

Safety evaluation of oral *Anethum graveolens* L total hydroalcoholic extract in mice

Sepideh Arbabi Bidgoli ^{1*}, Eghbal Taheri ², Hoda Baher ¹, Parisa Ziarati ³

1. Dept. of Toxicology & Pharmacology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran

2. Endocrinology & Metabolism Research Center (EMRC), Tehran University of Medical Sciences (TUMS), Tehran, Iran

3. Dept. of biological chemistry, Faculty of Advanced Sciences and Technology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran

ABSTRACT

Anethum graveolens L. (dill) is used widely in Asian food and folk medicine but its safety profile for further clinical studies has remained unclear. After administration of total hydroalcoholic extract to mice in acute, subacute and sub-chronic treatment periods, toxic responses were recorded by clinical, biochemical, hematological and pathological examinations. Doses up to 2000 mg/kg in acute study did not cause any mortality and doses up to 1000 mg/kg didn't cause any toxic effect in subacute study. Following to daily administration of doses of 1000 mg/kg/day as Maximum Tolerated Dose (MTD) and doses of 50 (1/20 MTD), 500 (1/2MTD) and 1000 mg/kg (MTD) in 45 days regimen, significant falls in white cell counts was reported after 3 weeks (P-value < 0.05) in high (P-value = 0.042) and intermediate dose (P-value = 0.018) groups of male animals. Dill extract caused significant reduction of FBS in high dose female animal group (P-value = 0.021). Portal mononuclear lymphoid and PMN leucocytes infiltration in three adjacent foci were seen in intermediate and high dose groups of both sexes which was clearly a dose dependent effect. Doses less than 50 mg/kg could be considered as safe dose in both genders of mice with the good potential for further antihypoglycemic or antihyperlipidemic clinical studies.

Key words: *Anethum graveolens* L, dill, safety, toxicity, MTD

*Corresponding Author : Dr. Sepideh Arbabi Bidgoli, Pharm D., Ph.D., Associate Prof. of Toxicology & Pharmacology, Department of Toxicology & Pharmacology, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), No 99, Yakhchal, Golhak, Dr. Shariati, Tehran-Iran
Tel: +98 21 22600037 Fax: +98 21 22002040
e-mail: arbabi.s@iaups.ac.ir

1. Introduction

Anethum graveolens L. (dill) is used as an anti-hypercholesterolaemic plant in Iranian folk medicine. Literature supports the therapeutic values of dill as a widely used medicinal herb in experimental models but its clinical efficacy remained controversial (Delaquis *et al.*, 2002). Recent animal studies shows that different fractions of this plant, besides its hypolipidaemic property (Hajhashemi and Abbasi, 2008; Nakano *et al.*, 1998), could protect the liver against the oxidative damages (Bahramikia and Yazdanparast, 2009) moreover the antibacterial efficacy of dill (Yazdanparast and Alavi, 2001) has provided a scientific basis which validates its traditional uses as homemade remedies (Kaur and Arora, 2009).

A. graveolens L or dill is a member of the Umbelliferae family which may show other pharmacological effects such as antiproliferative activity milk production, menstrual regulation and infertility effects (Monsefi *et al.*, 2006) in animal studies by oral routes. Although diverse studies on the biological activities of this extract have been performed; by now, no detailed toxicological assessment of dill has been reported to show its safety for long term clinical studies. For this reason present study has investigated the toxic effects of *A. graveolens* L total extract in both genders of mice as well as the pathological changes in different possible target organs of mice in acute and subchronic treatment models. This study aimed to find the Maximum Tolerated Dose (MTD) and No Observable Adverse Effect Level (NOAEL) of extract by analyzing for drug formulation and further clinical studies.

2. Materials & Methods

2.1. Test material

A. graveolens were purchased locally from herbal dealers in Tehran and all plant parts were authenticated and compared with their receptive standards in the herbarium maintained by department of pharmacognosy, Tehran University of Medical Sciences (TUMS) by expert pharmacognosist. Their samples were preserved in the laboratory of mentioned department for later studies. Total hydroalcoholic extract of *A. graveolens* L was provided by Food and drug research laboratory of Endocrinology and Metabolism Research Center (ENRC) of Tehran University of Medical Sciences.

