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Investigation of cerebroprotective effect of *Bixa orellana* on sodium nitrite induced hypoxic neurotoxicity

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Abstract

The present study was carried out to evaluate the cerebroprotective effects of ethanolic extract of *Bixa orellana* leaves in hypoxic neurotoxicity induced rats. Hypoxia was induced by treatment of rats with sodium nitrite in drinking water for 30 days. *Bixa orellana* attenuated cognitive deficits in sodium nitrite induced rats which was studied by Morris water maze test. The extract restored the decreased levels of dopamine and increased levels of acetylcholinesterases and glutamate to the normal levels which can be compared with standard Pyritinol. The present study reveals that the ethanolic extract of *Bixa orellana* leaves confers cerebroprotection against sodium nitrite induced hypoxic neurotoxicity in rats.
Keywords: Cerebroprotective, Hypoxia, *Bixa orellana*.

1. Introduction

Bixa orellana commonly known as annatto and Lipstick tree is a species of shrub in the Bixaceae family which is widely cultivated throughout the tropical world, since long back. Annatto has been used for centuries in many parts of the world for the prevention and management of a number of health disorders such as constipation, fevers, heartburn, asthma, scabies, ulcers, diarrhea, stomach upset, skin diseases, measles, anecdotal treatment of diabetes, allergy, leprosy, infectious diseases, burns, measles, gonorrhea, diarrhea, asthma, angina, tumors, skin problems, and urinary infections (oral and topic) [1]. The preliminary phytochemical screening of *Bixa orellana* showed the presence of carbohydrates, steroids, alkaloids, proteins, flavonoids, terpenoids, phenolics, tannins and glycosides [2]. *B. orellana* seeds contain bixaghanene, bixein, bixin, bixol, carotenoids, delta tocotrienol, ishwarane, isobixin and norbixin apart from this it also contains phenylalanine, salicylic acid, threonine, tomentosic acid and tryptophan. The leaf extract showed the presence of ishwarane, phytol, polyprenol, and a mixture of stigmasterol and sitosterol [3].

The different parts of plant has been screened for pharmacological activities and reported to possess antibacterial[4], antifungal[5], antigonorrhoeal[6], antileishmanial[7], antimalarial [8], cardioprotective[9], anti-inflammatory [10], antiulcer[11], hepatoprotective[12], diuretic[13], analgesic[14], hypoglycemic[14], Antidiarrheal[15], hypnotic[15], anticonvulsant[15], anticarcinogenic [16], antioxidant [17] and free radical scavenging activity[18]. The present study has been carried out to explore the cerebroprotective effect of *Bixa orellana* on sodium nitrite induced hypoxic rats.

2. Materials and Methods

2.1. Collection of Plant Material

Bixa orellana leaves were procured from local area of Warangal, Telangana, India in the month of January 2017. The identification and the authentication of this plant were done in the Department of Botany, Kakatiya University.

2.2. Preparation of plant extract

After collection, leaves were washed very carefully and clearly with water and dried under shade. The dried leaves were powdered in an electrical processor. 50 gram of dried leaf powder material was extracted in a soxhlet apparatus with 200 ml. of ethanol. The ethanolic extract was then distilled, evaporated and dried in vacuum to get semisolid resinous extract. All the extract were kept in desiccator and stored in a refrigerator for further pharmacological experiment.

2.3. Animals used for experimentation

Wistar rats of either sex, weighing about 180-200 grams were used in experiments. Animals were housed in polypropylene cages with not more than three animals per cage and maintained under standard condition (12 hours light / dark cycle; temperature 25± 3 ^{0} C; relative humidity 55 ± 5%) and had free access to standard pellet feed (Hindustan Lever Ltd., India) and water *ad libitum*. All the animals were acclimatized to laboratory condition for a week before commencement of experiment. The experiments on animals were conducted in accordance with CPCSEA and our protocols were duly approved by the Institutional Ethical Committee.

2.4. Acute Toxicity Studies

The acute toxicity study to carry out the gross behavioral effects and safety effects of the ethanolic extract of *Bixa orellana* leaves were carried on mice weighing about 20-25gm as per as per ICH Topic S7A guidelines and OECD 423 guidelines. Overnight fasted mice received the test extract at a dose of 5 mg/kg bodyweight orally and mortality was observed for first 24 hours, with special attention for the first 4 hours and daily then, for a total of 14 days. If no mortality was observed for any mice, then the procedure was repeated again with doses of 50, 300 and 2000 mg/kg orally. The extract was well tolerated by the mice without any explicit signs of toxicity even at the highest dose [19].

2.5. Experimentation

2.5.1. Induction of hypoxia

Sodium nitrite, which reduces the oxygen carrying capacity of the blood by changing normal hemoglobin to methemoglobin, was used to induce hypoxia. Hypoxia was induced by administration of sodium nitrite water (60 mg/kg body weight of sodium nitrite dissolved in normal water) by oral gavage (5 mL/kg dosing volume) for 30 days except the control group, which was provided with normal water [20].

