ORIGINAL ARTICLE

Indigofera tinctoria: Preliminary experimental study evaluating its analgesic and behavioural activities in animals

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Indigofera tinctoria Linn. (Family: Fabaceae) is traditionally used for the treatment of various ailments including nervous disorders. In homoeopathy too, *I. tinctoria* is used in the treatment of epilepsy and other nervous disorders, but no data has been scientifically documented to establish its central nervous system activities. In the present preliminary study, the different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* administered at a dose of 0.5 ml/rat/day were evaluated for their analgesic (hot plate, ice plate and Randall -Selitto tests) and behavioural (rota rod and open field tests) activities. The results revealed that all the four potencies of *I. tinctoria* had increased the latency time required to raise and to lick the fore or hind paw for thermal sensation on hot plate test and for cold sensation on ice plate test. They had also increased the quantum of threshold pressure to mechanical induced pain on Randall -Selitto test but depressed the motor coordination and locomotor activities. The observed activities suggest that the homoeopathic formulations of *I. tinctoria* possess CNS depressant property. However, further studies are required for a definitive conclusion.

Keywords: homoeopathic medicine; I. tinctoria; potencies; analgesic activity; behavioural effect; albino rats.

INTRODUCTION

Indigofera tinctoria has been extensively used in various folklore and traditional medicine systems for treatment of several disorders. In ayurveda and siddha, it is prescribed for chronic bronchitis, asthma, ulcers, skin diseases and constipation. Roots used for anti poison, giddiness, colic, gonorrhoea, urinary complaints and hepatitis. Leaves used for jaundice, vatha, fever and blennorrhagia.[1] In unani medicine, a past made from the seeds is applied topically on blemishes, freckles, pimples and other skin affections. Extract of whole plant is used in the treatment of liver ailments, epilepsy and other neurological disorders. [2, 3]

The ethanol extract of this plant was found to be useful in controlling lithium/pilocarpine induced status epilepticus in albino rats[4] and possessed analgesic activity against acetic acid induced writhing in mice[5] and also possessed antioxidant properties[6], whereas, chloroform residue of its alcoholic extract showed a significant decrease in plasma triglycerides, total cholesterol, glycerol and free fatty acids accompanied by an increase in high density lipoproteins concentrations.[7] Likewise, methanol extract of the aerial part of this plant showed significant decrease in blood glucose levels in alloxan-induced diabetic rabbits[8] and inhibited the proliferation of human Non-Small Cell lung cancer-549 cells.[9] It also possessed anthelmintic[10] and anti-inflammatory activities.[11] A bioactive fraction (indigitone) of its petroleum ether extract showed significant hepatoprotective activity against CCl4 induced liver injury in mice and rats.[12]

In homoeopathy too, *I. tinctoria* is being prescribed for the treatment of epilepsy and other nervous disorders [13], but lacks scientific claim. Therefore, it was considered worthwhile to evaluate the analgesic and behavioural activities of homoeopathic formulations (3x,

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6x, 12x and 30c potencies) of I. tinctoria in experimental animal models in order to provide scientific basis for its use in homoeopathy.

MATERIALS AND METHODS

Plant collection

Indigofera tinctoria Linn. (Family:Fabaceae) whole plant was collected in August,2007 from the Nilgiris Hills, Tamil Nadu, India and taxonomically identified/ authenticated by the Survey of Medicinal Plants and Collection Unit, Udagamandalam, Tamilnadu, India.

Drugs

Homoeopathic formulations of *I. tinctoria* in 3x, 6x and 12x potencies were prepared in decimal scale while 30c was prepared in centesimal scale by a reputed manufacturer of homoeopathic medicines (M/S. Bahola Laboratories, Puducherry, India)

Animals

Albino rats (120-140 g) obtained from National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad were used for the study. They were housed in polypropylene cages and maintained with 12:12 hrs, light/dark cycles, and room temperature 22-24ºC. The animals were fed with standard pellet (Hindustan Lever, Kolkata, India) diet and water was given ad - libitum. The animals were acclimatized to standard laboratory conditions for 10 days and thereafter they were accustomed to respective test procedures by giving them three test trials at 10 minutes intervals on each day for three consecutive days before starting the experiment.