2.2. Experimental animals and housing conditions

Experimental male and female healthy mice were obtained from Pasteur Institute of Iran at 8-10 weeks of age and 25-40 g body weight. Each six mice from each sex of animals were housed in stainless steel cages and

allowed to adapt to the conditions of the animal house for 14 days before the experiments. The animals were maintained on a 12 h dark/light cycle at about 22 ± 3 °C and allowed free access to standard laboratory diet (Pars Co.) and tap water *ad libitum* during the experiments. This study was conducted in accordance with Good Laboratory Practices (GLP) as defined in 40 CFR 792: US EPA Good Laboratory Practice Standards: TSCA; 21 CFR 58: US FDA and with Health Effects Test Guidelines, OPPTS 870.1100 (1998).

2.3. Acute test

Present investigation included an acute toxicity test first. In this preliminary study, single oral doses of *A. graveolens* L total extract (5, 50, 500, 1000, and 2000, mg/kg) were administered by oral gavages to 10 male and 10 female mice in each dose group. Mice were observed for mortality and signs of toxicity for 14 hours. In the second step mice were administered daily doses of 5, 50, 500, 1000 and 2000 mg/kg for 14 days. This test yielded information on the dose toxicity relationship, including an estimation of the maximum tolerated dose (MTD) for the main study and weight gain pattern during the second 14 daily treatments. Mice were observed for mortality; signs of toxicity and weight changes during 14 daily treatments. As the first four doses were considered safe without any significant weight reduction or signs of toxicity therefore doses of 50, 500 and 1000 mg/kg were considered as three different dose levels for subchronic study. Control groups received equal volumes of distilled water daily.

2.4. Subchronic toxicity study

Eighty mice (40 males and 40 females) were randomly divided into 8 groups (10 animals/sex/group). Table 1 shows the animal groups and their daily regimens of *Anethum* extract.

All the groups were administered orally from 1 g/ml solutions of plant extract on the basis of their body weights once daily for 6 days per week over a period of 45 days.

2.5. Clinical examinations

Clinical signs were observed and weights were recorded once daily. The recording items were divided to three categories:

Cageside Observations involved home-cage activity, feces amount, feces color, feces consistency, urine amount, urine color and behavior while removing from Cage.

Neurological Examination involved tail elevation, abnormal gait, ataxic gait and head Position.

Physical Examination involved death, hair coat, mucus membrane/eye/skin color, body temperature, respiratory rate, respiratory character, lacrimation, salivation amount and Eye Prominence.

Table 1. Animal groups and dosing manual.

Test groups	dose to animals (mg/kg body-weight/day)	Number of males	Code of Males	Number of females	Code of Females
Control	0	10	AMC	10	AFC
Low	50	10	AML	10	AFL
Intermediate	500	10	AMI	10	AFI
High	1000	10	AMH	10	AFH

2.6. Hematological studies

Various hematological parameters including Hematocrit (Hct), Hemoglobin Conc. (Hb), Mean Corp. Hb. (MCH), Mean Corp. Hb. Conc. (MCHC), Mean Corp. Volume (MCV), Total Leukocytes (WBC) and Platelet Count were determined in three animals/sex/group by automated blood analyzer in the main laboratory of Imam-Khomeini hospital at days 21 and 45 of the present study.

2.7. Biochemical assays

Biochemical parameters measured in three animals/sex/group with an automated biochemical analyzer in the same laboratory and consisted of Albumin, Total Cholesterol, LDL, HDL, Total protein, Fasting Triglycerides total protein (TP), Creatinine, Urea Nitrogen, Aspartate Aminotransferase (AST or SGOT), Alanine Aminotransferase (ALT or SGPT), Alkaline Phosphatase, (ALP), Total Bilirubin, Glucose, Calcium, Phosphorus Potassium, Sodium in days 21 and 45 of study.

2.8. Pathological studies

Different organs from Digestive tract (Esophagus, Stomach, Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum, Gall Bladder, Liver, Pancreas) Respiratory tract (Trachea, Lung), Cardiovascular tract (Heart), Reticulo-endothelial/hematopoietic tract (Lymph Nodes, Spleen, Thymus), Urogenital tract (Kidneys, Ovaries and fallopian tubes, Corpus Uteri, Cervix Uteri, Prostate, Testes, Urinary Bladder, Vagina), glandular organs (Adrenals, Pituitary Glands, Thyroid/Parathyroid Glands, Thymus), Bone (Femur), Skeletal Muscle, Skin, Epididymis were removed from 3 animals/sex/group whose blood and serums were assayed for hematological and biochemical studies. Organ weights were recorded and absolute and relative organ weights were compared in each group with related control. The tissues were fixed in 10% buffered formalin and dehydrated in graded series of alcohol, cleared in xylene and embedded in paraffin wax. Multiple sections from each block were prepared at 5 μ m and stained with haematoxylin and eosin (H&E).

