Acute and Subchronic Toxicity Assessment of the Hydroalcoholic Extract of *Stachys lavandulifolia* in Mice

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Abstract- *Stachys lavandulifolia* is used as the herbal tea and its wide and potent medical effects have been reported for the extract in animal studies. This study aimed to find the safety profile of the extract to find the appropriate doses for further human studies. The aerial parts of the plant were air-dried and the hydroalcoholic extract was obtained and concentrated by percolation method with 140 mg/ml concentration. To assess the toxicity profile of this extract, 60 female mice (30 cases, 30 controls, 24.8±2.1 g, 4-6 weeks) were administered the extract by oral gavages in acute (24 hrs), subacute (14 days) and subchronic (45 days) models. All clinical, hematological, biochemical and histopathological changes were assessed in appropriate midpoints and endpoints and compared with control group. Doses up to 140 mg⁄kg were recognized as maximum tolerated dose in subchronic model. Abnormal changes in kidney and liver weight in treatment groups as well as the significant elevation of biochemical parameters in 45 days study has suggested the possible hepatic and renal toxicity potentials of this extract with doses upper than 140mg⁄kg. Doses up 70 mg⁄kg could be considered as no observable adverse effect level (NOAEL) and could be used in further clinical trials on the possible therapeutic effects of this plant.

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Introduction

The sub cosmopolitan genus *Stachys* is represented by more than 270 species and is recognized as one of the largest genera of the Labiatae (1). Plants of this genus have long been applied to treat genital tumors, sclerosis of the spleen, inflammatory tumors and cancerous ulcers (2). Out of 34 species of *Stachys* which grows in many parts of Iran, Iraq and Turkey (3-5), *Stachys lavandulifolia* is used as the herbal tea in gastrointestinal disorders in Iran (6).

It is suggested that the extract of *Stachys lavandulifolia* possessed anxiolytic effect with relatively lower sedative activity than diazepam (7). After investigating the anxiolytic effects of different fractions of *Stachys lavandulifolia*, it is suggested that this effect could be related to flavonoids, phenylpropanoids or terpenoids contents of the aerial parts of this plant (8). Preliminary phytochemical studies showed that the aerial parts of the genus *Stachys* contain flavonoids which may be responsible for their antibacterial activity (9).Further phytochemical investigations of *Stachys lavandulifolia* species have shown the occurrence of flavonoids, diterpenes, phenyl ethanoid glycosides and saponins (10).

Other than the published effects of *Stachys lavandulifolia* in traditional folk medicine as anxiolytic, antiderpressive, appetite stimulant and analgesic agent (11) some local and unpublished reports from Qazvin province of Iran shows its antispasmodic effects as well as its strong efficiency in the control of primary dysmenorrhoea.

Although the plant was given in traditional methods for three menstrual cycles widely in this region, to our knowledge no detailed toxicological assessment on *Stachys lavandulifolia* or other species of *Stachys* has been reported to show the safety profile for short term and long term clinical studies. For this reason present study aimed to evaluate the toxic effects of *Stachys lavandulifolia* hydroalcoholic extract in female mice in acute and subchronic models.

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Materials and Methods

Test material

The plant material was collected from Qazvin province, Alamout region in June 2010. A voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences (herbarium no. 6695). Samples were preserved in the Pharmacognosy laboratory for later studies**.**

The aerial parts of the plant were air-dried in 5 days and the total weight of plant was reduced from 500 g to 300 g. The hydroalcoholic extract was provided by method of percolation using 80% Ethanol and 20% water during 6 days. After filtration of extract, the alcoholic extract was removed by rotary evaporator in 45 ^{o} C. The method of extraction was adjusted and the concentration of dried residue was settled to get the maximum concentration at 140 mg/ml (14%). The yield of oil was 0.6% in present extract.

Experimental animals and housing conditions

Experimental female healthy mice were obtained from Pasteur Institute of Iran at 4-6 weeks of age and 24.8 ±2.1 g body weight. Each six mice were housed in stainless steel cages and allowed to adapt to the conditions of the animal house for 10 days before the experiments. The animals were maintained on a 12 h dark/light cycle at about 22±3 ^{o}C and allowed free access to standard laboratory diet (Pars Co.) and tap water 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).