Experimental design

Albino rats were divided in to 5 groups of 18 each which were further divided into 6 sub-groups of 3 each. Different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* were administered orally at a dose of 0.5 ml/ rat/day for 30 days. Two groups of parallel controls, one receiving equivalent volume of alcohol (91.5%v/v; used as a vehicle for preparation of different potencies of *I. tinctoria*) and other receiving equivalent volume of normal saline were also run simultaneously. The response of drug was measured after 30 mintues of its administration on 10^{th}, 20^{th} and 30^{th} day. Readings taken just before administration of the drug/alcohol/ normal saline on day 1 of the study were considered as the initial control value in the same group for comparison. The experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) of Department of Zoology, Osmania University, Hyderabad (Reg.No.383/01/a/CPCSEA). All the experiments were performed in an isolated and noiseless air conditioned room between 10.00 -15.00 hrs.

Assessment of analgesic activity

Hot plate test

The hot plate latency assay was carried out as described by Eddy et al.[14] 30 minutes after the administration of drug, alcohol or saline, the rats were gently placed individually on a hot plate maintained at a temperature of 55 ± 2ºC. The reaction time (latency time: in seconds) of rats taken for licking of fore or hind paw or jumping was recorded. The reaction time of the rats to thermal noxious stimulus was taken on day 1 before the administration of drug, alcohol or normal saline was considered as initial value for comparison. A cut-off time of 15 sec [15] was selected to avoid tissue damage.

Ice plate test

Rats were gently placed individually on the ice cubes (0 - 4 ºC) filled in a container (20 x 20 x 20 cm) and covered with a plastic cover. The reaction time in seconds to lick the fore or hind paw to cold sensation was noted after 30 minutes of administration of test drug, alcohol or saline. The reaction time (latency time) of the rats to cold sensation was taken on day 1 before the administration of drug, alcohol or normal saline was considered as initial value for comparison. A cut-off reaction time of 15 sec was chosen in order to avoid physical injury to the animals. Percentage of analgesia was calculated as described in hot plate technique.[14]

Randall - Selitto test

The analgesic activity of drug against mechanical induced pain was carried out as described by Randall - Selitto. After 30 minutes of drug, alcohol or saline administration, the paw of the right foot of the rat was placed on the rubber base of the apparatus (Randall - Selitto apparatus, Ugo Basile, Italy) and pressure (in ponds; expressed in g) was applied either on 2^{nd} – 3^{rd} or 3^{rd} – 4^{th} metatarsal region through a pointed tip and increased gradually until vocalization elicited which was considered as threshold pressure to mechanical induced pain. Threshold pressure to mechanical induced pain taken on day 1 before the administration of drug, alcohol or normal saline was considered as initial value for comparison.[16]

Assessment of behavioural activity

Rota - rod test

Motor coordination, grip strengths of the rats were measured by using the automated rota rod apparatus (DolphinTM instrument).[17] The rotor was divided

into three compartments which allowed three rats to test simultaneously at a time. The rats capable of remaining on the rota rod for 60 seconds or more, in three successive trials were selected for the study. Thereafter, 30 minutes after the administration of drug, alcohol or normal saline, rats were placed on the horizontal rod with the head directed opposite to the direction of rotating rod at a speed of 5rpm[17] and fall off time i.e. when animals falls from the rotating rod, was recorded, which was taken as grip strength. The grip strengths of the rats were measured on day 1 just before administration of the drug, alcohol or normal saline were considered as initial value for comparison.

Open - Field test

Open field test was used for recording locomotor activity.[18] The apparatus consisted of a wooden (96x96x6cm) box. The floor of the box was divided into 36 equal squares which was painted alternatively with block and white colours and illuminated with low intensity diffuse light (40 W) placed at a height of 100 cm. The animals were placed gently in the centre of the apparatus one after another where they were free to walk and to get adapted to the new environment. After completion of their training individually, the animals were treated with test drug, alcohol or saline and 30 min later the animals were placed individually in the apparatus and the number of squares crossed in 5 min was recorded. The floor of the box was cleaned after every trial.

