NEUROPHARMACOLOGICAL PROFILE OF *PIPER BETEL* LEAVES EXTRACT IN MICE

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Summary

The present investigation deals with the neuropharmacological investigations of different doses (100,200,400 mg/kg.) of hydroalcoholic extract of leaves of *Piper betel.* The acute administration of extract reported increase in discrimination index in object recognition test, muscle relaxation, reduced locomotor activity, potentiation of haloperidol induced catalepsy, amphetamine antagonism.

The result points towards the potential activity of the *Piper betel* extract as a nootropic and also having property to attenuate amphetamine induced increased motor performance.

The study suggested that, the aforementioned effects are mediated via facilitation of cholinergic transmission and inhibition of dopaminergic as well as nor adrenergic transmission.

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Introduction

Stress involves complex biochemical, neural and immunological mechanisms and plays a crucial role in the genesis and progression of variety of diseased states (1). In today's life of stress and strain, there is a direct need for agent having neuroprotective and neuropharmacological activity enhancing learning, memory and overall performance of the brain (2).

In the recent years there have been a phenomenal rise it the interest of scientific community to explore the neuropharmacological actions and to confirm the veracity of claims made in official books of Ayurveda (3). A large number of plants claimed for their activity have been documented so far (4, 5, 6).

In the Indian traditional system of medicine leaves of *Piper betel* have been claimed for its neuropharmacological action especially for its nootropic effect. (7). The objective of the present work is to investigate the neuropharmacological actions of the hydroalcoholic extract of leaves of *Piper betel.*

Materials and methods

Plant material

The plant material in the form of hydroalcoholic extract prepared by the following procedure was received as gift sample (PBT-4031) from Green Chem. Herbals, Bangalore, India.

Preparation of extract

Leaves were extracted with 50% aqueous alcohol and concentrated. The concentrated mass was washed with petroleum ether several times to remove the resinous matter. Then the mass was diluted with 25% aqueous alcohol, filtered and concentrated, dried to get the powdered form of the extract.

Chemicals and drugs

Pentylenetetrazole, haloperidol, amphetamine, 5HTP, (Rajesh chemicals, Mumbai) and sodium nitrate, (Loba chemicals, Mumbai) were purchased from respective vendors. Diazepam and pentazocin injection, piracetam suspension, Clonazepam and Phenytoin tablets were purchased from the local market.

Preparation of drug solution

Accurately weighed quantity of powdered extract was dissolved in the distilled water to prepare the appropriate stock solution of the drug i.e. 10 mg/ml, 20mg/ml and 40 mg/ml respectively. The doses were administered orally by selecting the appropriate concentration of the stock solution. Clonazepam and Phenytoin were suspended in 1% w/v acacia.

Animals
Swiss male albino mice (18-22g) were used. They were maintained at 25 ± 2° C and relative humidity of 45 to 55% and under standard environmental conditions (12 h light 12 h dark cycle). The animals had free access to food (Chakan Oil Mills, Pune, India) and water ad libitum. Institutional Animal Ethical Committee approved the protocol. All experiments were carried out between 12:00- 16:00 h.

Acute toxicity test
Healthy adult male albino mice (18- 22g) were subjected to acute toxicity studies as per guidelines (AOT 425) suggested by the organization for economic co-operation and development (OECD-2001). The mice were observed continuously for 2 h for behavioral and autonomic profiles and for any sign of toxicity or mortality up to a period of seven days (8).

Behavioral effects
Behavioral effects of PB (100, 200 and 400 mg/kg) were assessed by visual observation 60 min after administration of vehicle or PB for next 2 h for behavioral changes (9). The observation parameters consisted of body position, alertness, reactivity to touch stimuli, righting reflex and lacrimation.

Effect on motor coordination
The motor coordination was assessed using digital rota rod (Inco- Ambala, India). Mice were trained by placing them on a rotating rod (20 rev/ min), twice daily for three consecutive days before the experiment. 30 min interval was kept between two trails. Only those mice which have demonstrated their ability to remain on the rotating rod for at least 2 min were selected**.** These selected mice were divided into five groups with 6 animals in each group. The Mice were then tested for motor coordination to record basal fall of time followed by respective drug treatment. One hour following the administration of vehicle or drug, mice were placed again on the rotating rod and the fall off time per 300 sec was recorded. The difference between mean fall of time before and after drug treatment was considered for evaluation. Diazepam (2 mg/ kg i.p.) was used as a reference standard (10, 11).

