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GC-MS ANALYSIS AND ANXIOLYTIC ACTIVITY OF ESSENTIAL OIL OF *SKIMMIA ANQUETILIA* OF KASHMIR REGION

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ABSTRACT: Medicinal plants essential oils in aromatherapy, complementary medicine or in folk medicine, is known for a long time or is gaining importance as a significant alternative method for the treatment of various ailments. In the last few years a lot of scientific studies have been conducted to investigate the effects and mechanisms of action of these natural compounds and their influence on the central nervous system (CNS). In the present study essential oil obtained from the *Skimmia anquetilia* leaves (Rutaceae) was subjected to chemical analysis and the oil was investigated for its anxiolytic and antidepressant effects on rats. Chemical analysis of the leaf oil (1.2%) by gas chromatography and mass spectrometry (GC/MS) whereas, anxiolytic and antidepressant properties were evaluated by employing the social interaction test and hole board in rats. GC/MS studies of *S. anquetilia* oil indicated the presence of important anxiolytic phytoconstituents like linalool (10.085%), geranyl acetate (5.588%), geraniol (1.463%) and limonene (1.362%). The anxiolytic studies of SAO were conducted at doses of 25 and 50 mg/kg, body weight. The SAO significantly increased head-dipping counts and its duration in hole board test and anxiolytic effects in social interaction test. The results of *in-vivo* pharmacological studies are indicative that SAO has a wide range of anxiolytic potential.

INTRODUCTION: *Skimmia anquetilia* (S. *anquetilia*) is an aromatic gregarious shrub belonging to family Rutaceae. It is mostly found in Western part of Himalayas and Kashmir in India.

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Traditionally, the leaf infusion of *S. anquetilia* is taken for treatment of headache, freshness and general fever. Traditionally, the leaf infusion of *S. anquetilia* is taken for treatment of headache, freshness and general fever.

The leaves are aromatic and known to contain linalool, geraniol, pinene, scopoletin, skimmianine, umbelliferone [1]. The aim of this paper is to explore the *Skimmia anquetilia* oil as anxiolytic agent. 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, the onset of action of 5-HT receptor ligands is slow [2, 3].

In this study, a possible anxiolytic and antidepressant property of *Skimmia anquetilia* essential oil were investigated using Social interaction test and hole board test using rats. Some other model like elevated plus maze is one of the most extensively used models of anxiety and is based on the aversion of rodents for open spaces and anxiolytics have been found to increase the proportion of time spent on the open arms [4].

Another test, Light and dark model based on spontaneous responses reflects a type of anxiety linked with uncontrollable stress (depressive anxiety) because animals are exposed by force to a novel and/or aversive environment from which they cannot escape [5].

Anxiolytics have been found to increase locomotion and time spent in the light zone, whereas anxiogenics decrease them [6]. The social interaction test is a useful animal model for evaluating anxiolytic compounds, which are prescribed for treating social phobia, social failure impairments, and emotional immaturity [7].

In the hole board test, anxiolytic increases head dipping time and its duration. Because the above tests have been validated pharmacologically, behaviourally and physiologically, as models of experimental anxiety in consideration to aforementioned reported psychopharmacological activities, it was thought prudent to investigate the anxiolytic potential of *Skimmia anquetilia*oil with the aim of finding agents that are safer and suitable for long-term treatment in anxiety like disorders.

MATERIALS AND METHODS:

Plant Material: The plant *Skimmia anquetilia was* collected from Gulmarg, Jammu & Kashmir, India. The plant was identified and authenticated by Dr. Zulfiqar Ali Bhat, from Department of Pharmaceutical Sciences, University of Kashmir, Srinagar-190006, India (Voucher specimen number - KUST03).

Extraction of Skimmia Oil: Dried leaves were submitted to hydro distillation in a Clevenger-type apparatus for 3 hours. After distillation the oil was collected, 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) was obtained.

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° C, oven temperature 40° C to 250° C at 3°C/ 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.

