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Deciphering the role of Bixin isolated from Bixa orellana L., in epileptic and psychotic experimental models in rodents

Potraj B
Department of Pharmacology, Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka

Mansoor Ahamed Sayed
Department of Pharmacology, Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka

Shravan Narasimha
Sree Siddahanga College of Pharmacy, Tumakuru, Karnataka

Suhas D. S
Sree Siddahanga College of Pharmacy, Tumakuru, Karnataka

Veerapur V. P
Department of Quality Assurance, Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka, veeresh36@gmail.com

See next page for additional authors

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Authors

Potraj B, Mansoor Ahamed Sayed, Shravan Narasimha, Suhas D. S, Veerapur V. P, and Vijayakumar S

Research Article

Deciphering the role of Bixin isolated from Bixa orellana L., in epileptic and psychotic experimental models in rodents

Potraj B, Syed Mansoor Ahamed, Shravan Narasimha, Suhas D S, Veerapur V P*, Vijayakumar S

Email: veeresh36@gmail.com

Abstract

Background: Epilepsy and psychosis have a complex and interesting relationship. The drugs used in the treatment of epilepsy have the chances of reducing the symptoms and the risk of psychosis. Tardionally, seeds of Bixa Orellana L. were used to treat epilepsy in Brazilian tribes. Objective: To explore the anti-epileptic and anti-psychotic activity of bixin on experimental animals. Methodology: The anti-epileptic activity was assessed by pentylenetetrazol (PTZ) and maximal electro shock (MES)-induced convulsions. The effect of bixin against psychosis was assessed by an apomorphine-induced stereotyped behaviour in rats along with the climbing activities in mice. Bixin (2.5 and 5.0 mg/kg, p.o.) was pre-treated for a period of fifteen days. Bixin was administered one hour prior to the respective induction (MES/PTZ/apomorphine). After the induction, the assigned rodents were monitored for the different stages of convulsions as well as behavioural changes with respect to stereotyped and climbing behaviours in experimental animals. Result: Pre-treatment of bixin exhibited dose dependent prevention of seizures in the MES and PTZ models at $P<0.001$ significant level. The high dose of bixin showed complete inhibition of tonic-extension phase seizure and improved the mean time latency in the onset of tonic-clonic convulsions. The bixin pre-treatment also showed a significant ($P<0.01$) deterrence in stereotyped and climbing behaviour dose dependently. Conclusion: The present study provided the scientific footage to the traditional usage of the herb in the manifestation of convulsion and psychosis.

Key words: *Bixa Orellana*; Maximal electro shock; Pentylenetetrazol; Seizures; Stereotype; Climbing behaviour.

Introduction

Epilepsy is one of the most dangerous neurological disorders that affect the brain due to a change in the electrical function, and by an increase in the seizures due to an abnormal cortical neuronal activity. Epilepsy and mood disorders have a complex and intriguing relationship[1]. Understanding the pathophysiology and association among psychosis and epilepsy is helpful for searching treatment strategies for neurological disorders[2]. A patient with epilepsy generally develops psychiatric symptoms; especially temporal lobe epilepsy patients develop psychiatric disorders with extinct schrizopenic symptoms. An estimated study among the general population tells that the occurrence of psychosis in epileptic patients is about 2 to 7%[3,4]. Plant based phytoconstituents and herbal remedies with ethanomedical or folk-lore claims against neuropsychiatric and neurodegenerative diseases with lesser side effects had shown a promising outcome[5]. *Bixa orellana*. (Family Bixaceae) is a small tree also called achiote, found in Brazil and which grows in the tropical regions of South and Central America[6]. Bixin is an apo-carotenoid - isolated from *Bixa orellana* seeds and it contains a higher concentration of bixin as high as 80% of the total pigments. The leaves, roots and seeds of *Bixa orellana* were used in the treatment of epilepsy by Brazilian tribes[8]. Bixin is a natural dye that is widely used in food, cosmetic and textile industries due to their non-toxic effects. World

Potraj B[1], Syed Mansoor Ahamed[1], Shravan Narasimha[1], Suhas D S[1], Veerapur V P[2], Vijayakumar S[1]

