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Anxiolytic effects and Chemical profile of leaf oil of Skimmia *anquetilia*

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Abstract
Skimmia anquetilia (Rutaceae) leaf has been used in traditional medicines for various disorders, no attempt has been carried out to scientifically investigate the plant for its traditional claims. The current investigation was carried out to isolate oil from leaves of *Skimmia anquetilia* (Rutaceae), chemically characterized and investigated for its anxiolytic potential. The anxiolytic properties were evaluated by elevated plus maze and light/dark arena tests in rats. GC/MS analysis of *Skimmia anquetilia* oil (SAO) indicated the presence of major phytoconstituents like vetiverol (37.315%), pregeijerene (15.136%), linalool (10.085%), β-fenchyl-alcohol (7.715%), geranyl acetate (5.588%), terpinyl acetate (3.300%), α –pinene (3.726%), β-phallendrene (2.624%), neryl acetate (2.433%), (Z.E) - α-farnesene (2.012%), geraniol (1.463%) and limonene (1.362%). The anxiolytic studies of *S. anquetilia* oil was conducted at dose level of 25 and 50 mg/kg, body weight. The SAO shown significantly dose dependent increased number of entries and time spent in open arms on the elevated plus maze test while as in the light/dark arena test, SAO showed an increase in number of crossings and time spent in light arena. The current studies are indicative that SAO has validated its traditional claim as anxiolytic potential.

Keywords
Skimmia anquetilia; elevated plus maze test, light and dark arena

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1. INTRODUCTION
Skimmia anquetilia (*S. anquetilia*) is an aromatic gregarious shrub of Rutaceae family. It is mostly found in Western part of Himalayas and Kashmir in India. Traditionally, the leaves infusion of *S. anquetilia* is taken for treatment of headache, freshness and general fever. The leaves are aromatic, contains linalool, geraniol, pinene, scopoletin, skimmianine, umbelliferone (Kumar etal., 2012). Synthetic anxiolytic drugs are amongst the most frequently prescribed drugs, as the disease is highly prevalent in the society. However, existing anxiolytic agents are associated with several limitations such as sedation, tachycardia, insomnia, decreased libido, cognitive impairments, alcohol interaction, addiction with benzodiazepines, and generally, lower the onset of action of 5-HT receptor ligands (Tripathi, 2004; Nemeroff, 2004). In this study, a possible anxiolytic property of leaves of *S. anquetilia* essential oil were investigated using elevated plus maze and Light dark arena test for anxiety using rats.

2. MATERIALS AND METHODS
2.1. Plant material
The plant *Skimmia anquetilia* collected from Gulmarg, Jammu & Kashmir, India. The plant identified and authenticated by Dr. A. R. Naqshi from Department of Botany, University of Kashmir, Srinagar-190006, India (Voucher specimen number - KUST03).

2.2. Extraction of *S. anquetilia* Oil

Dried leaves were hydro-distillated in a Clevenger-type apparatus. After distillation, the oil dried with anhydrous Na_2SO_4, and kept in a vial at a temperature of - 2°C for further analysis. Total yield of oil (1.2 % v/w) obtained.

2.3. Gas chromatography-mass spectrometry analysis of the essential oil of Skimmia anquetilia

GC/MS analysis was performed on a Varian Gas Chromatograph series 3800 fitted with a VF-5 ms fused silica capillary column (60m x 0.25mm, film thickness 0.25µm) coupled with a 4000 series mass detector under the following conditions: injection volume 1 µL with split ratio 1:60; Helium as carrier gas at 1 mL/min constant flow mode, injector temperature 230^0 C, oven temperature- 40^0 C to 250^0 C at 3^0C/ min. Mass spectra: electron impact (EI+) mode, 70 ev and ion source temperature 2500C. Mass spectra were recorded over 50-500 amu range. Peak identification was accomplished by comparison of MS with those reported in NIST 05 and WILEY libraries.

2.4. Experimental animals

Male Wistar rats (150-200 g/b.w.) were purchased from Central Animal House of Indian Institute of Integrative Medicine, Jammu (J &K), India. They were housed in polypropylene cages in standard laboratory conditions of temperature (25±2°C) with 12h/12h light and dark cycle. They had free access to food and water *ad libitum*. Department was approved for animal studies (App. No.801/03/ca/CPCSEA) and current protocol was approved by Institutional Animal Ethical Committee (Approval no. F-IAEC/Pharm. Sc./Approval/2011/02).