Statistical analysis

The data were expressed as Mean ± S.E.M. The difference between mean values of groups were statistically analysed by student's't' test. p-values < 0.05 were considered as statistically significant.[19]

Results

Assessment of analgesic activity

Hot plate test

The results of the analgesic effect of *I. tinctoria* using hot plate assay are presented in Table-1. There was an increase in the latency time (4.89 to 5.74 sec.) to thermal noxious stimulus when measured 30 minutes after the administration of different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* at a dose of 0.5 ml/rat/day on 10th day. A significant ($p<0.05$) increase in latency time was observed with those rats treated with 6x and 30c potencies of *I. tinctoria* as compared to initial latency time taken just before administration of drug on 1 day of the study. However, the increase in the duration of latency time to thermal noxious stimulus was tapered off gradually on 20th day and 30th day on continuation of the treatment. The initial latency time recorded on day 1 before administration of drug, alcohol or normal saline and 30 minutes after the administration of normal saline on different days of experimentation to noxious thermal stimulus was more or less constant (3.38 to 3.61 sec) (Table.1).

Ice plate test

The results of the analgesic effect of different potencies of *I. tinctoria* by ice plate assay are shown in Table-2. There was an increase in the latency time (7.56 to 8.57sec) to cold sensation when measured 30 minutes after the administration of different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* at a dose of 0.5 ml/rat/day on 10th day. The difference in the increase in latency time to cold sensation was significant ($p<0.05$) with those animals which were treated with 30c potency of *I. tinctoria*. Thereafter, the increase in the duration of latency time to cold sensation was tapered off

Table 1. Analgesic effect of IT(*Indigofera tinctoria)* (0.5ml/rat/day) on hot plate test

Group	Latency time to pain response in seconds On days of treatment			
	Initial	10th day	20th day	30th day
Control (Normal saline)	3.45 ± 0.52	3.53 ± 0.34	3.46 ± 0.36	3.38 ± 0.39
Vehicle (91.5% alcohol)	3.58 ± 0.61	4.89 ± 0.46	4.59 ± 0.32	4.05 ± 0.40
IT3x	3.56 ± 0.44	5.26 ± 0.49	5.01 ± 0.44	4.05 ± 0.39
IT6x	3.61 ± 0.39	5.70 ± 0.48*	5.18 ± 0.51	4.44 ± 0.35
IT12x	3.58 ± 0.52	5.29 ± 0.41	4.38 ± 0.36	4.06 ± 0.43
IT30c	3.43 ± 0.50	5.74 ± 0.46*	5.08 ± 0.55	4.44 ± 0.36

Values are mean ± SEM; n=3 in each group; * significantly different at p <0.05

gradually on 20th day and 30th day on continuation of the treatment. The initial latency time to cold sensation recorded on day 1 before administration of drug, alcohol or normal saline and 30 minutes after the administration of normal saline on different days of experimentation was more or less same (5.68 to 6.12 sec) (Table 2)

Table 2. Analgesic effect of IT(*Indigofera tinctoria)* (0.5ml/rat/day) on ice plate test

Group	Latency time to pain response in seconds On days of treatment			
	Initial	10th day	20th day	30th day
Control (Normal saline)	5.91 ± 0.45	6.09 ± 0.37	5.80 ± 0.55	6.04 ± 0.43
Vehicle (91.5% alcohol)	5.85 ± 0.51	7.76 ± 0.56	7.36 ± 0.48	6.44 ± 0.37
IT3x	6.05 ± 0.39	8.00 ± 0.45	7.38 ± 0.48	6.60 ± 0.36
IT 6x	6.12 ± 0.51	8.04 ± 0.51	7.45 ± 0.51	6.42 ± 0.40
IT12x	6.01 ± 0.52	7.87 ± 0.47	7.48 ± 0.42	6.40 ± 0.31
IT30c	5.68 ± 0.42	8.57 ± 0.52*	6.56 ± 0.46	6.17 ± 0.44