1Department of Pharmacology
2Deartment of Quality Assurance,
Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka

* Corresponding Author

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Health Organization (WHO) has only some dyes that are well accepted, and bixin is one among them[9]. Bixin is a carotenoid, and is one of the most efficient quenchers of biological reactive oxygen species. Studies revealed that they act by interfering with the production of reactive oxygen species against cisplatin induced cytotoxicity in PC12 neuronal cell lines[10]. Bixin possesses promising defensive effect on genotoxicity, mutagenicity and carcinogenicity in the tested neuronal cell lines and it also showed deterrent effects on both colon cancer (HCT-116) and SF-268 CNS cell growth[10]. In addition to this, bixin possesses an anti-inflammatory, cardioprotective[11], hepatoprotective[12] activities. There was no reported scientific data of bixin against epilepsy and psychosis disorder. Based on these above scientific data, the experiments were designed to explore efficacy of bixin in experimental animal models.

Materials and Methods

Chemicals

Pentylenetetrazol, apomorphine (Sigma-Aldrich, St. Louis, USA), phenytoin (Zydus neuroscience), diazepam (Ranbaxy), ethyl acetate, chloroform (Anugraha chemicals, Bangalore), and tween 80 (s d fine Chemicals) were utilized in the present study. Further, analytical grade chemicals were utilized.

Isolation and characterisation of Bixin

Fresh seeds of *Bixa orellana* (10 g) (without powdering) were refluxed with 75 ml of ethyl acetate at 35-40˚C for 4 hours. Then, the solution was evaporated to half of its volume using the rotary evaporator. Pure crystals were separated out on cooling on the ice bath. The crystals were collected by filtration and re-crystallized using dichloromethane and chloroform (1:4) in order to get a pure bixin. The crystals were completely dried and its chromatographic and spectroscopic studies were carried out[13]. The experimental doses of bixin (2.5 and 5 mg/kg) was selected based on reported literature[14].

Experimental animal and research protocol approval

Wistar rats (150-200 g) and albino mice (25-30 g) of both sexes were obtained from Sree Siddaganga College of Pharmacy, Tumakuru, Karnataka, and approval no SSCP/IAEC/Clear/72/2009-2010, based on the approved guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India. The animals were maintained as per the guidelines. Animals with abnormal conditions (severe aggression/unnecessary licking/self-injury and any physical abnormalities) were exempted from the experiment. Animals were allowed to acclimatize for seven days and were randomized according to their body weight for further studies.

Investigation of anti-epileptic activity

Maximal electro shock (MES)-induced convulsions

This model was used to assess the effect of bixin in the partially occurring seizures. The MES was induced using electro-convulsometer (Inco Industries). Phenytoin was used as the standard drug. The rats were randomized and assigned into groups of four (n=6). Group 1: Oral administration of vehicle (Tween 80 1% v/v) along with the MES; Group 2: Intra peritoneal administration of 90 mg/kg of phenytoin with MES; Group 3 & 4 received the oral dose of bixin (2.5 and 5 mg/kg) along with MES. The animals were pre-treated with bixin and the standard in respective doses for fifteen days. Then the rats were subjected to MES 1 h after the particular day dose as per the reported method[15]. After the induction, the various stages and durations of epilepsy were observed and noted.

Pentylenetetrazol-induced Convulsions

Pentylenetetrazol (PTZ) is used to assess the outcome of drugs on generalized seizures, particularly petit-mal type of seizures. Diazepam is used as the standard against the PTZ induction. The rats were randomized according to its body weight, and the grouping was done as the same as the first model. The rats were administered with two doses of bixin (2.5 and 5 mg/kg, p.o) and standard (4 mg/kg, i.p.) for the period of fifteen days before induction. On the 15th day, the PTZ (60 mg/kg, i.p.) was administered one hour post treatment of the particular day's dose. The occurrence and duration of the onset of tonic-clonic seizures were recorded, and finally the death rate was noted, and the percentage of death of each group was calculated[16].