2.5. Acute Toxicity Study

Acute toxicity study was conducted as per the OECD guidelines 425 (OECD, 2001). *S. anquetilia* oil at a dose level of 1600 mg/kg was found safe. Doses of 25, 50 mg/kg b.w. were selected in this study.

2.6 Drugs

Standard drug Diazepam was obtained from Ranbaxy Lab. Ltd., Baddi, Solan (India). Sodium carboxy methyl cellulose was purchased from CDH-Laboratory Reagent Pvt. Ltd. Post Box. No. 7138, New Delhi-110002 (India). Diazepam and *Skimmia anquetilia* oil (SAO) were suspended in a 1% sodium carboxymethyl cellulose solution. All drugs were prepared immediately before oral administration. Control group rats received only 1% aqueous sodium carboxy methyl cellulose solution. The effect of the drugs was estimated 60 minutes after drug administration. Drug dose, pre-treatment time and selection of 1% sodium carboxy methyl cellulose solution as vehicle were based on findings in preliminary experiments or taken from the literature. Tests were performed only after the rats had been acclimatized to the above environment for at least 7 days. All experiments were carried out between 09:00 and 16:00 h. In each experiment apparatus was cleaned using 5% ethanol before introducing the next animal to preclude the possible cueing effects of odors left by previous subjects.

2.7. Anxiolytic activity

2.7.1. Elevated plus-maze test

The wooden maze consists of two open arms (50 x 10 cm) and two closed arms of the same size enclosed with 40 cm high walls and a central square of 10 x 10 cm. The maze was 60 cm above the floor. A slight raised edge on the open arms (0.25 cm) provided additional grip for the animals, whereas open arm activity was further encouraged by testing under dim red light (4 x 25 W). The experiment was conducted as described (Pellow et al., 1985) in soundproof room during the dark phase of the light cycle (9 –16:00 h). To facilitate adaptation to new surroundings, rats were transported to the laboratory at least 1 h prior to testing. The trial was started by placing an animal on the central platform of the maze facing an open arm. Standard 5-min test duration was used and between subjects, the maze was thoroughly cleaned by 5% ethanol with damp and dry towels. Rats were randomly allocated to the following groups: vehicle control, Diazepam(2 mg/kg) p.o., SAO (25 and 50 mg/kg po). Similarly, study was carried out on 1^{st}, 3^{rd} and 7^{th} day for acute, subacute and chronic model. In acute study 60 min. after the first dose, in subacute 60 min. after the 3^{rd} dose and in chronic study 60 min. after the last dose on the 7^{th} day of drug or vehicle administration. The animal was placed at the central platform of the maze facing an open arm. Standard 5-min test duration was used and between subjects, the maze was thoroughly cleaned with damp and dry towels. The experiments were performed with an observer unaware of the treatment of the rats inside the room. The following parameters are classically measured in this test: frequency and duration (s) of arm visits, separately for open and closed arms. A mouse was considered to have entered an arm when all four paws were on the arm. The percentage of entries into open arms (open arm entries/total arm entries×100; % open arm entries) and the percentage of time spent in open arms (open arm time/total arm time×100; % open arm time) are used as traditional indices of the anxiety. In addition, head dips (exploratory head/shoulder movement over sides of maze) and closed arm returns (exiting from an arm with only two paws, and then returning/doubling back into the

same arm) were also recorded. Head dips were further differentiated as protected (occurring on or from the relative security of the closed arms or central platform) or unprotected (occurring on or from the open arms). Data were presented as percentage protected scores (protected/total×100; % Head-dips) described (Kumar et al., 2012).