Locomotor Activity
The locomotor activity (horizontal activity) was measured using a digital actophotometer (Space-lab, India). Each mouse was placed individually in the actophotometer for 05 min and basal activity score was obtained. Subsequently animals were divided into five groups and treated with test drugs. 60 min after dosing, the mice were placed again in the actophotometer for recording the activity score as described earlier. The results were reported as mean change in the locomotor activity. Diazepam (2 mg/ kg, i p**)** preparation was used as reference standard (12).

Analgesic activity

The analgesic effect was studied using digital hot plate (Columbus- USA) method wherein the reaction time(paw licking, jumping or any other sign of discomfort) was recorded at 0, 60, and 120 min after administration of vehicle (10 ml/ kg) or PB extract (100, 200 and 400 mg/kg). The temperature of the plate was maintained at 55°C ± 01° C. A cut off reaction time of 30 s was chosen in order to avoid injury. Pentazocin (30 mg/kg) was used as a reference standard (6).

Elevated plus maze (EPM)

Locally fabricated elevated plus maze consisting of two open arms (35 × 6 cm) and two enclosed arms (35 × 6 × 15 cm) was used. The maze was elevated to the height of 40 cm. Mice were placed individually in the center of the EPM facing an enclosed arm. The time spent by the mouse during the next 05 min on the open and enclosed arm was recorded. The animals received vehicle (10 ml/ kg) or PB (100, 200 and 400 mg/kg) 60 min before and diazepam (1 mg /kg i.p.) 30 min before their placement on the maze. Increased exploratory activity in the open arm was taken as an indication of anxiolytic activity (13, 14).

Object recognition test

The apparatus fabricated locally consisted of white colored plywood (70 × 60 ×30 cm) with a grid floor. It was illuminated by a 40 W lamp suspended 50 cm above the apparatus. The object to be discriminated was also made of plywood in two different shapes of 10 cm height and colored black.

One day before the test, mice were allowed to explore the box without any object for 02 min. On the day of test, in the first trial (T_1) conducted 60 min after administration of vehicle (10 ml/kg) or PB (100,200,400 mg/kg) or piracetam (150 mg/kg) two identical objects were presented in opposite corners of the box and the time taken by each mouse to complete 20 s of object exploration was recorded (Exploration was considered as directing the nose at a distance less than 2 cm to the object and/or touching with nose). Second trial (T_2) was performed 90 min after first (T_1) and a new object replaced one of the objects presented in T_1 and mice were left in the box for next 05 min. The time spent for exploring the familiar (F) and the new object (N) was recorded separately and discrimination index (D) was calculated as (N-F)/ (N+F). The object was changed randomly and apparatus was cleaned with hydrogen peroxide after each trial to avoid place preference and the influence of olfactory stimuli respectively (5).

Double unit Mirrored chamber

The mirrored chamber apparatus fabricated locally consisted of a mirrored cube (30× 30× 30cm), open on one side and placed in square box. The container box (40×40×30 cm) has a white floor and black wall making 5cm corridor completely surrounding the mirrored chamber. A sixth mirror was placed on the wall of the box, positioned to face the open side of the mirror chamber. The latency to enter the mirror chamber and time spent in mirror chamber during 5 min observation period was recorded 60 min after the drug administration. Diazepam (1mg/kg/i p) was used as a reference standard. These mice were not exposed to the apparatus before the test and evaluated only once to avoid

habituation problem. The apparatus was washed after each evaluation to eliminate potential cues such excreta, urine left by the previous occupant (16, 17).

5HTP-induced head twitches

Mice were pretreated with pargyline (75 mg/kg, i.p) in order to prevent the rapid degradation of 5-HTP. Thirty minutes later, vehicle or PB was administered. Sixty minutes thereafter, 5-HTP (50 mg/kg, i.p.) was injected in the mice and head twitches were counted for ten minutes after the onset of head twitches (18, 19, 20).