Acute test

Present investigation included an acute toxicity test first. In this preliminary study, single oral doses of *Stachys lavandulifolia Vahl* total extract (5, 50, 500, 1000, and 2000 mg/kg) were administered by oral gavages to 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.

Subchronic toxicity study

Forty female mice were randomly divided into 4 groups (10 animals in each group). Low dose group administered 7 mg/kg, medium dose group administered 70 mg/kg and high dose group administered 140 mg/kg from hydroalcoholic extract on the basis of their body weights once daily for 6 days per week over a period of 45 days. Distilled water was administered to negative control group of animals.

Clinical examinations

Clinical signs were observed and weights were recorded once daily in acute study and two times weekly in subchronic study. The recording items were divided to three categories:

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

Neurological Examination contained tail elevation, abnormal gait, ataxic gait and head position.

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

Hematological studies

Various hematological parameters including hematocrit (Hct), hemoglobin concentration (Hb), mean corpuscular hemoglobin (MCH) , mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), total leukocyte count (WBC) and platelet count were determined in three animals/sex/group by automated blood analyzer in the main laboratory of Imam Khomeini university hospital at days 21 and 45 of present study.

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, 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.

Histopathological studies

Different organs from digestive tract (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, gall bladder, liver, pancreas), respiratory system (trachea, lung), cardiovascular system (heart), reticulo-endothelial/hematopoietic system (lymph nodes, spleen, thymus), urogenital system (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 µm and stained with haematoxylin and eosin (H&E).

Statistical analysis

Values were expressed as mean ± 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 Kruskall-Wallis H test for comparison of more than two variables were used. A significant difference was accepted with $P<0.05$. All statistical methods were performed by SPSS 16.

Results

Acute oral toxicity

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 LD_{50} value for alcoholic total extract of *Stachys lavandulifolia* 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 higher than 10% in >500 mg/kg groups, this dose was considered as maximum tolerated dose (MTD) and doses of 7, 70 and 140 mg/kg were considered as three dose levels for subchronic toxicity study.

Subchronic toxicity study

Food and water consumption and weight changes

Food consumption was not significantly different among control and treatment groups ($P=0.095$) but increased levels of water consumption were observed in all dose groups ($P=0.02$). Details of food and water consumption in each treatment and control groups were considered in table 1. Weights of animals from all three stachys treated groups were recorded two times weekly. No significant weight change was detected for the duration of the study.

Table 1. Food (g/week) and water (l/week) consumption in cases and control groups

Dose Groups	Mean	Std. Deviation	*P*-value
Food (g/week)			
Control	137.12	19.03	
Low Dose	170.75	22.05	0.06
Medium Dose	163.22	17.11	0.074
High Dose	162.50	10.41	0.058
Water (l/week)			
Control	190.00	45.46	
Low Dose	280.00	35.59	0.021*
Medium Dose	305.00	42.03	0.032*
High Dose	352.50	60.76	0.05*

Table 2. Organ weights in treatment and controls after 45 days of study

	Groups	Mean(g)	Std. Deviation	Std. Error Mean	*P*-value
Lungs	control	0.1700	0.02449	0.01095	0.09
	treatment	0.2050	0.03450	0.01408	
liver	control	1.0580	0.30161	0.13489	0.034*
	treatment	1.4250	0.18097	0.07388	
spleen	control	0.1100	0.03536	0.01581	0.308
	treatment	0.1550	0.08666	0.03538	
kidney	control	0.2420	0.01924	0.00860	0.02*
	treatment	0.2983	0.02317	0.00946	
reproductive	control	0.4840	0.07701	0.03444	0.512
	treatment	0.4300	0.16444	0.06713	
heart	control	0.1080	0.00837	0.00374	0.152
	treatment	0.1172	0.01059	0.00432	

Survival and clinical signs

All animals were survived during this study in cases and control groups. Out of different daily cage side observations no clinical sign of toxicity was recorded in dose groups and control.