2.7.2. Light / dark box test

Light/dark test The test apparatus consisted of a wooden box (50 cm long x 30 cm high x 35 cm wide) divided in to two equal- size compartments by a barrier possessing a door way (10 cm high x10 cm wide) on the floor level at the centre of the partition. One of the compartments was painted black and covered with a lid, whereas the other was painted white and illuminated with a 60-W light bulb set 40 cm above the box (Gong et al., 2006). On the day of the test, rats were transported to the darkened testing room and left in their home cages for 2 h. Animals were then placed in the middle of the lit compartment, facing away the dark chamber. Rats could freely explore the box for 5 min. Study was carried out on 1st, 3rd, 7th day for acute, subacute, and chronic model. In acute study 60 min. after the first dose, in sub acute 60 min after the 3rd dose and in chronic study 60 min. after the last dose on the 7th day of drug or vehicle administration, the animal was placed at the centre of the brightly lit arena in the light and dark box. The number of entries into and the time spent in the bright arena, the number of rears in the bright and dark arenas were noted. Following each trial, the apparatus was cleaned by 5% ethanol to mask the odour left by the animal in the previous experiment. Hand operated counters and stop watches were used to score the behaviour of animals and experiments were performed with an observer inside the room (Kumar et al., 2012; Thippeswamy et al., 2011).

3. RESULTS

3.1. GC-MS analysis

GC-MS of *Skimmia anquetilia* oil indicated the presence of mainly 19 constituents. The constituents were identified by comparing with the NIST library of mass spectrometry at Indian Institute of Integrative Medicine, Jammu (J & K), India. Major chemical constituents of *Skimmia anquetilia* oil were identified as vetiverol (37.315%), pregeijerene (15.136%), linalool (10.085%), β-fenchyl-alcohol (7.715%), geranyl acetate (5.588%), terpinyl acetate (3.300%), α –pinene (3.726%), β-phallendrene (2.624%), neryl acetate (2.433%), (Z.E)- α-farnesene (2.012%), geraniol (1.463%) and limonene (1.362%). The entire major and the minor constituents are depicted in Fig. 1**.**

3.2. Anxiolytic activity

3.2.1. Elevated plus-maze test

The data observed from EPM test indicated that the anxiolytic effect of SAO at a dose of 50 mg/kg (1st 3rd, 7th day) significantly increased the percentage of time spent and percentage of open arm entries (*P < 0.05, **P < 0.01; Table 1) while as decreased the closed arms entries, % head-dips, closed arm returns (*P < 0.05, **P < 0.01; Table 1). Similarly, diazepam shows increased the percentage of time spent and percentage of arm entries in the open arms (*P < 0.05, **P < 0.01; Table 1).

3.2.1 Light / dark box test

The data observed from Light-Dark box test with SAO, at doses 25 and 50 mg/kg treated rat showed significant increase (*P <0.05, *P <0.01) in the time spent in the light arena and the number of crossing (*P <0.05, **P <0.01) between the light and dark arena. The animals treated with diazepam have also significantly increased the time spent (*P <0.05, **P <0.01) in the light arena as well as number of crossing (*P <0.05, **P <0.01, Table 2) between the light and dark arena, whereas the time spent in dark arena (**P <0.01) and duration of immobility (**P <0.01) were significantly reduced. However, the time spent in dark arena (**P <0.01) and duration of immobility (**P <0.01) were significantly reduced as compared to control (Table 2).

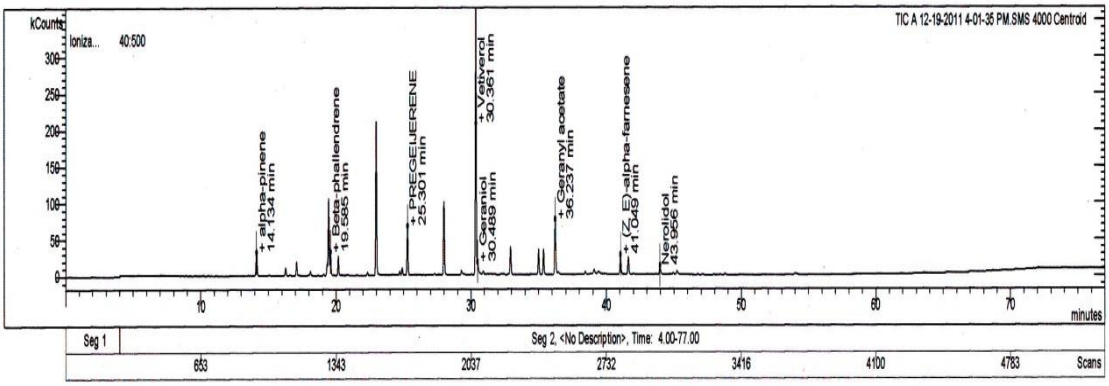
Print Date: 26 Dec 2011 12:03:10

Sample Report for a 12-19-2011 4-01-35 pm.sms

INSTRUMENTATION DIVISION, IIIM, JAMMU

Vijender Kumar (Sample-V-KU-02-A)

