsattroo *et al*., 2012). Basically, sexual behaviors are the result of stimulation of the central nervous system (CNS). Previously, Monsefi *et al.* (2006) demonstrated that AG extract changed the estrous cycle and increased milk production during lactation in female rats, suggesting that dill stimulated some regions of CNS, especially via the hypothalamic axis (Benelli *et al*., 1995; Cruz-Casallas *et al*., 1999). In the same vein, AG extract may also stimulate the part of the CNS that is particularly responsible for the mounting behavior in male rats, as shown in the present study (Fig. 1). Similar to *Moringa oleifera* leaves extract activity (Prabsattroo *et al.*, 2012), our results also showed a significant increase of mounting frequency with a low dose of AG on Day 1 and declining on Day 14, as compared with the control group (Fig. 1). Since this frequency was not a dose-dependent phenomenon, it is possible that the mounting frequency in rats might be responsible for AG administration at the low dose and short time period. However, the actual mechanism of mounting frequency stimulated by low AG is being investigated. At the end of experiment (Day 14), the testes and epididymis of all groups were examined to observe the morphologic alterations. In Figs. 2–4, the normal gross and histological results suggested that AG extracts were not harmful to any of the tissues studied (i.e., testes and epididymis).

The increasing levels of posttranslational proteins are involved in sperm maturation and testosterone production (Jones *et al*., 2008; Fardilha *et al*., 2011; Yamashita *et al*., 2011). In the present study, AG extract enhanced the protein phosphorylation level in testicular lysate (Fig. 5), suggesting that AG might stimulate tyrosine phosphorylation of testicular proteins involved in spermatogenesis and androgen synthesis. Although sperm concentration in the AG-treated group did not increase (Table 1), it is possible that they may be involved in testosterone synthesis. However, the testosterone levels in this study have not been measured. We assumed that the androgen levels in the serum of rats treated with AG extracts existed at a very small quantity, since there were no differences in mounting frequency on Day 14 (Fig. 1). If the androgen serums in this group were collected on Day 1 or 4, the testosterone levels responsible for male libido would be significantly increased. Similar to the morphological results, Fig. 6 and Table 1 revealed that AG extract did not affect the sperm physiology, especially sperm acrosome reaction status, suggesting that it may not cause male infertility. Taken together, AG extract is harmless to sperm production and acrosome exocytosis.

In conclusion, *Anethum graveolens* (AG) Linn. extract can enhance the mounting frequency and testicular protein phosphorylation in male rats. Indeed, this vegetable extract is not toxic to testes, epididymis, sperm production, or sperm physiology.

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