Haloperidol induced catalepsy

Mice were divided into four groups. The control group received vehicle (10 ml/kg p.o.) whereas the other group received PB (100, 200 and 400 mg/kg) 60 min before haloperidol (1 mg/kg i.p). After the treatment, the forepaws of the mice were placed on rod of 0.9 cm diameter set at 2.5 cm from top. Duration for which the mice retains the forepaws on the elevated rod was noted down at 0, 15, 30, 60, 90 and120 min. the cut off time was 300 sec. The animals were tested twice at each time interval and only the greater duration of time was recorded. Between measurements, the mice were returned to their home cages (21, 22).

Sodium nitrite induced respiratory arrest

Mice were divided into four groups and were treated with vehicle (10 ml/kg) or PB (100, 200, 400mg/kg). Sixty min later, all mice were subjected to sodium nitrite treatment (250 mg/kg i.p). The time between injection of sodium nitrite and death was recorded (23).

Amphetamine antagonism

Mice were divided in four groups and treated with either vehicle or PB. Sixty min later all animals received amphetamine (1 mg/kg i.p.) and thirty min thereafter, the locomotor activity was measured for five min duration using digital actophotometer (24).

Clonidine induced hypothermia

Mice were taken in groups of six each and rectal temperature was recorded using digital telethermometer (Dolphin, India) every sixty min after clonidine (0.1 mg/kg i.p.) till 180 min. vehicle (10 ml/kg) or PB (100, 200 and 400 mg/kg) were administered 60 min before clonidine (25).

Pentylenetetrazole induced seizure (PTZ)

Clonic seizures were induced 60 min after respective drug treatment in mice by subcutaneous injection of 80mg/kg Pentylenetetrazole. The latency to the onset of seizures in non-protected mice and lethality during the following 24 h was recorded and compared with those of control mice to assess the anticonvulsant activity of the extract. Clonazepam (0.1mg/kg i.p.) was used as a reference standard (26, 27, 28).

Maximal electroshock induced seizures (MES).

Tonic clonic convulsions were induced 60 min after the respective drug treatment by giving maximal electroshock seizures (MES) (40mA for 0.2sec) using an electroconvulsiometer (INCO, Ambala, India) via crocodile ear clip 60 min after

administration of either vehicle(10 ml/kg) , PB (100, 200 and 400 mg/kg) or Phenytoin (20 mg/kg). The number of animals protected from tonic hind limb extension seizure (abolition of tonic hind limb extension within 10 sec after delivery of the electroshock was considered as protected mice.) and duration of tonic hind limb extension seizure was determined in each dose group (26, 29).

Statistical analysis

The results are expressed as mean ± SEM**.** Comparison between the groups was made by one way analysis of variance (ANOVA) followed by Dunnett's test.

Results

Acute oral toxicity test

All mice were free of any toxicity up to the dose of 2 gm/kg however sedation was noted above the dose of 1gm/kg. From this data, three different doses 100,200,400 mg/kg were selected for further study**.**

Behavioral assessment

The mice were observed for 2 hr; 60 min after oral administration of vehicle or PB (100,200,400 mg/kg). The observations are summarized in table 1.

Table 1. Behavioral assessment of *PB* extract in mice

Behavioral signs	**Vehicle (10ml/kg)**	**PB (100 mg/kg)**	**PB (200 mg/kg)**	**PB (400 mg/kg)**
Alertness	-	-	-	↓
Body position	-	-	-	-
Reactivity to touch stimuli	-	-	-	-
Righting reflex	-	-	-	-
Lacrimation	-	-	-	↑

--Normal, ↑: Increased, ↓: Decreased.

Effect of motor- co-ordination

Reduction in the mean change in fall off time was reported with PB-200(14.5± 1.05) and 400 (15.00± 1.15) compared to that of vehicle treated mice (07.50 ± 0.84). The lower dose (100mg/kg) of PB did not cause any significant change.