Sample ID:	A	Operator:	Varian	Instrument ID:	Varian GC/MS #1
Vial:	TR: Tray1 VL: 24	Acquisition Date:	12/19/2011 4:01 PM	Data File:	...12-19-2011 4-01-35 pm.sms
Calculation Date:	12/26/2011 12:02 PM	Method:	c:\varianws\oils slow method.mth		
Inj. Sample Notes:	None			Injection:	1
Volume:	0.50 uL				



Target Compounds

Cmpd. Number	RT (min)	Peak Name	Area	Amount/RF
1	14.134	alpha-pinene	31025	3.726
2	16.273	Sabinene	10224	1.228
3	17.080	Beta-pinene	16966	2.038
4	18.095	Alpha-phallendrene	7069	0.849
5	19.337	Llimonene	11338	1.362
6	19.585	Beta-phallendrene	21851	2.624
7	22.333	Alpha-caryophyllene	3360	0.404
8	22.978	Linalool	83981	10.085
9	25.301	PREGEIJERENE	126037	15.136
10	27.988	Beta-fenchyl-alcohol	64243	7.715
11	29.298	Cis-geraniol	2949	0.354
12	30.361	Vetiverol	310723	37.315
13	30.489	Geraniol	12185	1.463
14	35.011	Terpinyl acetate	27478	3.300
15	35.373	Neryl acetate	20264	2.433
16	36.237	Geranyl acetate	46529	5.588
17	41.049	(Z, E)-alpha-farnesene	16754	2.012
18	41.640	Bicyclo germacrene	12984	1.559
19	43.956	Nerolidol	6739	0.809

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Injection Method Notes

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c:\varianws\oils slow method.mth
1ml/min Helium gas as career, injector 280deg C, split ratio 1:150, column oven 50 deg C for 5min,
250 deg @ 3deg/min, hold for 7 min, ca
pillary column CP-Sil-8, 30m X 0.25mm X 0.25u thick film.

Figure.1. GC-MS studies of *Skimmia anquetilia.*

Elevated plus-maze test

Table 1: Effects of Skimmia *anquetilia* oil in the elevated plus-maze test on rats

Group	% Open arm time			% Open arm entries			Total arm entries			Closed-arm entries			% Head-dips			Closed-arm returns		
Day	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th
Control	13.1± 1.37	14.2± 1.44	14.8± 2.11	13.3± 1.33	13.9 ± 1.43	14.2 ± 1.66	14.2± 1.54	14.9± 1.32	15.4± 1.76	11.9± 1.12	12.6± 1.70	13.8± 1.19	46.1± 3.72	46.8± 3.32	48.2± 4.12	3.22 ± 1.15	3.30 ± 0.90	3.44 ± 1.42
DZ 2 mg/kg	41.2± 2.61**	48.8± 2.55**	57.9± 3.48**	41.4± 2.32**	47.2 ± 2.10**	51.6 ± 2.64**	22.3± 2.53**	23.9± 2.30**	24.6± 2.54**	10.3± 1.28	8.9± 1.32*	8.4± 0.55**	28.2± 1.45**	27.8± 1.53**	27.6± 2.38**	1.82 ± 0.22	1.70 ± 0.18	1.54 ± 0.54
SAO (25 mg/kg)	24.2± 1.88	30.2± 1.48**	33.2± 2.37*	26.3± 1.34**	32.0 ± 2.00**	39.1 ± 1.56**	17.2± 1.77	18.0± 1.48	18.9± 2.24	13.1± 2.34	12.4± 1.26	10.4 ± 2.27*	39.1± 1.22	38.1± 2.64	33.8± 2.41**	2.54 ± 0.52	2.10 ± 0.22	1.72± 0.74
SAO (50 mg/kg)	29.5± 1.99**	38.1± 1.77**	44.2± 2.78**	28.2± 1.51**	36.4 ± 1.71**	40.2 ± 2.62**	18.1± 2.28*	20.5± 2.86	23.1± 2.90*	12.5± 1.61	10.7 ± 2.13	9.3 ± 1.39*	32.2± 2.71*	30.8± 1.52**	29.1± 2.03**	2.11 ± 0.22	1.82 ± 0.12	1.31 ± 0.42

Results are expressed as means ± S.E.M. (n=6). *P <0.05, **P <0.01, compared with control. Statistically analysed by ANOVA followed by Dunnet's test.