Investigation of anti-psychotic activity

Apomorphine-induced stereotyped behaviour in rats

The term stereotype is used to define various disciplines of psychosis. Apomorphine is known to induce stereotype behaviour in the experimental animals such as licking, biting of cage and atypical locomotor activities. A total of 24 rats were allocated into four groups (n=6). Group 1: vehicle (1% v/v tween 80 in water, p.o.) + Apomorphine (1.5 mg/kg, i.p.); Group 2 received Haloperidol (1 mg/kg, i.p.) + Apomorphine (1.5 mg/kg, i.p.); Group 3 & 4 received bixin (2.5 and 5 mg/kg respectively) + Apomorphine (1.5 mg/kg, i.p.). Bixin and haloperidol were pre-treated for fifteen5 days. On the behavioural parametre assessment day, apomorphine was administered one hour after their respective treatments. Then, the rats were shifted to individual observation cages, and the intensity of stereotype behaviour was measured every 10 min for a 90-min period using a scoring system[17].

Apomorphine-induced climbing behaviour in mice

Subcutaneous injection of apomorphine exhibited an unusual climbing behaviour, predominantly mediated by mesolimbic dopamine pathway. The mice were placed into four groups of six. Group 1: vehicle (1% v/v tween 80 in water, p.o.) + Apomorphine (3 mg/kg, s.c.); Group 2: Intra-peritoneal injection of 100 µg/kg of haloperidol + Apomorphine; Group 3: Oral dose of bixin (2.5 mg/kg) + Apomorphine; Group 4: Oral administration of bixin (5 mg/kg) + Apomorphine. All the experimental mice were trained for a climbing activity. Bixin and haloperidol were pre-treated for a period of fifteen days. Different doses of bixin were administered 1 hour before the administration of apomorphine. The mice were placed in individual cages and the climbing behaviour was recorded every ten minutes for a period of 30 minutes and scored as described[17].

Statistical analysis

The Chi-square test was performed for a PTZ-induced convulsion model between groups (number

Table 1. Effect of different doses of bixin on MES-induced convulsions in rats

Treatment	Various phases of convulsions (sec)				
	Flexion	Extension	Clonus	Stupor	Recovery / Death
Vehicle (1 ml/kg, p.o.)+ MES	4.167 ± 0.30	7.66 ± 0.61	19.17 ± 0.94	70.17± 1.83	Recovery
Phenytoin (90 mg/kg, i.p.) + MES	1.66 ± 0.21[c]	0[c]	5.83 ± 0.60[c]	19.67 ± 1.33[c]	Recovery
Bixin (2.5 mg/kg, p.o.) + MES	2.16 ± 0.30[c]	1.33 ± 0.33[c]	12.50 ± 0.42[c]	50.67 ± 1.35[c]	Recovery
Bixin (5 mg/kg, p.o.) + MES	2.16 ± 0.30[c]	0	9.50 ± 0.84[c]	29.83 ± 1.22[c]	Recovery
F	15.750	111.54	60.048	239.51	
Df	3, 20	3, 20	3, 20	3, 20	

Values are expressed in mean ±S.E.M. where n=6, [c]P<0.001 compared with MES alone treated group. Statistical analysis was done by one-way ANOVA followed by Tukey's post hoc test.

Table 2. Effect of different doses of bixin on pentylenetetrazol (PTZ)-induced convulsions in rats

Treatment	Onset of clonic action (sec)	Onset of tonic action (sec)	Number of rats recovered/used	Time to death in min	Number of deaths[Y]	Protection against mortality (%)
Vehicle (1 ml/kg, p.o.) + PTZ (60mg/kg, i.p.)	342.7 ± 15.41	508.2 ±12.89	0/6	28.17 ± 1.79	6	0.00
Diazepam (4 mg/kg, i.p.) + PTZ (60 mg/kg, i.p.)	AC	AC	6/6	Nil	0**	100.00
Bixin (2.5 mg/kg, p.o.) + PTZ (60 mg/kg, i.p.)	531.7 ± 11.00[c]	694.2 ± 14.86[c]	5/6	98.00 ±2.60	1**	83.33
Bixin (5 mg/kg, p.o.) + PTZ (60 mg/kg, i.p.)	654.3 ± 16.62[c]	801.2 ± 10.26[c]	6/6	Nil	0**	100.00

Treatment	Onset of clonic action (sec)	Onset of tonic action (sec)	Number of rats recovered/used	Time to death in min	Number of deaths[Y]	Protection against mortality (%)
F	902.87	1025.2		852.79		
Df	3, 20	3, 20		3, 20		

Values are expressed in mean ±S.E.M. where n=6, cP<0.001 compared with PTZ alone treated group. AC means absence of convulsion. Statistical analysis was done by one-way ANOVA followed by Tukey's test. □ Chi-square test was performed **P<0.01 compared to PTZ alone group.