2.5.2. Experimental design

The animals were divided into three groups containing six rats in each group.

Group I served as control and received normal saline.

Group II served as hypoxic rats and received sodium nitrite water for 30days.

Group III animals received ethanolic extract of *Bixa orellana* leaves (500 mg/kg; p.o) suspended in 1% gum acacia and sodium nitrite water for 30 days.

Group IV animals received Pyritinol 100 (mg/kg, p.o.) suspended in 1% gum acacia and sodium nitrite water for 30 days.

2.5.3. Morris water maze *task*

The Morris water maze task has been used extensively to explore spatial learning and memory in rodents. Maze is a circular pool of diameter 110 cm and height of 30 cm filled with water to a depth of approximately 20cm. The water is made opaque by adding nontoxic white dye and milk. The pool was divided into four equal quadrants and marked north, South, east and west; and a platform was submerged 1 cm below the opaque surface in the center of one of the quadrants. The many cues external to the maze were visible from the pool which could be used by the rats for spatial orientation was kept at constant position throughout the task.

The mice were released into the water and allowed for 90 seconds to find the platform. Animals received 2 trials per day with 20 minutes inter-trial interval for 4 days, the latency to find the platform was low (< 10 sec). During each trial, the escape latencies of mice were recorded. Once the mouse located the platform, it was permitted to remain on it for 20 sec. If the mouse did not locate the platform within 120 sec, it was placed on the platform for 20 sec and then removed from the pool. After several trails, the test was conducted on the 30th day of sodium nitrite water drink and time required to escape on to the platform was recorded [21].

2.5.4. Dissection of Brain

The conscious mice were decapitated and the brain was removed within 30 sec from the skull. The skin covering the skull was cut along the midline and removed to expose the dorsal skull plates. The plates were split by introducing one blade of the paired scissors along the midline. The plates were then twisted and turned across the lateral border to expose the brain. The membrane covering the brain was removed with the help of fine forceps. The brain then was taken out using spatula was washed in cold saline.

2.5.4.1. Estimation of Dopamine

Whole brain was homogenized in 0.1 ml hydrochloric acid -butanol, (0.85 ml of 37% hydrochloric acid in one liter *n*- butanol) for 1 min in a cool environment. The sample was then centrifuged for 10 min at 2,000 rpm. 0.08 ml of supernatant phase was removed and added to an Eppendorf reagent tube containing 0.2 ml of heptane and 0.025 ml 0.1 M hydrochloric acid. After 10

min of vigorous shaking, the tube was centrifuged under same conditions to separate two phases.

Upper organic phase was discarded and the aqueous phase (0.02 ml) was used for estimation of dopamine assay. The assay represents a miniaturization of the trihydroxide method. To 0.02ml of hydrochloric acid phase, 0.005ml of 0.4M hydrochloric acid and 0.01ml Ethylenediaminetetraacetic acid /Sodium acetate buffer (pH 6.9) were added, followed by 0.01ml 0.1 M iodine solution in ethanol for oxidation. The reaction was stopped after two minutes by addition of 0.01ml sodium sulphite in 5m sodium hydroxide (0.5 g sodium sulphite in 2 ml deionized water and 18 ml 5 M sodium hydroxide). Acetic acid (0.01 ml, 10 M) was added 1.5 minutes later. The solution was then heated to 100^{o} C for 6 minutes. When the sample again reached room temperature, excitation and emission spectra were read in the microcuvette at 330-375nm for dopamine uncorrected instrument values [22].

2.5.4.2. Estimation of acetylcholinesterase (AChE)

About 20 mg of brain tissue per ml of phosphate buffer (0.1 M; pH 8) was homogenized in a tissue homogenizer. A volume of 0.4 ml aliquot of brain homogenate was added to the cuvette containing 2.6 mL of 0.1 M phosphate buffer. Exactly 100 µl of dithiobis-nitrobenzoic acid reagent was added to the cuvette and the absorbance was measured at 412 nm and then 20 µl of acetylcholine chloride was added. A change in the absorbance/min was now calculated. The values of AchE are expressed in µmol of Ach Hydrolyzed/min/mg protein [23].

2.5.4.3. Estimation of Brain glutamate

Weighed quantity of the brain tissue was homogenized with two parts by weight of perchloric acid and centrifuged for 10 min at 3000 rpm. 3ml of Supernatant liquid was adjusted to pH 9.0 with 1 mL phosphate solution. It was then allowed to stand in an ice bath and filtered through a fluted filter paper. Absorbance was measured at 340 nm. Similarly a blank reading was measured at 340 nm. The level of glutamate was expressed as µmol/g tissue [24].

2.6. Statistical Analysis

Values were expressed as Mean ± Standard Deviation. The Significance of differences among the treated groups was evaluated using one way analysis of variance (ANOVA). The test followed by Dunnett's multiple comparisons test of significance. p values less than 0.05 were considered as statistically significant.

