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Nootropic activity of butanolic extract *Passiflora ncarnata* leaves

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Abstract

Alzheimer's disease (AD) is the most common cause of dementia among people of age 65 and older. Nootropics are a class of psychotropic drugs that enhance learning, acquisition and reverse learning impairments in experimental animals. Numerous plants have been used to treat cognitive disorders in the traditional medicine.

Passiflora incarnata Linn (Family: Passifloraceae) has been widely used in traditional medicine as sedative, anxiolytic, antispasmodic, analgesic, anticonvulsant, and wormicidal. Till date there are no reports on nootropic activity of *Passiflora incarnata*. Hence, in the present study, nootropic activity of *n*-butanol extract of *P. incarnata* leaves (BEPI) was studied using elevated plus maze and object recognition test.

The results of the study signified that *n*-butanol extract of *P. incarnata* leaves containing flavonoids possessed nootropic activity indicated by shortening of transfer latency (TL) and an increase in discrimination index. Pretreatment with BEPI protected the animals from memory deficits produced by scopolamine.

The results of present study confirmed that nootropic effect of BEPI is mediated through facilitation of brain cholinergic neurotransmission and major phytoconstituent like flavonoids, alkaloids and phenolic compounds may be responsible for nootropic effect of *P. incarnata*.

Keywords: Nootropic, elevated plus maze test, object recognition test.

Introduction

Cognitive dysfunction, a major health problem in 21st century, is one of the most functionally debilitating aspects of many neuropsychiatric disorders and neurodegenerative disorders, such as Alzheimer's disease, schizophrenia, depression, dementia, cerebrovascular impairment, seizure disorders, head injury and Parkinsonism [1]. Alzheimer's disease (AD) is the most common cause of dementia among people of age 65 and older. AD is an irreversible, progressive disorder in which brain cells deteriorates, resulting in the loss of cognitive functions, primarily memory, judgment and reasoning, movement coordination and recognition [2].

Nootropics are a class of psychotropic drugs that enhance learning, acquisition and reverse learning impairments in experimental animals. In traditional practices of medicine, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases such as Alzheimer's disease and other memory related disorders. The plants with potential cognitive enhancement activity includes *Bacopa monnieri,* Celastrus paniculata, Albizzia lebbeck[3], Clitoria ternatea [4], Acorus calamas, Lawsonia inermis[5], Curcuma longa, Ginkgo biloba, Glycyrrhiza glabra, Galanthus nivalis (galanthamine) and many more[6].

Passiflora incarnata Linn (Passifloraceae) has been widely used in traditional medicine in West India, Mexico, Netherland, South America, and Argentina. It has been used as an anxiolytic and sedative-hypnotic since ancient time. The main constituents of leaves of *P. incarnata* are flavonoids such as vitexin, isovitexin, orientin, isoorientin, apigenin and kampferol [7]. *P. incarnata* is known to possess antitussive, analgesic, anticonvulsant, antiasthmatic, aphrodisiac, and anti-inflammatory activity [8]. However, there is a lacuna with respect to the scientific evaluation of *P. incarnata* in preclinical animal models of learning and memory. Hence, the present investigation is undertaken to study nootropic activity of *P. incarnata* leaves.

Neurochemical analysis of Alzheimer's disease has revealed that there is a marked reduction in the acetylcholine (ACh) content of the cortical and hippocampal regions of the human brain. Centrally acting antimuscarinic drugs (like scopolamine) impair learning and memory of rats and human beings. The amnesia induced by scopolamine represented the main symptom of Alzheimer's disease, and piracetam reversed this effect in rodents [9]. Hence, scopolamine induced amnesia model is explored in the present study using elevated plus maze to assess cholinergic involvement.

Materials and methods

Plant material

The fresh leaves of *P. incarnata* were collected in the months of June, July and August 2010 from local nursery in Pune, Maharashtra, India. The plant was identified and authenticated by Dr. Dinesh Shirodkar, Botanical Survey of India (BSI), Pune, India. A voucher specimen has been deposited in BSI (Voucher Specimen No: PASSIN 3)

Preparation of extracts

Shade dried leaves (1000 g) were powdered and macerated with ethanol for 48 hrs. The extract was evaporated to dryness. The extract was suspended in water and extracted successively with hexane, chloroform, ethyl acetate and *n*- butanol. The percentage yield of extracts was 11.7% w/w, 1.1% w/w, 0.9% w/w and 22.5% w/w respectively. *n*- butanol extract of *P. incarnata* leaves (BEPI) was used for further study.

Drugs and Chemicals

Piracetam and Scopolamine were obtained from Alkums Drugs and Pharmaceutical Ltd, Haridwar. Ethanol (Baker, Germany), hexane, chloroform, ethyl acetate and n-butanol used in this study were procured from S.D. Fine Chem. Limited, Mumbai, Maharashtra, India.