Table 3: Effect of different doses of bixin on apomorphine-induced stereotypy in rats

Treatment	Stereotype Score								
	10 min	20 min	30 min	40 min	50 min	60 min	70 min	80 min	90 min
Apomorphine (1.5 mg/kg, i.p.)	3.33±0.55	3.66±0.71	4.00±0.73	4.66±0.21	4.66±0.66	5.16±0.74	5.50±0.42	4.33±0.49	4.00±0.96
Haloperidol (1 mg/kg, i.p.) + Apomorphine (1.5 mg/kg, i.p.)	0.16±0.16[c] (95.20)	0	0	0	0	0	0	0	0
Bixin (2.5 mg/kg, p.o.) + Apomorphine (1.5 mg/kg, i.p.)	3.00±0.51 (9.90)	3.50±0.42 (4.40)	3.33±0.33 (30.00)	2.83±0.47[b] (39.27)	2.66±0.42[c] (44.20)	2.50±0.42[b] (51.55)	3.00±0.63[b] (45.45)	2.00±0.36[b] (53.81)	2.16±0.47 (46.00)
Bixin (5 mg/kg, p.o.) + Apomorphine (1.5 mg/kg, i.p.)	2.00±0.57 (39.93)	2.83±0.54 (35.33)	2.00±0.57[a] (50.00)	2.50±0.42[b] (46.35)	2.16±0.30[b] (53.64)	2.16±0.54[b] (58.13)	1.83±0.47[c] (66.72)	1.50±0.42[a] (65.75)	1.00 ± 0.36[b] (75.00)
F	8.899	11.939	12.864	33.302	47.189	17.63	18.216	23.61	9.275
Df	3, 20	3, 20	3, 20	3, 20	3, 20	3, 20	3, 20	3, 20	3, 20

Values are expressed in mean ±S.E.M. where n=6, [a]P<0.05, [b]P<0.01, [c]P<0.001 compared with apomorphine alone treated group. Statistical analysis was done by one-way ANOVA followed by Tukey's test. Values mentioned in parenthesis indicate the percentage protection compared to apomorphine control.

Table 4: Effect of different doses of bixin on Apomorphine-induced climbing behaviour in mice

Treatment	Climbing behaviour scores		
	10 min	20 min	30 min
Apomorphine (3 mg/kg, s.c.)	1.33 ± 0.21	1.67 ± 0.21	1.67 ± 0.21
Haloperidol (0.1 mg/kg, i.p.) + Apomorphine (3 mg/kg, s.c.)	0	0	0
Bixin (2.5 mg/kg, p.o.) + Apomorphine (3 mg/kg, s.c.)	0.67 ± 0.21 (49.62)	1.00 ± 0.26 (40.00)	0.67 ± 0.21[b] (59.90)
Bixin (5 mg/kg, p.o.) + Apomorphine (3 mg/kg, s.c.)	0.50 ± 0.22[a] (62.40)	0.50 ± 0.22[b] (70.05)	0.50 ± 0.22[b] (70.05)
F	8.837	5.943	14.362
Df	3, 20	3, 20	3, 20

Values are expressed in mean ±S.E.M. where n=6, [a]P<0.05, [b]P<0.01 compared with apomorphine alone treated group. Statistical analysis was done by one-way ANOVA followed by Tukey's test. Values mentioned in parenthesis indicate the percentage protection compared to apomorphine control.

of deaths). All other data was expressed as mean ± S.E.M and n=6. Statistical study was performed by a one-way ANOVA Tukey's post hoc test, with the help of Graph Pad Prism Version 5.0, USA.

