Research Paper

Pharmacological evaluation of the extracts of *Sphaeranthus indicus* flowers on anxiolytic activity in mice

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ABSTRACT

Objective: The objective of the study was to investigate the anxiolytic activity of petroleum ether, alcohol and water extracts, obtained from the flowers of *Sphaeranthus indicus* Linn, in mice.

Materials and Methods: Elevated plus maze (EPM), open field test (OFT) and foot-shock induced aggression (FSIA) were the screening tests used to assess the anxiolytic activity of the extracts on mice. Diazepam (1 mg/kg) served as the standard anxiolytic agent.

Results: The animals receiving extracts or diazepam (1 mg/kg) showed an increase in the time spent, percent entries and total entries in the open arm of the EPM; increased ambulation, activity at centre and total locomotion in the OFT; and decreased fighting bouts in the FSIA, suggesting anxiolytic activity. Petroleum ether extract (10 mg/kg), alcoholic extract (10 mg/kg) and water extract (30 mg/kg) resulted in prominent activity in the mice. Petroleum ether extract (10 mg/kg) resulted in more prominent anxiolytic activity in the EPM and OFT than ethanolic or water extracts, but was less than that produced by diazepam (1 mg/kg).

Conclusion: Petroleum ether extract of *S. indicus* flowers produces prominent anxiolytic activity in mice.

KEY WORDS: *A*nxiety, *Gorakhmundi*

Introduction

Sphaeranthus indicus Linn belongs to family *Asteraceae*. The plant is commonly known as *Gorakhmundi* in Hindi. It is an annual spreading herb, which grows approximately 15-30 cm in height. The plant is distributed through out the plains and wet lands in India, Sri Lanka and Australia.[1]

All the parts of the plant have medicinal uses. In folk medicine, the plant is reportedly used in treating epileptic convulsions, mental illnesses and hemicranias.[2] The whole herb is used in ayurvedic preparations to treat epilepsy and mental disorders.[3] It is used to treat vitiated conditions of hemicranias, jaundice, hepatopathy, diabetes, leprosy, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia and skin diseases. It is also used as a nervine tonic. The oil prepared using the plant roots is reportedly useful in treating scrofula and as an aphrodisiac. The external application of a paste of this herb is beneficial in treating pruritus and edema, arthritis, filariasis, gout and cervical adenopathy. It also treats piles and hepatitis.[4]

A large number of constituents have been isolated from the extracts of the whole herb, flowers and leaves. Essential oil, obtained by steam distillation of the whole herb, contains ocimene, α-terpinene, methyl-chavicol, α-citral, geraniol, α-ionone, ß-ionone, d-cadinene, p-methoxycinnamaldehyde[5] and an alkaloid sphaeranthine.[6] The alcoholic extract of powdered caputula contains stigmasterol, ß-sitosterol,[7] hentriacontane, sesquiterpene lactone,[8] sesquiterpine glycoside, sphaeranthanolide,[9] flavone and isoflavone glycosides.[10]

Recently, many medicinal properties have been attributed to the extracts, fractions and isolated constituents of *S. indicus* flowers, which include hypotensive, peripheral vasodilatory and cathartic activity of alcoholic extract,[11] antimicrobial activity of alkaloidal and nonalkaloidal fractions of alcoholic extract[12] and sesquiterpine isolated from petroleum ether extract.[13] Essential oil,[14] obtained from leaves, possesses antifungal properties.

There is a paucity of scientific data about the anxiolytic activity of the extracts of flowers of *S. indicus*. Hence we set out to investigate the same in mice.

Materials and Methods

Plant collection and extraction

The flowers of *S. indicus* were collected at Raigad, Maharashtra. They were authenticated by Dr. Mujumdar (Head, Dept. of Botany), Agharkar Research Institute, Pune. A voucher

specimen was deposited at the herbarium in the institute.

The powdered plant material (500 g) was soaked in petroleum ether (2000 ml) and allowed to stand for 48 h, with occasional shaking. The macerate was decanted and filtered, through cloth, and then, through Whatman filter paper (No.1). This process of extraction was repeated with the same volume of petroleum ether. The macerates were pooled and evaporated to yield a dark greenish yellow, waxy mass (yield 3.2%). The residue, called 'marc,' after the extraction with petroleum ether) was dried and extracted with ethanol (90%) by the same procedure to yield a greenish brown semisolid (yield 4.0%). The water extract was prepared by macerating marc after alcohol extraction, using the same procedure and evaporated at 40^{0}C to yield a dark brown solid mass (yield 11.6 %).