Organ weight

In subchronic study increased size and weight of liver and kidney were detectable in treatment groups. Increased weight of liver was 1.058±0.301 g *vs* 1.425±0.18 g (*P*=0.034) and increased weight of kidney was 0.242±0.019 g *vs* 0.298±0.0231 g (*P*=0.02). Exact weight of organs after 45 days of study in high and medium doses were showed in table 2.

Hematological studies

Blood samples of animals were analyzed at days 21 and 45 of study and compared at each endpoint with controls. Significant changes were collected in table 3. Although no changes of WBC, total erythrocyte count, Hb, Htc and MCHC were recorded in all dose groups, MCV (*P*=0.001), MCH (*P*=0.005) and platelet count (*P*=0.001) showed significant raises in high dose (140 mg/kg) group of animals during the 45 days study.

Table 3. Significant hematological changes in 21 and 45 days of study

Groups	21 days Study			45 days study		
	Mean	Std. Deviation	*P*-value	Mean	Std. Deviation	*P*-value
Mean Corp. Volume (MCV)						
Control	41.2	0.5		44.93	0.05	NS
Low Dose	42.5	0.7	NS	44.21	0.07	NS
Medium Dose	42.9	0.91	NS	42.4	1.08	NS
High Dose	42.6	1.9	NS	30.92	19.34	0.001**
Mean Corp. Hb. (MCH)						
Control	13.39	0.68		15.7	0.96	
Low Dose	14.6	0.2	NS	14.8	0.32	NS
Medium Dose	13.96	0.56	NS	15.05	0.98	NS
High Dose	14.3	1.00	NS	26.5	9.4	0.005**
PLT/ Platelet Count 10^9/l						
Control	1167.7	272.2		896 .33	225.2	
Low Dose	946	48.1	NS	989	38.1	NS
Medium Dose	1227.9	199.7	NS	1115.9	142.7	NS
High Dose	1354.7	62.9	NS	492.1	55.6	0.001**

* *P*<0.05, ** *P*<0.01

Table 4. Significant biochemical changes in high dose group in 45 days study

	N	Groups	Mean	Std. Deviation	*P*-value
FBS (mM)	6	treatment	150.60	10.04	0.040*
	6	Control	115.33	5.62	
Urea	6	treatment	52.0000	28.91	0.197
	6	Control	32.0000	6.00	
Creatinine	6	treatment	0.72	0.38	0.002*
	6	Control	0.93	0.11	
Cholesterol (mM)	6	treatment	94.6000	12.87	0.264
	6	Control	76.6667	25.16	
Fasting TG (mM)	6	treatment	114.40	54.13	0.040*
	6	Control	66.66	13.31	
HDL	6	treatment	24.20	2.94	0.120
	6	Control	6.66	1.15	
LDL	6	treatment	92.6	73.45	0.011*
	6	Control	124.00	23.57	
Aspartate Aminotransferase (AST or SGOT; U/l)	6	treatment	223.602	26.847	0.029*
	6	Control	51.0000	9.89	
Alanine Aminotransferase (ALT or SGPT; U/l)	6	treatment	177.40	12.48	0.169
	6	Control	144.67	58.79909	
Alkaline Phosphatase (ALP) (U/l)	6	treatment	21.4800	19.83260	0.178
	6	Control	15.0000	13.38208	
Calcium (mM)	6	treatment	8.9000	5.92	0.01*
	6	Control	3.8000	2.10	

Biochemical studies

Serum samples of animals were analyzed at days 21 and 45 of study and compared at each endpoint with controls. Although no significant abnormality were recorded in all dose groups during 21 days study, significant rise in calcium and creatinine levels was recorded in low dose 45 days study (*P*=0.04 and 0.002 respectively). More biochemical abnormalities were detected in high and medium doses groups in 45 days of study. Table 4 showes the biochemical changes in 45 days study. Abnormal rise in FBS (p=0.04), creatinin (*P*=0.002), TG (*P*=0.04), LDL (*P*=0.011), AST (*P*=0.029) and calcium (*P*=0.01) showed significant metabolic effects of this extract in long term administration.

Urine analysis

No significant changes were detected between treatment and control groups. Data is not showed.