Results

Characterisation of bixin

The melting point and TLC profile of the isolated compound was matched with an authentic sample of bixin. The melting point was found to be (197-199 °C). In order to carry out Co-TLC profiling of the isolated and reference standard bixin, several solvent systems were tried, and one containing chloroform: methanol (94:6) gave good results with R_f value of the isolated compound matching with that of the reference standard (R_f = 0.5). Further, the overlay spectrum of the isolated bixin and

reference standard was found to be matching with λmax of 503 and 473 nm.

Maximal electro shock-induced convulsions

Pre-treatment of bixin for fifteen days to MES-induced rats showed significant reduction in all the observed phases of convulsions. Further, the bixin showed the marked (P<0.001) reduction in the hind limb extensor stage in a dose dependent way. A higher dose of bixin exhibited a significant (P<0.001) defence against the MES induced seizures by the complete inhibition of tonic-extension phase seizure, as compared with a lower dose [Table 1].

Pentylenetetrazol -induced convulsions

The pre-treatment of bixin showed a significant (P<0.001) protection against the PTZ induced convulsions. The pre-treatment of bixin increased the mean time latency at the onset of a tonic-clonic action. The maximum effect of bixin was observed at a higher dose with 100 % protection against the mortality compared to a lower dose [Table 2]. The effect of bixin (5.0 mg/kg) was comparable with that of diazepam (4.0 mg/kg).

Apomorphine-induced stereotype behaviour in rats

Apomorphine administered alone showed a stereotyped behaviour categorized by licking, sniffing, rearing, gnawing in a repetitive and compulsive manner over the period of 90 minutes. Pre-treatment with bixin 2.5 mg/kg exhibited a significant reduction (30 to 46%, respectively) in a stereotyped behaviour in a 40-minute (P <0.01) to 80 (P <0.01) minimum time period. Bixin 5 mg/kg showed a marked reduction (50.00 to 75%, respectively) in a stereotyped behaviour from 30 (P <0.05) to 90 min (P <0.01) minute interval. Haloperidol treated rats did not show any stereotyped behaviour from 20 minutes to the end of the assessment period with 100% protection [Table 3].

Effect of Bixin on apomorphine-induced climbing behaviour in mice

A subcutaneous injection of apomorphine to the group of mice revealed a marked increase in the climbing behaviour with first rearing and then full climbing activity. Pre-treatment with bixin (2.5 & 5 mg/kg) showed a significant reduction 49.62 to 59.90% & 62.40 & 70.05% protection against apomorphine control group, respectively. The treatment of Bixin 2.5 mg/kg showed a decrease in the climbing behaviour at 30 minutes (P<0.01), while a higher dose of bixin 5mg/kg reduced the climbing behaviour from a 10-minute (P<0.05) to 30-minute (P<0.01) interval [Table 4].

In all the tested parameters and models, the F-ratio was found to be higher, and it suggested that the null hypothesis was found to be false and the alternative hypothesis was true.

Discussion

The anti-convulsion effect of bixin was investigated using the MES and pentylenetetrazol induced seizures. The maximal electro shock (MES)-induced seizures is an established procedure for the assessment of anti-epileptic drugs to protect against the hind limb extension seizure (HLE), similar to grand-mal epilepsy in humans[18]. The standard drugs such as phenytoin, valproate, felbamate and lamotrigine, have protective effect against MES-induced seizures, by either blocking the seizure spread, or by slowing down the voltage activated sodium channels, thus inhibiting the HLE seizures[19]. A higher dose of bixin (5 mg/kg) and phenytoin exhibited 100% abolition of HLE. However, the molar concentration of bixin was found to be lower (12.7 μmoles) than phenytoin (360 μmoles). Therefore, bixin was considered to be a better candidate than phenytoin in reversing the MES-induced convulsions. Thus, bixin can restrict the repetitive firing of the action potential by slowing down the voltage activated sodium channels.