The extracts of petroleum ether and alcohol were stored in air-tight glass bottles, at room temperature. The water extract was stored in air-tight glass bottles in a refrigerator.

Preparation of dosage form

The emulsion of petroleum ether extract (SIP) was prepared by triturating (blending) the accurately weighed quantity of the extract with 2.5% polysorbate 80 (Tween 80) in a glass mortar, with the gradual addition of water for injection (WFI), to make up the required volume. The emulsion of the alcohol extract (SIA) was prepared by triturating the accurately weighed quantity of the extract with 2.5% polysorbate 80 and 0.5% carboxy methylcellulose (CMC) in a mortar, with the gradual addition of WFI, to make up the required volume. The accurately weighed quantity of the aqueous extract (SIW) was dissolved in WFI to prepare the clear solution. Diazepam injection, (Calmpose®, Ranbaxy Laboratories Ltd. India), was diluted with WFI.

Vehicles, to be administered to respective control groups, were prepared using the same procedure without the addition of extracts.

Storage

The dosage forms of the extracts were prepared freshly on the day of the experiment and kept, at room temperature, in air-tight, amber coloured vials to protect them from light.

Phytochemical analysis

The petroleum ether (SIP), alcohol (SIA) and water (SIW) extracts of *S. indicus* were tested for the presence of carbohydrates, proteins, amino acids, alkaloids, flavonoids, glycosides, saponins, tannins, steroids and oil, using standard procedures.[15]

Animals

Swiss albino male mice (22-25 g) were obtained from the Serum Institute of India Ltd., Pune. The animals were housed in groups of 6-8 per cage and maintained at 24^{0}C±1^{0}C, with relative humidity of 45-55% and 12:12 h dark/light cycle. The experiment was carried out between10:00 to 17:00 h. The animals had free access to food (Standard chew pellets, Chakan Oil Mills, Sangli) and water, *ad libitum*. Food, not water, was withdrawn 3 h before and during the experiment.

The Institutional Animal Ethics Committee of Poona College of Pharmacy, Pune approved the pharmacological and acute toxicity protocol.

Acute toxicity

Acute toxicity study was performed in mice. The extracts were administered intraperitoneally (i.p.) at doses of 175, 550, 2000 mg/kg. They were then observed for signs of toxicity, continuously for 2 h, and for mortality up to 24 h, after injection.[16]

Assessment of anxiolytic activity

Treatment schedule

Elevated plus maze and open field test: The animals were divided into 13 groups, consisting of 6 mice per group. Groups 1, 5 and 9 received vehicle 1- (2.5% Tween 80), vehicle 2- (0.5% CMC+2.5% Tween 80) and vehicle 3- (WFI), respectively. Groups 2, 3 and 4 received SIP 10, 30 and 100 mg/kg; Groups 6,7 and 8 received SIA 10, 30 and 100 mg/kg; Groups 10, 11 and 12 received SIW 10, 30 and 100 mg/kg; and Group 13 received diazepam 1 mg/kg.

Foot shock induced aggression (FSIA)

In the FSIA test, the animals were divided into 13 groups of 12 mice (6 pairs of male mice) per group. The treatment was allotted to the groups as above, using doses 30, 100 and 300 mg/kg of all extracts (SIP, SIA and SIW). The group receiving vehicle 1 and vehicle 2 served as control for SIP and SIA, respectively, while vehicle 3 was the control group for SIW and diazepam. All the vehicles or extracts were administered, i.p., to mice 45 min before the start of the experiment and diazepam was administered, i.p., 30 min before the start of experiment. The volume of dosage administered was 5 ml/kg of body weight.