Experimental Animals: Male Wistar rats (150-200 gm body weight) were used for anxiolytic activity study. Rats were obtained 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 of Pharmaceutical Sciences (University of Kashmir) is approved for carrying out animal studies (Approval No.801/03/ca/CPCSEA) and the protocol for the present study was approved by Institutional Animal Ethical Committee (Approval no. F-IAEC/Pharm. Sc./Approval/2011/02).

Acute Toxicity Study: Acute toxicity study was conducted as per the internationally accepted protocol drawn under the OECD guidelines 425 (OECD, 2001). *Skimmia* oil at a dose level of 1600 mg/kg was found safe. Doses of 25, 50 mg/kg b.w. were selected as the study dose of *Skimmia* oil.

Drugs: Diazepam was obtained from Ranbaxy Lab. Ltd., HPSIDC-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 both suspended in a 1% sodium carboxy methyl cellulose solution.

All drugs were prepared immediately before use and were given orally. Control rats received 1% aqueous sodium carboxy methyl cellulose solution only. 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.

Anxiolytic Activity:

Procedures:

Social Interaction test: This model is based on the observation that animals tend to reduce or even suppress their interaction with other animals under conditions of new environments or excessive light.The test procedure for social interaction test was similar to that described by File and Pellow (1985) [4].

The test arena was a black Plexiglas box, 60 × 60 × 35 cm, with the base divided into 9 cm squares by lines of white tape. Two test conditions were performed: high light (380 lx), unfamiliar arena (HLUF) and high light, familiar arena (HLF).

On day 1 of testing, each rat was randomly assigned according to body weight (<10 g difference) to an unfamiliar partner in groups of 12 animals (six pairs) which were subsequently administered the appropriate drug. These rats were then replaced into their home cage until testing. Following appropriate pre-treatment time, members of each pair of unfamiliar rats were placed in opposite corners of the arena and observed for social interaction behaviors for 10 min.

Social interaction time (s) per pair of rats was measured as time of sniffing, mutual grooming, adjacent lying, climbing over and crawling under the partner, and approximation [8], Aggressive-type behaviors (e.g. kicking, aggressive grooming, biting, boxing and jumping [9] were also scored.

These behaviours were treated as separate entities and same were modulated by different pharmacological agents than social behaviors [10]. After day 1, test rats were returned to their home cages.

On days 2 and 3, the rats were placed individually, without drug, in the same box for 10 min per day to familiarize them with the apparatus. On the fourth day, the same pairs of rats were once again placed in the test arena for 10 min and the same test procedure was carried out [11-12].

Pairs of rats were allocated randomly to the following test groups: vehicle control, diazepam (1 mg/kg po), as standard and SAO (25 and 50 mg/kg p.o.) were evaluated as test drug for this research activity. All the results were statistically analyzed (*P <0.05) vs. control. Data statistically analyzed by ANOVA followed by Tucky test.

Hole-board test: The hole-board apparatus is an open-field with four equally spaced holes of 3.8 cm in diameter in the 60 × 60 × 35 cmfloor area, with the base divided into 9 cm squares by lines of white tape. The light intensity of the arena floor was 100wt bulb. The centre of each hole was 10 cm from the nearest wall.

The floor of the box was positioned 25 cm above the ground and divided into squares of 10×10 cm^2 with a water-resistant marker. Rats were randomly allocated to the following groups: vehicle treated as control, diazepam (1 mg/kg/ po) as standard and test drugs at different doses level.

The rats were administered with drugs or vehicle. Thirty minutes later, each animal was placed in the center of the hole-board, and allowed to freely explore the apparatus for 5 min. Total locomotor activity (numbers of squares crossed), number and duration of head dipping and latency to the first head-dip was recorded. A head dip was scored if both eyes disappeared into the hole [13-15].

The apparatus was wiped with a damp cloth after each trial and any faeces removed.

RESULTS:

Identification of Constituents of SAO: GC-MS of *Skimmia anquetilia* oil (SAO) indicated the presence of 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.

Important phytoconstituents like vetiverol (37.315%), 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%) areidentified as major constituents in *S. anquetilia* oil.