Values are mean ± SEM; n=3 in each group; * significantly different at p <0.05

Randall - Selitto test

The results of the analgesic effect of different potencies of *I. tinctoria* on Randall-Selitto assay are presented in Table-3. The results indicates that administration of different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* at a dose of 0.5 ml/rat/day for 30 days had increased the degree of threshold pressure (146.33 to 151.67g) to mechanical induced pain on 10th day. The increase in threshold pressure was significant (p<0.05) in those rats which were treated with 6x and 30c potencies. Such effect did not persist but gradually tapered off on 20th day and 30th day of experiments on further continuation of the treatment. On the other hand the quantum of threshold pressure required to elicit vocalization to applied mechanical pain was more or less same (131.33 to 133.33 g) on day 1 before administration of drug, alcohol or normal saline and 30 minutes after the administration of normal saline on different days of experimentation (Table.3).

Table 3. Analgesic effect of IT(*Indigofera tinctoria)* (0.5ml/rat/day) on Randall-Selitto test

Group	Latency time to pain response in seconds On days of treatment			
	Initial	10th day	20th day	30th day
Control(Normal saline)	132.00 ± 4.55	132.66 ± 3.89	131.33 ± 3.75	133.33 ± 4.40
Vehicle(91.5% alcohol)	132.66 ± 4.48	146.33 ± 3.57	142.33 ± 4.33	138.33 ± 4.63
IT3x	132.00 ± 4.58	147.33 ± 3.52	143.00 ± 4.72	136.00 ± 5.03
IT6x	132.66 ± 3.71	148.66 ± 4.33*	144.66 ± 4.17	137.33 ± 5.04
IT12x	131.33 ± 4.33	148.00 ± 4.61	142.33 ± 4.25	137.66 ± 3.84
IT30c	131.00 ± 3.21	151.67 ± 3.38*	144.66 ± 4.84	139.33 ± 4.97

Values are mean ± SEM; n=3 in each group; * significantly different at p <0.05

Assessment of behavioural activity

Rota - rod test

The results of different potencies of *I. tinctoria* on motor coordination activity of rats using grip strength test are shown in Table-4. The results obtained from rota-rod test showed decrease (29.98 to 37.47 sec) in the grip strength of the rats when measured 30 minutes after the administration of the different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* at a dose of 0.5 ml/rat/day on 10th day. The decrease in grip strength was significant ($p<0.05$) only with those rats treated with 12x potency of *I. tinctoria*. Afterwards, there was a progressive reversal in the grip strengths of drug treated rats on further continuation of the treatment as the rats stayed for longer duration but still for less duration on the Rota rod that was observed on day 1 before administration of drug (49.11 to 51.55 sec) when tested on 20th and 30th day of experiment (Table.4).

Table 4 . Analgesic effect of IT(*Indigofera tinctoria)* (0.5ml/rat/day) on ice plate test

Group	Latency time to pain response in seconds On days of treatment			
	Initial	10th day	20th day	30th day
Control(Normal saline)	51.53 ± 2.84	51.55 ± 2.39	49.97 ± 2.61	50.70 ± 3.45
Vehicle(91.5% alcohol)	50.15 ± 3.22	37.47 ± 3.50	39.92 ± 2.90	47.74 ± 2.46
IT3x	50.50 ± 3.86	35.91 ± 3.93	41.73 ± 3.34	47.83 ± 2.97
IT6x	50.22 ± 3.32	35.74 ± 4.33	42.58 ± 2.63	45.08 ± 3.07
IT12x	49.11 ± 3.44	29.98 ± 3.89*	41.66 ± 3.33	46.55 ± 2.28
IT30c	49.46 ± 3.68	34.80 ± 4.21	41.92 ± 2.76	46.59 ± 3.72