Animals

Swiss albino mice (25-30 g) of either sex were used. Animals were housed under standard conditions of temperature (24 ± 2°C) and relative humidity (30-70%) with a 12:12 hr light: dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. The institutional animal ethical committee approves all the experimental protocols.

Qualitative phytochemical analysis

The screening for presence of phytoconstituent was carried out in accordance with the standard protocol as described by Trease and Evans [10].

Quantitative phytochemical estimation

Total phenolic content determination

The total phenolic content of BEPI was determined by using the Folin–Ciocalteu assay. An aliquot (1 ml) of extracts (10mg/100ml of distilled deionised water) or standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l) was added to 25 ml volumetric flask, containing 9 ml of distilled deionised water (dd H_2O). A reagent blank using dd H_2O was prepared. One millilitre of Folin–Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7 % Na_2CO_3 solution was added to the mixture. The solution was diluted to volume (25 ml) with dd H_2O and mixed. After incubation for 90 min at room temperature, the absorbance against reagent blank was determined at 750 nm with an UV-Vis Spectrophotometer. Total phenolic content of BEPI was expressed as mg gallic acid equivalents (GAE)/100 g fresh weight. All samples were analyzed in triplicate [11].

Total flavonoid assay

Total flavonoid content was measured by the aluminum chloride colorimetric assay. An aliquot (1 ml) of extracts (10mg/100ml of alkaline methanol) or standard solution of chrysin (20, 40, 60, 80 and 100 mg/l) was added to 10 ml volumetric flask containing 4 ml of dd H_2O. To the flask was added 0.3 ml 5 % $NaNO_2$. After 5 min, 0.3 ml 10 % $AlCl_3$ was added. At 6th min, 2 ml 1 M NaOH was added and the total volume was made up to 10 ml with dd H_2O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of BEPI was expressed as mg chrysin equivalents (CE)/100 g fresh mass. Samples were analysed in triplicate [11].

Total alkaloid determination

Total alkaloid content was measured by Bromocresol Green colorimetric assay. Aliquots (0.4, 0.6, 0.8, 1 and 1.2 ml) of atropine standard solution (1mg/10ml in distilled water) were accurately measured and transferred each to different separating funnels. To it 5 ml phosphate buffer (pH 4.7) and 5 ml Bromocresol Green (BCG) solution was added. This mixture was shaken mixture with 1, 2, 3 and 4 ml of chloroform. An aliquot (1 ml) of extracts (1mg/10ml in distilled water) were collected in a 10 ml volumetric flask and then diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without atropine [12].

Acute toxicity studies

To evaluate the acute toxicity of BEPI after a single intraperitoneal dose, Swiss albino rats were fasted for 6 h with only water provided ad libitum. Mice were divided into experimental groups (n=6) and were treated intraperitoneally at doses of 150,300 and 600 mg/kg. Animals were then allowed free access to food and water. The animals were observed for any abnormal behavior, and mortality was noted for 14 days after the intraperitoneal administration for the acute toxicity. The control group was treated with normal saline (1ml/kg, i.p.) [13].

Nootropic activity

Elevated plus maze test (EPM)

An elevated plus maze consisting of two open arms (36x6 cm) and two enclosed arms (35x6x15 cm) was used. The maze was elevated at the height of 25 cm. Mice were placed individually at the end of the open arm and the time taken by the animal to enter into either of the enclosed arm (transfer latency, TL) was recorded. On the first day the mouse was allowed to explore the plus maze for 5 min and sent back to home cage after the first trial. After 24

hours mice were placed again on the elevated plus maze individually as before and TL was noted again. TL measured on first day served as parameter for acquisition. TL was expressed as retention scores after 24 hours or one week for each mouse by calculating the 'inflexion ratio'.

$$\text{Inflexion ratio} = (L_1\text{-}L_0)/L_0$$

Where L_0 = transfer latency after 24 hours/week in seconds.
L_1=initial transfer latency in seconds.
The mice were treated with vehicle, BEPI (150and 300 mg/kg, i.p.) and piracetam (100 mg/kg, i.p.) alone or 30 min before scopolamine (0.3 mg/kg, i.p.). Each group consisted of 6 animals [14].