Pentylenetetrazol (PTZ) is a popular chemoconvulsant, which is a selective inhibitor of the chloride channel and $GABA_A$ receptor. A PTZ-induced seizure is used to confirm the efficacy of the anti-epileptic drugs. A sufficiently higher dose of PTZ produces severe and continuous seizures that develop from myoclonic twitches of the face and forelimbs. This process happens without the loss of postural reflex to clonic seizures of limbs, and with the loss of postural reflex to full tonic extension of both forelimbs and hindlimbs[20]. Any drugs that hyperpolarize the postsynaptic membrane and

reduce its neuronal excitability by enhancing $GABA_A$ receptor mediated neurotransmission are considered as anti-convulsant. Diazepam, phenobarbital, valproate and felbamate acting by this mechanism can also be used to prevent PTZ-induced seizures. In contrast, the drugs that block T-type Ca^{2+} channels like ethosuximide can also be used to prevent this type of seizure[19]. The bixin showed a protective effect by an increase in the mean-time of latency at the onset of clonic and tonic action by blocking the entry of Cl^- into $GABA_A$ receptor. The higher dose of bixin at 12.7 µmoles (5 mg/kg, p.o.) showed a 100% protection against mortality, which was equivalent with the standard diazepam of 15 µmoles (4 mg/kg, i.p.). It is very well known that if F-ratio in ANOVA analysis was found to be nearly one, then the null-hypothesis is true, which means that there is no statistical difference between the groups. In the present study, statistical ANOVA comparison between groups in all the tested parameters was found to be more than one. Thus, the null-hypothesis was rejected and an alternative hypothesis was accepted.

Stimulation of dopaminergic neurotransmission in the neostriatum area of basal ganglia will induce stereotyped and repetitive behaviour in rodents. A dopaminergic drug like apomorphine, acts on dopamine D_2 receptors in a direct pathway, leading to the development of stereotyped behaviours like sniffing, gnawing, rearing and compulsive gnawing, through the stimulation of a dopamine transmission[20]. The same above-mentioned stereotyped behaviour was observed in the present study after the injection of apomorphine, and the intensity of stereotyped behaviour was typically increased for the entire observation period. Pre-treatment with bixin for fifteen days significantly reduced the stereotyped behaviour. A high dose of bixin 5 mg/kg exhibited a significant reduction in the stereotyped behaviour with 50-75% protection against an apomorphine treated group. However, the standard dopamine D_2 receptor blocker like haloperidol showed 100% protection against the stereotyped. Agents that decrease the stereotype and repetitive behaviour through a direct pathway by antagonizing dopamine D_2 receptors in the nigrostriatal system in the basal ganglia or by acting indirectly via a blockade of serotonergic, noradrenergic, and by showing an agonistic action on the GABAergic system, it will help to alleviate psychiatric symptoms[21].

Apomorphine-treated mice exhibited an abnormal climbing behaviour categorized by rearing and full climbing activity. The pre-treatment of bixin altered the climbing performance and rearing in mice. Both the doses of bixin (2.5 and 5 mg/kg) exhibited 60% and 70% protection compared to the apomorphine-treated group. The standard drug haloperidol exhibited a maximum protection (100%). The ability of agents to enhance the GABAergic transmission in an agonistic manner will reduce the climbing behaviour induced by apomorphine, and will be a neuroleptic agent for psychiatric disorders[22]. Overall, a high dose of bixin exhibited maximum protection by altering the stereotype and climbing behaviour, which might be through a direct pathway by inhibiting dopaminergic transmission or through the activation of GABAergic transmission. Further, the altered antioxidant defence system in the brain leads to the development of neurodegenerative disease. Several amino acids, plant based bioactive molecules and carotenoids with strong antioxidant properties, possesstherapeutic favourable effects against Alzheimer's, Parkinson's and neuroinflammation conditions in several animal models and cell lines[23]. Bixin is also a carotenoid with strong scavenging activity against a free radical generation, and it protects against a cisplatin-induced neuronal cell damage. This observed activity is correlated with anti-carcinogenic properties[10]. In line with these evidences, bixin also exhibited good anti-convulsion and anti-psychotic properties in the present study, probably by enhancing GABA-mediated transmission and the blockage of D_2 receptors. Thus, bixin provided a strong scientific data in support of its traditional claim for the treatment of epilepsy and neurodegenerative diseases[8] by reversing the altered behavioural changes. Furthermore, exploration of possible mechanism of bixin for epilepsy and psychosis is warranted to examine its specific action.

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