Elevated plus maze (EPM)

The EPM apparatus consisted of two open arms (30 x 5 cm) and two closed arms (30 x 5 x 20 cm) emanating from a common central platform (5 x 5 cm). The two pairs of identical arms were opposite to each other. The entire apparatus was elevated to a height of 50 cm above the floor level. The animals received the treatment as per the schedule, 45 min before the start of the session. At the beginning of the session, a mouse was placed at the centre of the maze, its head facing the closed arm. It was allowed to explore the maze for 5 min. The time spent in the open arm, percent entries in the open and closed arms and total entries were recorded. An entry was defined as the presence of all four paws in the arm. The EPM was carefully wiped, with 10% ethanol after each trial, to eliminate the possible bias due to the odour of the previous animal.[17]

Open field test (OFT)

The OFT apparatus consisted of a wooden box (68 x 68 x 45 cm^3), with a dark gray floor, subdivided into 16 equal fields. The experimental room was a sound attenuated, dark room. The OFT, illuminated with a 40W bulb, focusing on the field from a height of about 100 cm, was placed in the experimental room. After 45 min of treatment, the mice were placed individually in a corner square of the OFT and the ambulation (the number of squares crossed at periphery), total locomotion (total number of squares travelled) and activity in the centre (number of central squares crossed) was recorded for 5 min.[18]

Foot shock induced aggression in mice (Dolphin, India)

A pair of male mice was placed in a box with a grid floor consisting of steel rods with a distance of 6 mm. A constant AC current of 0.8 mA was supplied to the grid floor. A constant shocker delivered 60 Hz current for 5 sec, followed by 5 sec intermission. Forty-five minutes after the treatment, the total number of fights was recorded for 3 min. The fighting behaviour

consisted of vocalisation, leaping, running, rearing and facing each other with some attempts to attack by biting. Six pairs of mice were used for each treatment.[19]

Statistical analysis

All data are presented as mean±SEM and analysed by one-way ANOVA, followed by Dunnett's test. The groups treated with extracts were compared with the respective vehicle group. The diazepam treated group was compared with vehicle (WFI). *P* values <0.05 were considered statistically significant.

Results

Phytochemical analysis

The SIP showed the presence of steroids, fats and oils. The SIA showed the presence of carbohydrates, proteins, amino acids, tannins, phenols, steroids, fats and oils, while the SIW showed the presence of carbohydrates, proteins, amino acids, tannins, phenolic compounds, saponins and alkaloids. [Table 1]

Acute toxicity

All extracts were found to be safe in the doses used and there was no mortality up to a dose of 2 g/kg, i.p.

Assessment of anxiolytic activity

Elevated Plus Maze (EPM): In the EPM, the behaviour, which was observed, confirmed the anxiolytic activity of diazepam as reported previously.[20] The SIP and SIA, at doses of 10 and 30 mg/kg, significantly ($P<0.01$) increased the time spent in the open arm. [Figure 1A] The SIW, at 30 mg/kg, significantly increased the time spent in the open arm. Percent open arm entries were increased significantly at 10 mg/kg of SIP ($P<0.05$), SIA, SIW ($P<0.01$) and 30 mg/kg of SIA, SIW ($P<0.05$). [Figure 1B] Similarly, a significant decrease in the percent closed arm entries was found at the same dose levels.

Table 1

Results of the phytochemical analysis of the different extracts of *S. indicus*

Test	*SIP*	*SIA*	*SIW*
Carbohydrates	-	+	+
Reducing sugar	-	+	+
Monosaccharides	-	+	+
Pentose sugar	-	-	-
Hexose sugar	-	-	-
Proteins		+	+
Proteins containing tyrosine and tryptophan	-	-	-
Protein containing sulphur	-	-	-
Amino acids	-	+	+
Tannins and phenols	-	+	+
Glycosides	-	-	-
Cardiac glycosides	-	-	-
Anthraquinone glycosides	-	-	-
Saponins	-	-	+
Flavonoids	-	-	-
Alkaloids	-	-	+
Steroids	+	+	-
Fats and oil	+	+	-

+ positive test; - negative test; SIP – *S. indicus* petroleum ether extract; SIA – *S. indicus* alcoholic extract; SIW – *S. indicus* aqueous extract.