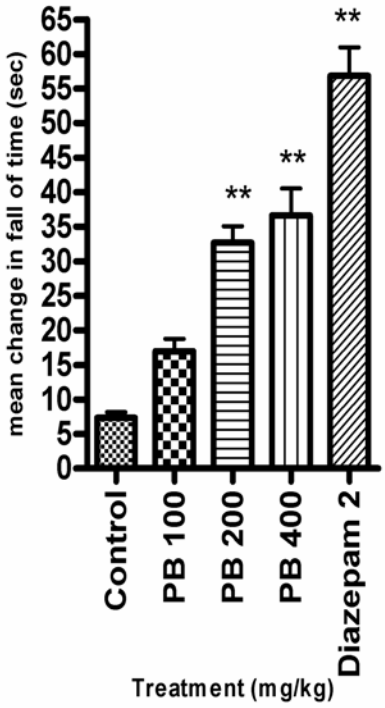

Figure 1: Effect of PB extract on the motor performance in mice. n=6, Data was analyzed by one-way ANOVA followed by Dunnetts *P<0.05, **P<0.01, ***P< 0.001

Effect of Locomotor activity

PB in a dose of 100 mg/kg did not produce any significant change in locomotor activity (16.66±2.88) as compared to control (10.16 ± 1.70). However next two doses i.e. (200 and 400 mg/kg) produced significant reduction (26.83±2.93; 25.83±1.92) respectively in locomotor activity.

Analgesic activity

There was no significant increase in reaction time in mice treated with different doses of PB at any time interval when compared against vehicle treated mice. Pentazocin was found to be effective in this regard (Data not shown)

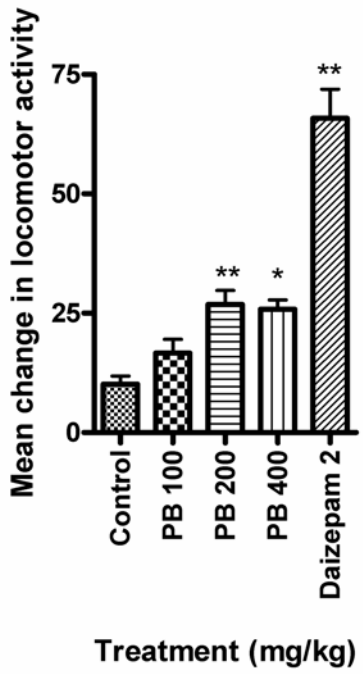

Figure 2: Effect of PB extract on the mean change in locomotor activity in mice. n=6, Data was analyzed by one-way ANOVA followed by Dunnetts *P<0.05, **P<0.01, ***P< 0.001

Elevated plus maze

The PB treatment did not show any significant effect on the time spent in open or enclosed arm when placed on EPM. Diazepam significantly increased time spent in open arm and thereby showed anxiolytic action.

Object recognition test

The mice in the first trial required 29.44±1.37 to 13.95± 1.21 sec to explore the objects. In the second trial, when new object-replaced one of the object, PB (200 and 400mg/kg) and piracetam treated mice required significantly less time to explore the familiar objects as compared with the new object. Both PB and piracetam significantly improved the discrimination index.

Double unit mirrored chamber

Pre treatment with different doses of PB did not affect latency to first entry or time spent in mirror chamber when compared to vehicle treated mice; Diazepam, a reference standard showed significant anxiolytic effect. (Data not shown).

Table 2: Effect of PB extract and Diazepam on anxiety induced using elevated plus maze apparatus.

	Time spent in sec (mean± SEM)	
Treatment (mg/kg)	**Open arm**	**Enclosed arm**
Vehicle (10ml/kg)	56.45 ± 4.69	225.45 ± 6.72
PB (100)	63.89 ± 4.20	225.34 ± 4.47
PB (200)	57.79 ± 3.24	228.24 ± 2.88
PB (400)	55.96 ± 3.11	236.44 ± 3.46
Diazepam (1)	78.37 ± 3.36**	196.42 ± 6.66**

n = 6 Data was analyzed by one-way ANOVA followed by Dunnett's *P<0.05, **P<0.01, ***P< 0.001

Table 3: Effect of PB extract and Piracetam on discrimination index in object recognition test.