2.9. Statistical analysis

Values were expressed as means $\pm$ SD. To compare groups, homogeneity of variances was evaluated first. When variances were not significantly different data were analyzed by one-way analysis of variance (ANOVA) and the Student's t-test. When variances were considered significantly different Man Whitney U test for comparison of two variables and Kruskal Wallis H test for comparison of more than two variables were used (Rober and Clarke, 2002). A significant difference was accepted with P-value < 0.05. All statistical methods were performed by SPSS 16.

3. Results

3.1. Acute oral toxicity studies

Doses of 5, 50, 500, 1000 and 2000 mg/kg were administered to animals. No deaths and no signs of toxicity were recorded in the first 24 hours of administration. Based on the lack of mortality in both genders of mice at the limit test, the LD₅₀ value for alcoholic total extract of *A. graveolens* L was recorded greater than 2000 mg/kg of body weight and this extract was categorized as practically non toxic agent. Doses were continued for the next 14 days of this study. Although all animals looked healthy with normal physical activities during the next 14 days of study but the highest dosing group lost weight when compared with other groups. No signs of toxicity, adverse hematological, biochemical and pathologic lesions were observed in any of the animals at necropsy but regarding the weight lose in our highest dosing group, 1000 mg/kg was considered as the Maximum Tolerated Dose (MTD) and the doses of 1000, 500 and 50 mg/kg were considered as three dose levels for subchronic toxicity study.

3.2. Subchronic toxicity study

3.2.1. Survival and clinical signs

Two animals in AHM (High dose male) and ACF (control female) in the first week of subchronic study

Table 2. Percent of eye prominence in treatment and control groups.

Dose Groups	Week 3		Week 4	
	Eye Prominence (+)	Eye Prominence (-)	Eye Prominence (+)	Eye Prominence (-)
ALF	28.6	71.4	16.7	83.3
AIF	42.9	57.1	16.7	83.3
AHF	50	50	50	50
ACF	50	50	0	100
ALM	14.3	85.7	14.3	85.7
AIM	42.9	57.1	14.3	85.7
AHM	14.3	85.7	20	80
ACM	20	80	0	100

and 3 animals from ACM(2) and AHF(1) were died in the third week of our study. In the fourth week of the present study two cases from AIF (intermediate dose female) and ALF (low dose female) showed ataxia and abnormal gait and died. The animals were immediately necropsied after death and no signs of toxicity were detected in mentioned groups (treatment and controls). As no treatment related deaths were detected in our preliminary test with higher doses and due to equal number of deaths in case and control groups without pathological signs of toxicity therefore these deaths were interpreted as no treatment related deaths. Out of different daily cage side observations eye prominence was recorded in different groups of animals from the 4th week of study. Table 2 shows the frequencies (%) of this eye disorder in all eight treatment and control groups. All treatment groups were compared with their related controls. Man –Whitney test showed no significant differences between the cases and the control. This abnormality was dose related in females but not in male groups. The abnormality removed in the 4th week of study from controls. Treatment groups were also recovered to normal state in the 5th week of this study.

3.2.2. Growth rate

Weights of animals from all three *Anethum* treated groups and controls of both sexes were recorded two times weekly. Although they did not showed any significant weight gain for the duration of the study but no statistically significant changes were also observed for sex matched groups compared to the control with respect to body weight at any time point evaluated. No significant differences were also recorded in food consumption of animals during this study.

3.3. Hematological Studies

Blood samples of animals were analyzed at days 21 and 45 of study and compared at each endpoint with controls. Results were collected in Table 3. Significant decrease

of WBC was recorded in AIM (P-value = 0.018) and AHM (P-value = 0.042) dose groups but MCV (P-value = 0.002) and MCH (P-value = 0.002) showed significant raises. Although all abnormal hematological changes recovered to normal state in next weeks but WBC in AHM (P-value = 0.031) and ALM (P-value = 0.02) dose groups remained significantly lower than controls. Raise of platelets were also recorded in the second endpoint in male animals (P-value = 0.051).