Table 2: Effects of *Skimmia anquetilia* oil in a light and dark arena test

Group	Time spent in lighted box			Time spent in dark box			Number of crossing			Duration of Immobility		
Day	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th	1st	3rd	7th
Control	71.4 ±6.22	79.2± 8.45	88.5 ± 7.11	179.1 ±8.54	177.8±9.96	168.3 ± 7.31	10.5 ± 2.53	11.8 ± 1.90	14.7 ± 2.60	48.6 ± 2.33	42.6 ± 2.86	43.2 ± 3.43
DZ 2 mg/kg	188.2± 7.44	191.3± 5.82	209.8 ± 6.39	91.6 ± 8.05	94.3 ± 9.57	76.9 ± 8.13	28.2 ± 1.84	33.6 ± 2.55	35.4 ± 2.93	20.9 ± 2.46	13.9 ± 1.71	13.3 ± 1.10
SAO (25 mg/kg)	158.2± 8.31	173.9± 7.22	184.4 ± 5.85*	119.7 ± 3.68**	107.8±4.24 **	98.3 ± 4.60	22.3 ± 4.11	24.9 ± 2.66	26.4 ± 2.84	22.1 ± 2.65	18.2 ± 3.11	17.3 ± 2.06
SAO (50 mg/kg)	165.7± 7.56*	195.9± 4.88*	204.6 ± 5.10**	113.3 ± 3.51**	89.1± 3.80**	80.9 ± 3.08**	25.1± 2.24**	27.6 ± 2.05**	31.3± 2.44**	21.1± 0.77**	18.5± 3.57**	14.6 ± 1.85**

Results are expressed as means ± S.E.M. (n=6). *P <0.05, **P <0.01, compared with control. Statistically analysed by ANOVA followed by Dunnet's test.

4. DISCUSSION

Elevated plus-maze is the simplest apparatus to study anxiolytic response of almost all types of antianxiety agents. Exposure of the animals to novel maze alley evokes approach avoidance conflict which is stronger in open arm as compared to enclosed arm. Rodents (rats and mice) have aversion for high and open spaces and prefer enclosed arm and, therefore, spend greater amount of time in enclosed arm. When animals enter open arm, they freeze, become immobile, defecate and show fear-like movements. The plasma cortisol level is also reported to be increased, as a true reflection of anxiety (Pathak et al., 2011). Essential oils, especially rutaceae family, are popularly used in therapies for mood states (anxiety, depression) and effect as mental relaxation. *Skimmia anquetilia* oil was evaluated for its constituents and was found to contain 19 constituents; some of these were in major quantity especially Vetiverol (37.315%), pregeijerene (15.136%), linalool (10.085%) and limonene (1.362%). This oil could be rich sources for isolation of vetiverol. The results shows anxiolytic type activities of SAO (25 and 50 mg/kg) Diazepam(2 mg/kg) increased both the percentage of time spent and entries into the open arms of the maze and decreased the percentage head-dips and in closed-arm entry. The activity is found more in SAO 50mg/kg treated rats as compared to SAO 25 mg/kg and the same exhibited on all the days of our recordings which clearly indicate a dose dependant anxiolytic activity. The results also indicated a slight increase in anxiolytic activity with the passage of time and dosing on each day. This is quite evident from the results with the activity reaching its maximum on the seventh day as compared to initial days. In the light/dark test is, the rats treated with SAO 25mg/kg, the time spent by the rats in the light box was insignificant as compared to either Diazepam(2 mg/kg) or SAO 50 mg/kg on all the days of the experiment. The activity of SAO 50 mg/kg treated rats was quite significant and comparable with diazepam on all the days of the treatment and experimentation time. The pharmacological mechanism that might account for the anxiolytic effect of SAO has not been clearly identified yet. SAO contain vetiverol, linalool, limonene and linalyl acetate which are well recognized potential anxiolytic agents and these can interact and modulate the $GABA_A$ receptors, N-methyl-D-aspartate (NMDA) receptor in cerebral cortex and nicotinic receptor at the neuromuscular junction. It can be suggested and concluded that the GABAergic system is at least partly involved in the anxiolytic activity of *S. anquetilia* oil in rats.

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