Treatment (mg/kg)	**Discrimination index**
Vehicle (10ml/kg)	0.185 ± 0.017
PB (100)	0.210 ± 0.020
PB (200)	0.271 ± 0.025*
PB (400)	0.266 ± 0.026*
Piracetam (150)	0.296 ± 0.018**

n = 6 Data was analyzed by one-way ANOVA followed by Dunnett's *P<0.05, **P<0.01, ***P< 0.001

5HTP-induced head twitches in mice

5 HTP induced 18.40±1.43 head twitches in 10 minutes in vehicle treated control mice. Pretreatment with PB significantly increased the number of twitches. The dose 200 and 400 mg /kg were found to be equally effective where as 100 mg /kg was not significant in this regard.

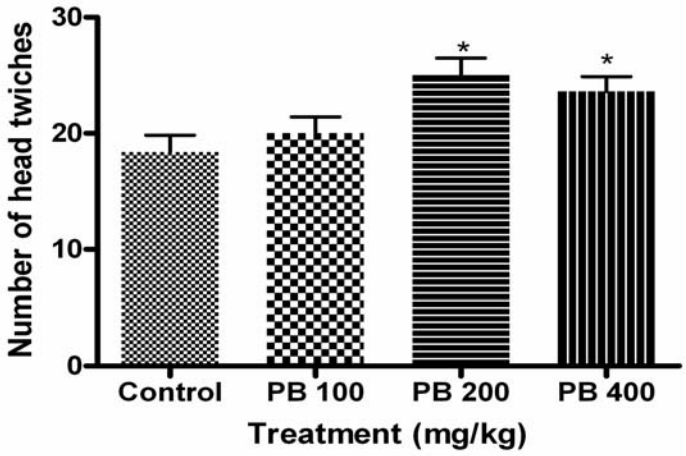

Fig 3: Effect of PB extract on number of head twiches induced by 5HTP n=5 Data was analyzed by one-way ANOVA followed by Dunnetts *P<0.05, **P<0.01, ***P<0.001

Haloperidol induced catalepsy

In haloperidol-induced catalepsy, maximum catalepsy was noted at 120 min. PB (400 mg/kg) treatment showed significant potentiation of epilepsy from 30 to 120 min. The maximum potentiation was noted at 60 min. the lower two doses of PB (100 and 200 mg/kg) did not show any significant potentiation.

Table 4: Effect of PB on duration of haloperidol- induced catalepsy in mice.

Time	Mean duration of catalepsy in sec			
	Vehicle(10ml/kg)	**PB(100 mg/kg)**	**PB (200 mg/kg)**	**PB (400 mg/kg)**
0 min	3.01 ± 0.13	2.88 ± 0.13	3.01 ± 0.16	3.32 ±0.13
15 min	15.84 ±0.72	15.59 ±0.87	15.02 ±1.07	16.72 ±1.04
30 min	28.87 ± 1.86	30.44 ±1.59	33.04 ±0.85	36.24 ± 2.01*
60 min	151.97 ± 8.23	144.65 ± 7.56	160.01 ± 7.13	189.87 ± 7.82**
90 min	236.46 ± 10.21	250.19 ± 12.11	250 ± 13.26	285.46 ± 5.43*
120 min	249.93 ± 9.47	252.90 ± 6.19	257.87 ± 10.73	280.94 ± 5.23*

n=6 Data was analyzed by one-way ANOVA followed by Dunnett's *P<0.05, **P<0.01, ***P< 0.001

Sodium nitrite induced respiratory arrest

Vehicle treated control mice died after 1257 ± 19.37 sec of sodium nitrite administration whereas; PB 200 and 400 mg/kg treatment delayed the onset of death to 1714.66 ± 24.99 and 1798.16 ± 25.47 sec respectively.

Amphetamine antagonism

Pretreatment with PB 200 and 400 mg/kg significantly reduced (317 ± 10.59 and 248.33 ± 9.65 respectively) the score compared to vehicle treated control mice (412.33 ± 6.64).