3.4. Biochemical Studies

Routine regimens of *A. graveolens* caused significant FBS reduction (from 333±38 to 192±46, p=0.021) in females at day 21. Table 4 shows the clinical chemistry values in mice fed *A. graveolens* for 45 days.

3.4.1. Urine analysis

No significant changes were detected between case and control groups in both genders (data not shown).

3.5. Necropsy studies

Absolute and relative weights of all organs e.g. brain, heart, kidney, liver, pancreas, spleen, testis, ovary, adrenal, thyroid, parathyroid, lymph nodes, bone, muscles etc remained unchanged from corresponding control groups and no gross changes was observed in this part of study.

3.6. Pathological Studies

Pathological studies were performed on H&E stained slides at baseline, day 21 and day 45 in both genders. No pathological lesion was detected in heart, brain, lungs, spleen, thymus, kidney, reproductive systems, digestive systems and nervous system of animals during the treatment periods. Portal mononuclear lymphoid and PMN leucocytes infiltration in three adjacent foci of male and female animals were detected around the central hepatic vein in both genders in intermediate and high dose groups. This pathological feature was considered as xenobiotic induced hepatitis which recovered to normal state after discontinuation of the extract in recovery period.

Table 3. Mean hematological values for mice fed *Anethum graveolens* for 45 days.

SEX		males				females			
Dose Groups		ACM	ALM	AIM	AHM	ACF	ALF	AIF	AHF
Number of Animals		3	3	3	3	3	3	3	3
Red blood cells									
Hematocrit (Hct)	%	40.6±6.4	44.3±2.3	42.8±5.2	44.1±3.2	45.2±3.7	46.2±3.2	42.2±1.8	45±2.2
Hemoglobin Conc. (Hb)	g/L	13.43±0.9	13.6±0.7	13.25±2	13.46±0.8	13.7±1	14.3±1	12.7±0.2	13.1±0.4
Mean Corp. Hb. (MCH)		15.3±0.5	15.3±0.5	15.5±0.7	15.3±0.5	15.6±0.5	15.5±0.7	15.3±0.5	15.5±0.7
Mean Corp. Hb. Conc. (MCHC)		33.3±3.2	30.6±1.5	31±1.4	30.3±0.5	30.3±0.5	31	30.3±1.1	29±2.8
Mean Corp. Volume (MCV)	L/L	46±3.6	51±1.7	50±1.4	50.3±1.1	52±1.7	50±1.4	50.3±1.1	53±2.8
Total Erythrocyte Count (RBC)	10 ¹² /L	8.3±0.8	8.7±0.7	8.6±0.8	8.7±0.4	8.7±0.5	9±0.5	8.3±0.1	8.4±0.5
White Blood Cells									
Total Leukocytes (WBC)	10 ⁹ /L	9.7±2.1	4.4±1.1*	6.1±2	3.7±2.3*	4.3±1.5	7.2±1	8±2.8	4.9±2.1
Clotting Potential									
Platelet Count	10 ⁹ /L	866±158	959±646	1246±46.6*	828±185	729±114	864±208	846±344	786±33

*Values <0.05

4. Discussion

Although there is a paucity of animal studies on the contribution of *A. graveolens* in the treatment of hyperlipidemic and hypercholesterolemic conditions (Kojuri *et al.*, 2007), HCl-induced gastric lesions (Hosseinzadeh *et al.*, 2002), regulation of corticosteroid-induced type 2 diabetes mellitus (Panda, 2008), prevention and treatment of type 2 diabetes mellitus (Fodor and Keye, 2006), regulatory properties of the menstrual cycle and antifertility effects (Monsefi *et al.*, 2006) the safety profile of this traditional remedy has not been clarified yet. According to the EMEA 2006 guideline (EMA Guideline 2006) for many herbal preparations contained in well-established or traditional herbal medicinal products an adequate safety profile, may be confirmed by their long-term medicinal and/or food use. However, in cases where a safety concern is recognized or suspected, non-clinical investigations may be needed. The lack of some specific non-clinical studies (e.g. genotoxicity studies) may also pose a safety concern (Morkunas, 2002). Although one investigation showed that essential oil extracted from the aromatic plant dill was not genotoxic for mouse bone marrow cells *in vivo* and even reduced the mutagenic effect of benzo (a) pyrene inducing micronuclei but in the SMART test, a dose-dependent increase in mutation frequency was observed for essential oils from dill herb (Lazutka *et al.*, 2001). On the

basis of existing documents it seems necessary to define the safety profile of the total extract for marketing authorization of dill as a possible future pharmaceutical agent. To support the future clinical studies of dill in EMRC with more understanding about its toxicity profile in long term administration, present study was conducted in experimental model for the first time and single doses up to 2000 mg/kg was not toxic therefore Dill could be categorized as unclassified toxic agent (category 5) on the basis of OECD toxicity classification method (OECD guideline 2009).