Histopathological studies

Histopathological studies were performed on H&E stained slides at baseline, 21 and 45 days after discontinuation of extract. Although no pathological lesion was detectable in heart, brain, kidney, lungs, spleen, thymus, ovaries, fallopian tubes, digestive systems, muscles, thyroid, parathyroid and nervous system of all treatment animals during the study periods, portal mononuclear lymphoid and polymorphonuclear (PMN) leucocytes infiltration in three adjacent foci of animals were detected around the central hepatic vein in high dose group after 45 days of study. This pathological feature was considered as xenobiotic induced hepatitis which recovered to normal state after discontinuation of extract in recovery period.

Discussion

Although *Stachys* species have been used as folk medicine throughout the world for centuries, *Stachys lavandulifolia* is of more interest to researchers and clinicians because of its specific, wide and potent therapeutic effects (13). As the safety profile of this plant in acute, subacute and subchronic tests are not determined yet, this research showed the safety of this plant in all three models of toxicity assessment. By observation of no deaths and no signs of toxicity in the first 24 hours of administration in doses up to 2000

mg/kg, this extract could be categorized as category 5 materials according to the Globally Harmonized System of Classification and Labeling of Chemicals criteria (14). In fact the criteria for Category 5 are intended to enable the identification of substances which are of relatively low acute toxicity hazard and this extract has anticipated having an LD_{50} higher than 2000 mg/kg bodyweight which is not hazardous in acute doses.

One recent study on the methanol extract of the aerial parts of *Stachys lavandulifolia* discovered a new phenylethanoid glycoside, 4,3',4'-trimethoxy-lavandulifolioside A, named lavandulifolioside B, together with three other known phenylethanoid glycosides, which made this plant more specific among other *Stachyss* species (15). By recent discovering of these compounds, new medical applications as well as many traditional uses (7,9,11) in long term doses could be expectable from oral administration of future dosage forms therefore it is necessary to record its safety profile in repeated dose studies. During the subacute (14 days) study we have calculated the maximum tolerated dose (MTD). MTD is expected on the basis of an adequate subchronic study to produce limited toxicity when administered for the duration of the test period. It should not induce 10% or greater retardation of body weight gain as compared with control animals (15). The MTD of this extract was found in doses <500 mg /kg and we started the subchronic study by **7**, 70 and 140 mg/kg/day. Although these doses were considered safe in 21 days study, the second half of study showed toxic effects even in low dose group. Mild liver and kidney toxicity signs by significant changes in organ weights were seen and biochemical abnormalities confirmed these toxic responses but in pathological studies no lesion was detectable in the kidneys of animals. In high dose (140mg/kg) group during the 45 days study period, portal mononuclear lymphoid and PMN leucocytes infiltration in three adjacent foci of animals were detected around the central hepatic vein after 45 days of study. This pathological feature was considered as xenobiotic induced hepatitis. It seems that doses up 70 mg /kg could be considered as no observable adverse effect level (NOAEL) and should be used in further clinical trials on the possible therapeutic effects of this plant.

Stachys lavandulifolia has been utilized as traditional medicine by the indigenous people of many parts of Iran (16) e.g. Chaharmahal va Bakhtiari (17), Kurdistan (18), Iraq and Anatolia (12,18). As the boiled extract obtained from the aerial parts of *S. lavandulifolia* are used as antipyretic, anti-inflammatory, spasmolytic and sedative medicament (4) we can conclude from present study that the extract of this plant in high doses even in repeated doses (<21 days study) could be safe without signs of toxicity. Anti-inflammatory and antibacterial effects, and wound healing activity of *S. lavandulifolia* extract have been shown in several pharmacological studies (1,17,20) and this study suggest daily doses of <70 mg /kg for long term administration of its extract in different oral dosage forms .

Since there is no academic study on *S.lavandulifolia* extract toxic effect, we conclude the safety and lack of toxicity of this extract for short term use in doses up to 70 mg/kg. Hence, it is necessary to establish the scientific basis for the therapeutic actions of this folk medicine as it may serve as the source for the development of more effective drugs.

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