Values are mean ± SEM; n=3 in each group; * significantly different at $p<0.05$

Open field test

The results of different potencies of *I. tinctoria* on locomotor activity of rats by open field test are shown in Table-5. There was a decrease in the locomotor activity of the rats (43.66 to 46.66 squares in 5 minutes) when measured on 10th day of the experiment, 30 minutes after administration of different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* at a dose of 0.5 ml/rat/day. The difference in locomotor activity was significant ($p<0.05$) with those rats treated with 6x and 12x potencies of the drug when compared to initial locomotor activity taken just before administration of drug on day 1 of the study. However, such depressant effect of drug on locomotor activity slowly vanished off on continuation of drug treatment when tested subsequently on 20th and 30th day of the study. The average locomotor activity as measured in terms of crossing of the squares of a open field apparatus during 5 min of observations on day 1 before administration of drug, alcohol or normal saline and 30 minutes after the administration of normal saline on different days of experimentation was 'more or less' same (63.00 to 65.66 squares in 5 minutes) (Table.5).

DISCUSSION AND CONCLUSION

The use of *I. tinctoria* in epilepsy and other neurological disorders are well documented.[2,3] In homoeopathy too, it is being prescribed for the treatment of epilepsy and other nervous disorders,[13] but no data has been documented to establish its CNS effects scientifically. Therefore, the present preliminary study was undertaken for the first time to evaluate the analgesic and behavioural activities of different (3x, 6x, 12x and 30c) potencies of *I. tinctoria* in albino rats.

Different potencies (3x, 6x, 12x and 30c) of *I. tinctoria* were evaluated in hot plate, ice pate and Randall - Selitto tests for analgesic activity because of the facts that these tests are very sensitive and reliable for screening of new analgesic drugs. The results indicated that all the four potencies (3x, 6x, 12x and 30c) of *I. tinctoria* had increased the latency times for both thermal noxious stimulus and cold sensation and had also increased the quantum of threshold pressure to mechanical induced pain when measured on 10th day of study 30 minutes after the administration of the drug. On the other hand, the latency time and the quantum of threshold pressure were constant in saline

Table 5 . Behavioural effect of IT(*Indigofera tinctoria)* (0.5ml/rat/day) in the open field test

Group	Latency time to pain response in seconds On days of treatment			
	Initial	10th day	20th day	30th day
Control(Normal saline)	64.00 ± 3.51	65.66 ± 4.70	64.66 ± 3.52	65.33 ± 4.80
Vehicle(91.5% alcohol)	63.00 ± 4.00	46.00 ± 4.93	53.00 ± 3.21	60.33 ± 4.17
IT3x	64.66 ± 4.97	45.66 ± 5.23	55.00 ± 3.46	60.66 ± 4.91
IT6x	64.33 ± 3.52	43.66 ± 3.17*	56.00 ± 4.61	59.33 ± 4.66
IT12x	63.66 ± 3.66	45.33 ± 4.33*	50.33 ± 3.84	58.66 ± 4.63
IT30c	64.00 ± 5.03	46.66 ± 4.70	49.33 ± 4.25	57.33 ± 4.09

Values are mean ± SEM; n=3 in each group; * significantly different at p <0.05

treated animals. The flavonoids and saponins have been reported to be responsible for analgesic activity. [5] The same constituents may also be responsible for the analgesic activity of *I. tinctoria* observed in the present study.

This drug in different potencies (3x, 6x, 12x and 30c) were also evaluated in rota rod as well as open field tests for its behavioural activity. The results showed that different (3x, 6x, 12x and 30c) potencies of *I. tinctoria* had decreased the grip strength and locomotor activity when measured on 10th day of study 30 minutes after the administration of the drug which indicates that *I. tinctoria* possesses CNS depressant effect.

The ability of drug to prolong the reaction (latency) time to the thermal and mechanical induced pain and also to decrease the locomotor and motor coordination is the sign of central nervous system depression.[20, 21] Wearing off such depression on prolonged and continuous use of the drug may be either due to decreased sensitivity of the central nervous system or due to increased metabolising enzymatic activity in the liver. Further study is required to conclude the cause of wearing off such depression. Alcohol used as a vehicle to prepare different potencies (3x, 6x, 12x and 30c) of the drug *I. tinctoria* is well known to have these effects on its prolonged use.

Based on this present study, it may be suggested that the homoeopathic formulations of *I. tinctoria* possess CNS depressant property; however, further studies are necessary for a definitive conclusion.

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