Figure 1. Results of Elevated Plus Maze test in mice administered with *S. indicus*. Values are mean±SEM, n = 6 in each group. **P*<.05, ***P*<.01 when compared to vehicle 1, #*P*<.05, ##*P*<.01 when compared to vehicle 2 and +*P*<.05, ++*P*<.01 when compared to vehicle 3. (One-way ANOVA followed by Dunnett's test). SIP-Petroleum ether extract; SIA-Alcohol extract; SIW-Water extract

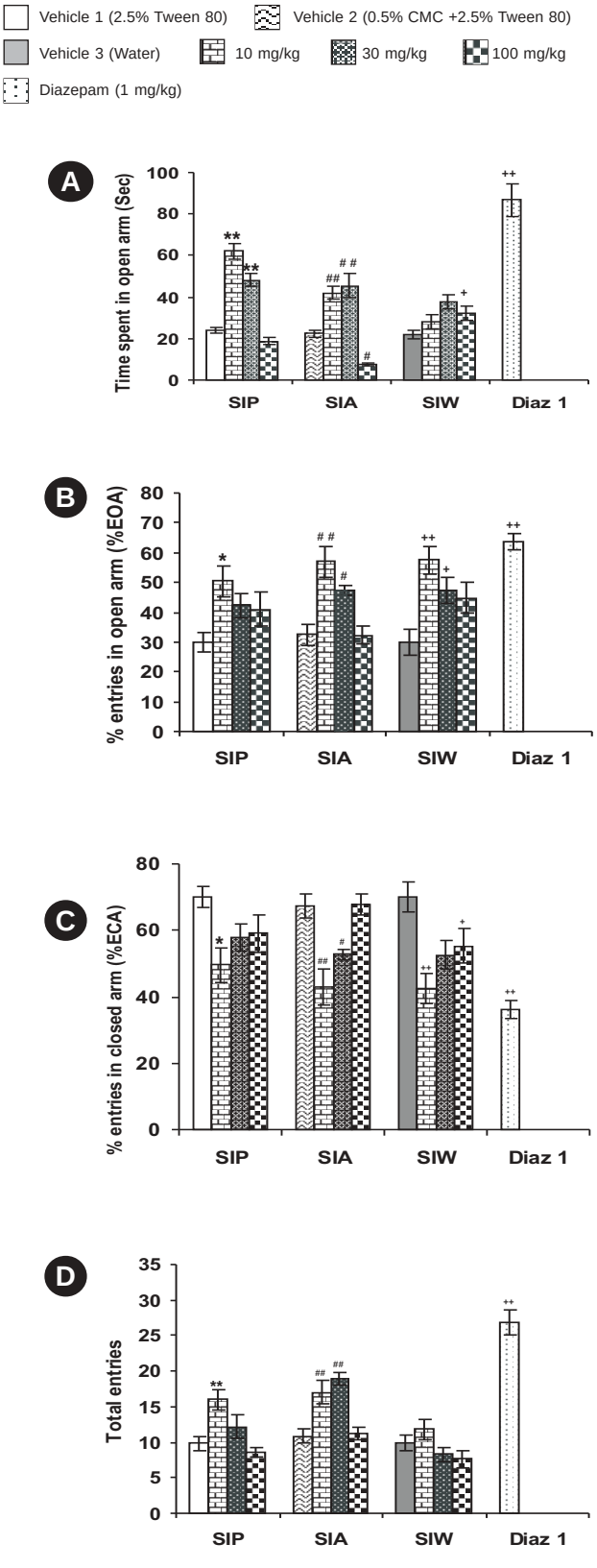


Table 2

Results of Open Field Test in mice administered with *S. indicus*

Group	Ambulation (number of squares crossed at periphery)	Activity at centre (number of central squares crossed)	Total locomotion (total number of squares travelled)
Vehicle1	48.33 ± 1.98	2.17±0.48	53.33±2.20
SIP 10	100.50 ± 10.51**	12.67±2.16**	113.17± 10.67**
SIP 30	96.00 ± 3.29**	8.67±1.12**	104.67±2.76**
SIP 100	1.83 ± 0.31**	0.00±0.00	1.83±0.31**
F	68.602	22.27	85.542
df	3, 20	3, 20	3, 20
P	0.0001	0.0001	0.0001
Vehicle 2	43.67 ± 1.28	2.00±0.37	45.67±1.52
SIA 10	79.67 ± 6.76##	7.50±0.76##	87.17±6.49##
SIA 30	103.83 ± 9.60##	5.00±0.73#	108.83±9.47##
SIA 100	76.83 ± 5.69##	5.50±0.76##	82.33±6.33##
F	14.229	11.273	15.783
df	3, 20	3, 20	3, 20
P	0.0001	0.0002	0.0001
Vehicle 3	47.50 ± 1.98	2.33±0.42	49.83±2.09
SIW 10	72.33 ± 7.43++	3.33±0.88	75.67±8.07++
SIW30	78.83 ± 2.83++	2.33±0.42	81.17±3.02++
SIW 100	9.00 ± 0.97++	0.00±0.00	9.00±0.97++
Diazepam 1	114.16 ± 8.06++	13.16±1.10++	127.33±7.84++
F	57.321	55.987	67.032
df	4, 25	4, 25	4, 25
P	.0001	.0001	.0001