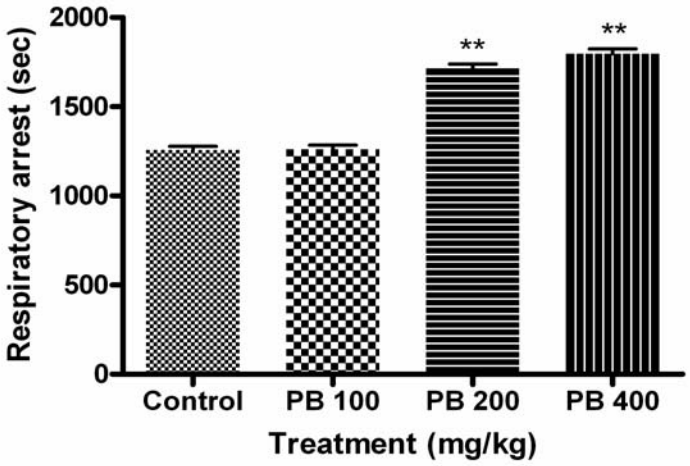

Fig 4: Effect of PB extract on Sodium nitrite induced respiratory arrest n=5 Data was analyzed by one-way ANOVA followed by Dunnetts *P<0.05, **P<0.01, ***P<0.001

Clonidine induced hypothermia

Clonidine induced hypothermia was not affected by the pre treatment of PB. However, a non significant potentiation of hypothermic effect was seen. (Data not presented)

Pentylenetetrazole induced seizure (PTZ)

In vehicle treated control mice, convulsions were produced after 191.66 ± 11.27 sec. The pretreatment with PB delayed this onset up to 345.83 ± 34.91. All the control mice died immediately after onset of convulsions while one of six was survived in each PB 200 and 400 mg/kg treated group. Standard clonazepam showed 100% protection.

Maximal electroshock induced seizures (MES).

The mean duration of hind limb extension in vehicle treated group was 25.02 ± 01.53 sec which was reduced to 17.94 ± 02.05 sec with pretreatment with 400 mg/kg PB. The dose 100 and 200 mg/kg were found to be ineffective in this regard. Considering the parameter of number of mice protected, only one mouse was protected with the pretreatment of 400 mg/kg PB.

Table 5: Effect of PB extract and clonazepam on PTZ induced convulsions in mice.

Treatment (mg/kg)	Onset of convulsions (sec.)	Mice convulsed/mice used	% Mortality
Vehicle (10ml/kg)	191.66 ± 11.27	6/6	100
PB (100)	294.5 ± 15.26	6/6	100
PB (200)	340.66 ± 23.82	6/6	83.33
PB (400)	345.83 ± 34.91	6/6	83.33
Clonazepam (0.1)	Nil	0/6	00

n=6 Data was analyzed by one-way ANOVA followed by Dunnett's *P<0.05, **P<0.01, ***P< 0.001

Table 6: Effect of PB extract and phenytoin on MES induced convulsions in mice.

Treatment (mg/kg)	Duration of hind limb extension (sec.)	Mice convulsed/mice used
Vehicle (10ml/kg)	25.02 ± 1.53	6/6
PB (100)	21.15 ± 1.38	6/6
PB (200)	18.72 ± 2.30	6/6
PB (400)	17.94 ± 2.05*	6/6
Phenytoin (25)	Nil	0/6

n=6 Data was analyzed by one-way ANOVA followed by Dunnett's *P<0.05, **P<0.01, ***P< 0.001

Discussion

Piper betel is claimed to be useful to improve learning and memory, in Indian traditional system of medicine yet not documented scientifically in this regard (7). Despite extensive research, the neurological basis of learning and memory remains controversial (30). Moreover, existence of cognitive problem in various neurological illnesses like Alzheimer's disease, depression, convulsions, etc is well known (31, 32). The modern therapy employed to treat these disorders further may result in cognitive impairments (33).

Phenytoin, a widely used anticonvulsant upon prolonged administration results in cognitive deficit (34, 35). The anticholinergic drugs useful to control Parkinson's disease may deteoriate cognitive behavior in co existed Alzheimer's disease (5). This complexicity of various disorders of CNS may be due to widespread role of different central neurotransmitters and hence it is worthwhile to investigate the drug for detailed neuropharmacological actions.