Anxiolytic Activity

Social Interaction Test: The time of active social interaction of the animals treated with both 25 and 50 mg/kg doses of SAO and diazepam (1 mg/kg po) significantly ($p<0.005$) increased social interaction time compared with the control group of animals. In the HLUF test condition there was a significant drug-induced increase in social interaction (***P*<0.01). Further analysis confirmed that both 25 and 50 mg/kg doses of SAO and diazepam (1 mg/kg po) significantly increased social interaction time compared with the control group and also markedly enhanced active social interaction (**P <0.01) **Table 1**.

In the HLF test condition there was again a significant drug-induced increase in social interaction (***P*<0.01) at doses of 25 & 50 mg/kg of SAO. Although the diazepam (1 mg/kg) had effect on time spent in social interaction but the same was less than the effect shown by 50 mg/kg of SAO **Table 1**. The data of varied behavioural categories are shown in **Table 2**.

The results showed that the increase of social interaction time was based on the enhancing duration of sniffing and mutual grooming and/or adjacent lying for rats (**P <0.01 or *P <0.05).

On the other hand there was a significant decrease in the duration of aggressive behaviourswith SAO at 25 and 50 mg/kg (**P <0.01 or *P<0.05) in both HLUF and HLF test conditions. However, diazepam also decreases the duration of aggressive behaviours, results are depicted in **Table 1.**

TABLE 1: SOCIAL INTERACTION TEST - ANXIOLYTIC AND ANTIDEPRESSANT EFFECTS OF SAO

Time spent in varied behavioral categories (seconds)	Control	Diazepam 1 mg/kg	SAO 25 mg/kg	SAO 50 mg/kg
HLUF test condition				
Sniffing and mutual grooming	27.43 ± 1.52	58.44 ± 2.27**	54.71 ± 1.62**	76.89 ± 4.27**
Adjacent lying	26.47 ± 1.45	81.60 ± 4.55**	37.64 ± 2.07*	51.42 ± 2.66**
Climbing over and crawling under	14.88 ± 2.16	40.18 ± 1.99**	23.90 ± 0.22	46.09 ± 2.68**
Approximation and follow	36.73 ± 2.62	55.76 ± 3.74**	39.87 ± 2.54*	52.13 ± 2.17**
Aggressive-type behaviors	11.12 ± 1.09	09.83 ± 0.54**	10.21 ± 2.56**	06.10 ± 1.65**
HLF test condition				
Sniffing and mutual grooming	44.83 ± 2.73	69.38 ± 3.92**	65.15 ± 2.50**	72.88 ± 2.76**
Adjacent lying	38.52 ± 1.93	60.87 ± 2.82**	42.57 ± 2.32*	56.47 ± 3.24*
Climbing over and crawling under	14.54 ± 2.46	32.10 ± 2.59**	20.07 ± 1.62	28.46 ± 2.86**
Approximation and follow	45.90 ± 1.86	66.21 ± 3.45	51.33 ± 2.62*	59.80 ± 2.42**
Aggressive-type behaviors	16.74 ± 0.58	08.67 ± 1.25**	09.68 ± 2.18**	08.10 ± 2.47**

*SAO- *Skimmia anquetilia* oil as test drug; Diazepam used as standard drug. Drug administered (n =6 pair/group) 40 min before (oral). Test conditions were high light unfamiliar (HLUF) and high light familiar (HLF). *P <0.05, **P <0.01, significantly different from control. Data's statistically analysed by ANOVA followed by Dunnet's test.

Hole-board Test: The rats were grouped and evaluated as vehicle control, diazepam (1 mg/kg po), as standard and SAO (25 and 50 mg/kg p.o.) were evaluated as test drug for various parameters of hole board activity. SAO (25 mg/kg/p.o.) treated rats showed a reduction in head-dip latency time, increase head-dip count, head-dip duration and rearing duration similar to standard diazepam (*P <0.01). However, at dose of 50mg/kg/p.o. of SAO and diazepam increased the locomotion time as compare to control untreated rats. Results show increased head dip latency as compared to lesser dose of test and standard drug **Table 2**. All the groups were compared with the control group. Data statistically analyzed by ANOVA followed by Dunnet test.