As doses up to 1000 mg/kg/day caused no more than a 10% weight decrement, as compared to the appropriate control groups, and does not produce mortality, clinical signs of toxicity, or pathologic lesions therefore mentioned dose was considered as Maximum Tolerated Dose (MTD) (15) and doses of 50 (1/20 MTD), 500 (1/2 MTD) and 1000 mg/kg (MTD) were considered as selective doses for subchronic study test (Hodgson, 2004). Following daily administration of mentioned doses, significant falls in white cell counts was reported after 3 weeks (P-value < 0.05) in high (P-value = 0.042) and intermediate dose (P-value = 0.018) groups as well as low dose group of male animals after 45 days of study. Other gender-specific hematotoxic effects of this extract e.g. raise of MCH and MCV were recovered to normal state in the second half of present study.

Surprisingly dill extract caused significant reduction in FBS in high dose female animal group (P-value = 0.021)

Table 4. Mean clinical chemistry values in mice fed *Anethum graveolens* for 45 days.

SEX		males				females			
Dose Groups		ACM	ALM	AIM	AHM	ACF	ALF	AIF	AHF
Number of Animals		3	3	3	3	3	3	3	3
Red blood cells									
Hematocrit (Hct)	%	40.6±6.4	44.3±2.3	42.8±5.2	44.1±3.2	452±3.7	46.2±3.2	42.2±1.8	45±2.2
Hemoglobin Conc. (Hb)	g/L	13.43±0.9	13.6±0.7	13.25 ± 2	13.46±0.8	13.7± 1	14.3± 1	12.7±0.2	13.1±0.4
Mean Corp. Hb. (MCH)		15.3±0.5	15.3±0.5	15.5 0.7	15.3±0.5	15.6± 0.5	15.5 0.7	15.3±0.5	15.5±0.7
Mean Corp. Hb. Conc. (MCHC)		33.3±3.2	30.6±1.5	31±1.4	30.3±0.5	30.3±0.5	31	30.3±1.1	29± 2.8
Mean Corp. Volume (MCV)	L/L	46±3.6	51±1.7	50±1.4	50.3±1.1	52± 1.7	50±1.4	50.3±1.1	53± 2.8
Total Erythrocyte Count (RBC)	10 ¹² /L	8.3±0.8	8.7± 0.7	8.6± 0.8	8.7± 0.4	8.7± 0.5	9± 0.5	8.3±0.1	8.4± 0.5
White Blood Cells									
Total Leukocytes (WBC)	10 ⁹ /L	9.7±2.1	4.4±1.1*	6.1±2	3.7±2.3*	4.3±1.5	7.2± 1	8.±2.8	4.9±2.1
Clotting Potential									
Platelet Count	10 ⁹ /L	866± 158	959±646	1246± 46.6 *	828±185	729±114	864±208	846±344	786±33

*Values <0.05

in the first half of present study, but the same effect was not recorded in the second half of this experiment. Although the aqueous extract did not cause any change on biochemical profile, absolute and relative weights of all organs eg brain, heart, kidney, liver, pancreas, spleen, testis, ovary, adrenal, thyroid, parathyroid, lymph nodes, bone, muscles etc but portal mononuclear lymphoid and PMN leucocytes infiltration in three adjacent foci were seen in intermediate and high dose groups of both sexes which was clearly in a dose dependent manner. This study suggests that single oral dose of 2000 mg/kg and long term oral multiple doses of this extract as a safe agent in most organs of mice except hepatotoxic effects of 500, 1000 mg/kg doses in the liver of both sexes and leucopenia with doses higher than 50 mg/kg in male animals. Mentioned hematotoxic effect should be confirmed by differential count of WBC in further studies. Due to present study doses less than 50 mg/kg could be considered as No Observable Adverse Effect Level (NOAEL) in both genders of the mice which is appropriate for further antihypoglycemic assessment of this extract or other necessary studies on its other possible therapeutic effects.

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