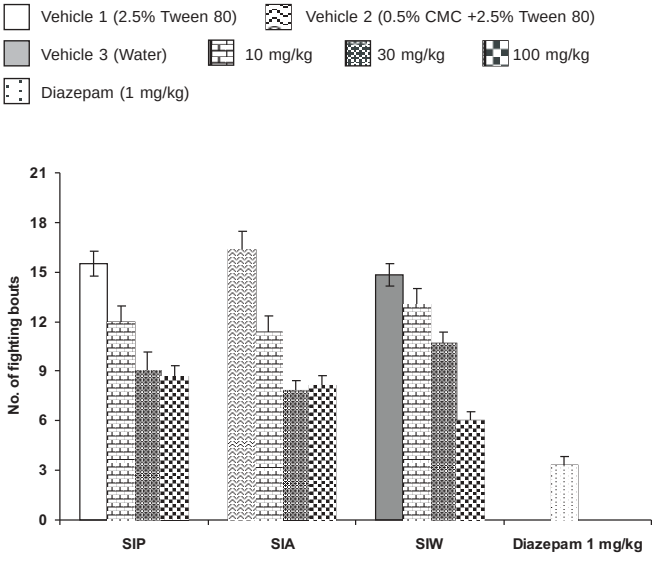
Values are mean±SEM, n = 6 in each group. Data analysed using one-way ANOVA, followed by Dunnett's test. **P*<.05, ***P*<.01 when compared to vehicle 1, #*P*<.05, ##*P*<.01 when compare to vehicle 2 and +*P*<.05, ++*P*<.01 when compared to vehicle 3.

[Figure 1C] The total arm entries were increased significantly at SIP 10 mg/kg ($P<0.01$), SIA 10 and 30 mg/kg ($P<0.01$). [Figure 1D] The SIA, at a higher dose of 100 mg/kg, showed a significant ($P<0.05$) decrease in time spent in the open arm, without any significant change in percent entries in the open arm, percent entries in the closed arm and total entries. Diazepam (1 mg/kg) significantly increased the time spent in the open arm, percent entries in the open arm and total entries, whereas the closed arm entries were significantly ($P<0.01$) decreased. [Figure 1]

Open field test

Diazepam 1 mg/kg significantly ($P<0.01$) increased the ambulation, activity at the centre and total locomotion. Similar results were exhibited by the extracts in the OFT. The SIP, SIA and SIW at 10 and 30 mg/kg showed a significant ($P<0.01$) increase in the ambulation and total locomotion. However, activity at the centre was increased significantly ($P<0.01$) by SIP (10 and 30 mg/kg) and SIA (10 mg/kg), whereas increased activity at the centre by SIA (30 mg/kg) was less significant ($P<0.05$). The SIW failed to produce any significant increase in activity at the centre. At higher doses, the SIP and SIW showed a decrease in all the parameters observed in the OFT [Table 2]

Figure 2. Effects of the extracts of *S. indicus* flowers on foot shock induced aggression in mice. Results are expressed as mean±SEM, n = 6 in each group. **P*<.05, ***P*<.01 when compared to vehicle 1, #*P*<.05, ##*P*<.01 when compared to vehicle 2 and +*P*<.05, ++*P*<.01 when compared to vehicle 3. (One-way ANOVA followed by Dunnett's test.)