3. Results

The ethanolic extract of *Bixa orellana* leaves was found to be safe at the maximum dose of 2000 mg/kg body weight by Oral route. Even after 14 days, rats were found to be well tolerated with no mortality and no signs of toxicity. Hence the dose of 500 mg/kg body weight was selected for the study.

Oral administration of 500 mg/kg body weight ethanolic extract of *Bixa orellana* leaves significant attenuated the escape latency when compared to increased escape latency in positive control hypoxia induced group (Table 1).

Table 1: Effect of *Bixa orellana* Extract on Morris Water Maze Task

S. No	Treatments	Escape latency (sec)
1	Group I-Control	19.33±4.27
2	Group II- Positive Control	38.29±5.63^{a**}
3	Group III-Extract Treated	29.87±5.75^{b*}
4	Group IV-Standard	18.92 ±6.42 ^{b**}
Values are mean ± SD; n=6; *p<0.05, **p<0.01; a- Group I vs. Group II; b-Group II vs. Groups III and IV		

In hypoxia induced group significant increase in the AChE activity and brain glutamate level was observed when compared with control group. In Extract treated group significant decrease in AChE activity and brain glutamate level was detected when compared to that of hypoxic control group. Dopamine levels in case of hypoxic control were decreased when compared to that of control group. The extract augmented the dopamine levels when compared to hypoxia induced group. (Table 2)

Table 2: Effect of *Bixa orellana* Extract on Brain Enzymes

S. No	Treatments	AChE (µmol of Ach Hydrolyzed/min/ mg protein)	Dopamine (pg/mg of wet tissue)	Glutamate (µmol / gm tissue)
1	Group I-Control	17.36± 0.31	633.47± 34.24	72.86± 2.92
2	Group II-Positive Control	20.14± 0.27^{a**}	513.33± 26.33^{a*}	87.17± 2.69**
3	Group III-Extract Treated	18.87± 0.33^{b*}	579.16± 33.83 ^{b*}	79.33± 3.17 ^{b*}
4	Group IV-Standard	17.42 ± 0.24 ^{b**}	634.67± 41.27 ^{b**}	73.04± 3.09 ^{b**}
Values are mean ± SD; n=6; *p<0.05, **p<0.01; a- Group I vs. Group II; b-Group II vs. Groups III and IV				

4. Discussion

This study has been carried out to assess the effect of ethanolic extract of *Bixa orellana* on sodium nitrite induced hypoxic rats. For the present study Pyritinol was used as standard drug. Pyritinol is an indirectly acting cholinergic drug in the Central nervous system and has been reported to significantly improve memory and

cognitive functions. Apart from this it also increases in cerebral glucose utilization and cerebral blood flow [25].

In the present study Sodium nitrite was used to induce hypoxia. Sodium nitrite in blood is highly reactive with hemoglobin, thus affecting hematopoiesis and causes induction of methemoglobinemia.

Elevated levels of methemoglobin can lead to anemic hypoxia a condition in which there is inadequate supply of oxygen to tissues [26].

Hypoxia leads to neuronal functional failure, cerebral palsy and neurodevelopmental delay with characteristic biochemical and molecular alterations resulting in permanent or transitory neurological squeal or even death [27]. Hypoxia causes alterations of neurotransmitters and cognitive impairment in terms of learning and memory. Impairment in memory function along with a decrease in acetylcholine levels, increase in acetylcholinesterase activity, down regulation of choline acetyltransferase, α-7-nicotinic acetylcholine receptor and M1 muscarinic acetylcholine receptor was noted in hypoxia [28].

In the present study extract significantly augmented the acetylcolinesterase levels which were decreased by the administration of sodium nitrite. The effect of hypoxia consists in a marked inhibition of dopamine release from rat striatum which was confirmed by the levels of dopamine observed in hypoxic group [29].

These decreased dopamine levels were improved significantly by the administration of *Bixa orellana* leaves extract. Glutamate is an excitatory neurotransmitter in brain primarily produced from glutamine by the action of phosphate-activated glutaminase, a mitochondrial enzyme. Hypoxiacauses increase in glutaminase activity and glutamate levels in dorsal and ventral brainstem regions in rats [30]. The Glutamate activity in the brain was increased in rats treated with sodium nitrite when compared with the normal; in addition, the sodium nitrite induced increase in Glutamate was attenuated by *Bixa orellana* extract treatment.

The treatment with ethanolic extract of *Bixa orellana* attenuated cognitive deficits in sodium nitrite induced rats. In water-maze test ethanolic extract decreased the escape latency almost to the normal level; it is possible that cerebroprotection plays a role in favorable effect of *Bixa orellana* on sodium nitrite induced cognitive effects.

5. Conclusion

In conclusion, the present observations provide evidence that ethanolic extract of *Bixa orellana* leaves significantly attenuated the hypoxic effects induced by sodium nitrite and this effect may be mediated because of its cholinergic effect.

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