In the present investigation, PB extract exhibited increase in discrimination index, muscle relaxation; reduction in basal as well as amphetamine induced increased locomotor activity. The extract also increased 5HTP head twitches; delayed sodium nitrite induced respiratory arrest and potentiated haloperidol induced catalepsy. The effect on time spent in open arm and mirror chamber in elevated plus maze and double unit mirror chamber respectively was not significant.

The improvement in discrimination index by the two doses (200 and 400 mg/kg) of extract proved that PB met major criteria for nootropic activity, improvement of memory in absence of cognitive deficit (36). Similar doses delayed the death due to sodium nitrite induced respiratory arrest. In this case, respiratory arrest is caused due to chemical hypoxia and thereby reduces oxygen carrying capacity. The drug that delay or abolish this arrest probably act by improved cholinergic transmission (23). The neurological basis of learning and memory established the role of cholinergic system (37, 38). The widespread use of scopolamine, a muscarinic blocker as an experiment model of amnesia, (39, 40) facilitation of learning and memory along with increase in central cholinergic transmission (41) have proven the role of cholinergic system in the learning and memory. The delayed respiratory arrest and improved discrimination index in the present study validate its traditional claim of nootropic and indicate possible role of central cholinergic transmission. Although the involvement of cholinergic system is well established, the role of other neurotransmitter can not be ignored (30).

It has been reported that serotonin (5HT) 1A receptors are involved in learning and memory processes and 5HT 1A agonist 8-hydroxy 2-(di-n-propilamino) tertaralin (8-OH-DPAT) improved consolidation of a conditioned response when injected after training (42). Another study reported improvement in cognitive performance of rats and patients suffering from Alzheimer's disease by the 5HT-1A receptor agonist (37). In this study, increase in 5HTP induced head twitches indicated enhanced serotonergic transmission.

The marked increase in turnover of nor- adrenaline (NA) by the piracetam, an established nootropic agent (43), reports regarding mental confusion as well as retardation of memory consolidation by the amphetamine, a drug that markedly augment central nor

adrenergic activity (44) indicated inverse relationship between noradrenergic transmission and cognitive performance. In the present study, PB significantly reduced amphetamine induced increased locomotor activity. Moreover extract potentiated (Although statistically not significant) hypothermic effect of clonidine, a presynaptic α adrenoreceptor agonist that produces hypothermia by reducing nor adrenergic transmission (25). The aforementioned results suggest the inhibitory effect of PB extract on nor-adrenergic transmission.

Haloperidol induced catalepsy appears to be due to blockade of dopamine (DA) transmission (45, 46, 47). The depletion of brain DA content after piracetam (48) and oil of *celastrus paniculatus* administration, (3) which possess nootropic activity has also been documented. The PB in the dose of 400 mg/kg has significantly potentiated haloperidol induced catalepsy between time intervals of 30 – 120 min. In addition, significant reduction in basal locomotor activity has also been observed. These finding suggest inhibition of DA transmission in the claimed activity.

Seizure is an outcome of an imbalance between excitatory and inhibitory neurotransmitters. (5) It has been stated that, MES induced hind limb extension can be prevented by inhibition of voltage dependent Na^+ channels or blocking glutamatergic excitation mediated by NMDA receptors. On the other hand, PTZ induced seizures can be prevented either by reducing T- type current or by facilitation of GABA–A receptor mediated inhibitory neurotransmission.(49) PB did not show anticonvulsant effect in PTZ and MES model.

The extract did not produce any significant anxiolytic activity when tested on EPM and double unit mirror chamber too. Generally most of the anxiolytic agents have an adverse effect on memory as seen with the benzodiazepines, commonly used anxiolytic (50). The lack of anxiolytic activity may indirectly boost its nootropic potential.

Conclusion

It is thus apparent that hydroalcoholic extract of *Piper betel* leaves exhibited improvement in the discrimination index, potentiation of haloperidol induced catalepsy, reduction in basal as well as amphetamine induced increased locomotor activity and delay in sodium nitrite induced respiratory arrest. These results suggest possible facilitation of cholinergic transmission and inhibition of dopaminergic as well as nor adrenergic transmission by the extract.

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