Foot shock induced aggression

The number of fighting bouts was decreased significantly by the SIP and SIA at 30, 100 and 300 mg/kg. However, the SIW showed similar results at 100 and 300 mg/kg only. Diazepam (1 mg/kg) significantly ($P<0.01$) decreased the number of fighting bouts. [Figure 2]

Discussion

The EPM test is based on a premise where the exposure to an EPM evoked an approach-avoidance conflict that was considerably stronger than that evoked by the exposure to an enclosed arm.[21] The decrease in aversion to the open arm is the result of an anxiolytic effect, expressed by the increased time spent and entries in the open arm. The SIP and SIA increased the time spent and percent entries in the open arm, with percent decrease in the closed arm. The increase in percent entries in the open arm, showed by SIA, was more, but the time spent in the open arm was less than the SIP (10 mg/kg), suggesting that the average time spent in the open arm per entry was less with the latter. The decrease in percent closed arm entries, by the extract, suggest that the increase in total arm entries is due to an increase in open arm entries rather than close arm entries. This finding can be correlated with the previous finding that close arm entries correlated to locomotor activity, which also indicate the exclusive stress alleviating effect of the extract.[22] Therefore, behaviour alterations induced by SIP (10 and 30 mg/kg), SIA (10 and 30 mg/kg) and SIW (30 mg/kg) were consistent with an anxiolytic activity. The SIA and SIW showed weak anxiolytic activity as compared to SIP (10 mg/kg).

In the OFT, the confrontation with the situation induces anxiety behaviour in rodents. The anxiety behaviour is triggered by two factors, i.e., individual testing (the animal was separated

from its social group) and agoraphobia (as the arena is very large, relative to the animals breeding or the natural environment). In such situations, rodents show thigmotaxic behaviour identified by spontaneous preference to the periphery of the apparatus and reduced ambulation.[18] Anxiolytic treatment decreases this anxiety induced inhibition of exploratory behaviour. The SIP (10 mg/kg) showed a more prominent effect on central activity as compared to the effects shown by the SIA and SIW. Even though the SIA (30 mg/kg) showed a more pronounced effect on ambulation, the effect on the central activity was less than the SIP (10 mg/kg). The higher number of total entries and the longer time spent in the open arm in the EPM agreed with the increased locomotion and the higher central activity, respectively recorded in the OFT, and suggested an anxiolytic activity.[23] The SIW increased ambulation, without any effect on central activity. Present results suggest that the SIP (10 mg/kg) possess more anxiolytic activity as compared to the SIA and SIW, which agreed with the results obtained in the EPM.

It is well known that extracts, plant sources.[24] isolated constituents from plants[25] and synthetic drugs such as benzodiazepines and phenobarbital[26] possess anxiolytic and sedative activities. At higher doses, diazepam (20 mg/kg p.o.) in rats[27] chlordesmethyldiazepam (benzodiazepine receptor full agonist) and desmethyldiazepam (benzodiazepine receptor agonist, metabolite of diazepam) at 5 mg/kg, i.p., in mice showed decreased activity in the OFT, which was concluded as a sedative effect.[28] A similar decrease in ambulation at higher dose of SIP and SIW (100 mg/kg) was found, which might be due to the sedative property of the extracts. In the EPM, the decrease in the time spent in the open arm, without a change in the open arm, closed arm and total entries at higher doses, may be attributed to the sedative effect of the extracts. This agreed with the earlier report by Sukma *et al* [29] in the case of barakol, a constituent of *Cassia siamia* Lamk.

The reduced number of fighting bouts indicated an anxiolytic activity in FSIA.[30] The SIP, SIA and SIW, showing a decrease in the number fighting bouts, support anxiolytic activity in the EPM and OFT. Not only anxiolytics, but also other classes of drugs such as sedative (meprobamate and phenobarbital), neuroleptics (perphenazine) and analgesic (methadone),[31] were found to be active in this test A higher dose (100 and 300 mg/kg) of all extracts showed prominent effects in this test which may be due to the sedative effect of the extracts, rather than an anxiolytic activity.

Earlier investigations of chemical constituents and their pharmacology reveal that saponins possess anxiolytic activity.[25] Tannins have been also shown to possess activity against many CNS disorders.[32]

The SIP showed the presence of fats, oils and steroids. There are a number of fatty acids, oils and steroids, isolated from various plants, but there is a paucity of scientific data regarding their anxiolytic activity. Therefore, we cannot assign anxiolytic activity to any one of them, without isolating the specific constituent. Similarly, the SIW showed the presence of saponins, but revealed weak anxiolytic activity as there are large numbers of saponins, *viz.,* steroidal saponins, triterpine saponins, and all saponins did not possess anxiolytic activity.

To summarise, the SIP (10 mg/kg) possesses more prominent anxiolytic activity than the SIA and SIW. Diazepam (1mg/kg) showed more activity than the extracts. The decreased activity at a higher dose (100 mg/kg) may be due to the sedative effect.

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