TABLE 2: EFFECTS OF LEAVES OF *SKIMMIA ANQUETILIA* OIL (SAO) IN HOLE-BOARD TEST IN RATS

Groups	Locomotion (Square crossed)	Head-dip counts	Head-dip duration (Sec.)	Head-dip latency (Sec.)	Rearing counts	Rearing duration (Sec.)
Control	26.7 ± 2.71	3.2 ± 0.22	4.4 ± 0.16	119.5 ± 5.85	13.7 ± 1.50	14.7 ± 2.91
Diazepam	25.2 ± 2.31	7.2 ± 2.11[a]	15.8 ± 2.63[a]	44.6 ± 2.74[a]	18.2 ± 2.43[a]	22.7 ± 2.12[a]
SAO 25	28.6 ± 2.53	4.9 ± 0.85	10.52 ± 1.76[a]	64.7 ± 3.73[a]	15.5 ± 2.36	18.24 ± 2.87
SAO 50	32.69 ± 1.12[a]	5.8 ± 0.43[a]	11. 90 ± 1.82[a]	52.81 ± 2.49[a]	19.76 ± 1.99[a]	21.38 ± 1.29[a]

Effects of SAO on anxiety in hole board test in rats; Results are expressed as means ± S.E.M., n = 6 per group. SAO25, and 50 means *S. anquetilia* oil at dose level 25, and 50mg/kg/p.o. respectively. The values [a]*P*<0.01 vs. control group. Statistically analyzed by ANOVA followed by Dunnetts test.

DISCUSSION AND CONCLUSION: Essential oils, especially those of Rutaceae family, are popularly used in therapies for their effects on mood states (anxiety, depression) and are believed to induce an effect of mental relaxation.

The results of present study suggest that the essential oil of *Skimmia anquetilia*, at the doses of 25 and 50 mg/kg/b.w./p.o., induced a decrease in the level of emotionality, evaluated in social interaction test models of anxiety.

Such response might be correlated with the activation of the olfactory system by the volatile components present in the oil, which suggests a possible central action. We would highlight the fact that anxiolytic drugs lessen fear and increase the exploratory activity of animals [15-16].

The anxiolytic and antidepressant effects on the central nervous system which are likely to be attributed by a specific component of the essential oil such as vetiverol, linalool, limonene and citronellal are scientifically recognized for such activity [17].

The social interaction test of anxiety was developed to provide an ethologically based test that was sensitive to both anxiolytic and anxiogenic effects. An increase in social interaction is indication of an anxiolytic effect, whereas a specific decrease in social interaction indicates an anxiogenic effect. This test provided a new approach to the neurobiological mechanisms underlying anxiety disorders.

The aversive test condition of the social interaction test (HLUF) increases 5-HT and DA turnover throughout the rat brain. In brief, the social interaction test is an extremely useful animal model for evaluating anxiolytic compounds, which are prescribed for treating social phobia, social failure/impairments and emotional immaturity [13, 18].

In Social interaction test, SAO and DZ decreased aggressive behaviors at the doses of 25, 50 mg/kg and 1 mg/kg respectively while the same doses significantly increased and prolonged social interaction time of the HLUF test condition.

Whereas, SAO (25 mg/kg/p.o.) treated rats in hole board test showed a reduction in head-dip latency time, increase head-dip count, head-dip duration and rearing duration similar to standard diazepam (*P <0.01). However, at dose of 50mg/kg/p.o. of SAO and diazepam increased the locomotion time as compare to control untreated rats.

The results indicate that SAO has a significant anxiolytic-like effect in rats. The activity is more in SAO 50mg/kg as compared to SAO 25 mg/kg and the same was exhibited on all the days of our

recordings which clearly indicate a dose dependant activity with maximum activity in SAO 50mg/kg.

The pharmacological mechanism that might account for the anxiolytic effect of SAO has not been clearly identified yet. In this study we have reported that 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 that the GABAergic system is at least partly involved in the pharmacological activity of SAO.

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 a rich sources for isolation of vetiverol.

Further investigations are going on to isolate bioactive components from *Skimmia anquetilia* oil to study the molecular pharmacological mechanism for its anxiolytic activity in animal models.

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CONFLICTS OF INTEREST: Nil

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