Object recognition test (ORT)

The apparatus was formed by the colored plywood (70x60x30 cm) with a grid floor that could be easily cleaned with hydrogen peroxide after each trial. The apparatus was illuminated by 40 W lamp suspended 50 cm above the box. The objects to be discriminated were also made of plywood in two different shapes of 8cm height colored black.
The day before test mice were allowed to explore the box (without any object) for two min. On the day of test in the first trial (T_1) two identical objects were placed in two opposite corners of the box and the amount of time taken by each mouse to complete 20 sec of object exploration was recorded. Exploration was considered directing the nose at a distance <2cm to the object and /or touching it with the nose. During the second trial (T_2, 90 min after T_1) one of the objects presented in trial T_1 was replaced by new object and the mice were left in the box for 5 min. The time spent for exploration of familiar (F) and the new object (N) were recorded separately and discrimination index (D) was calculated (N-F/N+F). Care was taken to avoid place preferences and olfactory stimuli by randomly changing the role (familiar or new object) and the position of the two objects during T_2 and cleaning the apparatus with hydrogen peroxide.
The mice were treated with vehicle, BEPI (150and 300 mg/kg, i.p.) and piracetam (100 mg/kg, i.p.) 30 minutes before the first trial. The second was performed 90 min. after the first trial. Each group consisted of 6 animals [15].

Statistical analysis

Statistical analysis of data received was performed using the software Primer of Biostatistics (Primer of Biostatistics, Version 4, Stanton A. Glantz). Results were expressed as mean ± SEM. Significant differences between groups were determined by analysis of variance test followed by Dunnett's test. Differences between data sets were considered as significant when p value was less than 0.05.

Results

Qualitative phytochemical screening

The preliminary phytochemical screening of BEPI showed presence of tannins, glycosides, alkaloids, flavonoids and phenolic compounds. The rest of compound did not test positive.

Quantitative phytochemical estimation

Total phenolic content determination

A linear calibration curve of gallic acid, in the range of 0-100 mg/l with coefficient of determination (r^2) value of 0.9967, was obtained (Fig. 1). Total phenolic content of BEPI was found to be 20.85±0.36 mg gallic acid equivalent /100 gm of dry weight (Table 1)

Table 1: Total phenolic, flavonoid and alkaloid content of butanolic extract of *Passiflora incarnata* leaves (BEPI)

Extract	Total phenolic (mg GAE/100 gm of dry weight)	Total flavanoid (mg CE/100gm of dry weight)	Total alkaloids (mg AE/100gm of dry weight)
BEPI	20.86	45.33	73.94

Total flavonoid content determination

A linear calibration curve of chrysin, in the range of 0-100 mg/l with coefficient of determination (r^2) value of 0.9911, was obtained (Fig. 2). Total flavonoid content of BEPI was found to be 45.33±0.20 mg chrysin equivalent /100gm of dry weight (Table 1).

Total alkaloid content determination

A linear calibration curve of atropine, in the range of 0-120 mg/l with coefficient of determination (r^2) value of 0.9959, was obtained (Fig. 3). Total alkaloid content of BEPI was found to be 73.94±0.10 mg atropine equivalent /100gm of dry weight (Table 1).

Acute toxicity study

In the acute toxicity study, intraperitoneal administration of BEPI at doses of 150, 300 and 600 mg/kg did not cause mortality in mice during 14-days observation. The mice did not show any signs of toxicity or change in general behavior or other physiological activities. Thus, 150 and 300 mg/kg dose of BEPI, which is a safe dose, was used for further animal study.

Nootropic activity

Elevated plus maze test

In elevated plus maze test, vehicle treated mice displayed TL of 37.17 ± 1.16 seconds on day 1, which decreased on day 2 and day 9. BEPI administered intraperitoneally at a dose of 150 and 300 mg/kg decreased TL on day 2 and day 9 significantly ($p<0.001$). Piracetam (100 mg/kg) also decreased TL on day 2 and day 9 significantly while inflexion ratio was increased significantly on day 2 and day 9 compared to vehicle treated animals. Scopolamine at a dose of 0.3 mg/kg, i.p. increased TL on day 2 and day 9 significantly, while inflexion ratio was decreased. Thus, scopolamine produced amnesic effect in animals. Pretreatment with BEPI (300 mg/kg, ip) decreased TL on day 2 and day 9 compared to scopolamine treated animals significantly. Figure 4 and 5 depicts the effects of BEPI on learning and memory in elevated plus maze test.

Object recognition test

In object recognition test, the mice in the first trial (T_1 session) required more time to explore the objects. In the second trial (T_2 session), when a new object replaced one familiar objects, BEPI at a dose of 150 and 300 mg/kg and piracetam at a dose of 100 mg/kg required significantly less time to explore the familiar object as compared with the new object. BEPI (300 mg/kg, i.p.) significantly ($P < 0.001$) reduced exploration time of familiar object from 133.50 ± 9.39 in first trial to 37.67±3.91 in second trial. Similarly, piracetam also reduced exploration time of familiar object in second trial. Thus, Piracetam and BEPI (150 and 300 mg/kg) significantly increased the discrimination index as compared to vehicle treated group which is indicative of its nootropic activity. Figure 6 and 7 depicts the results of BEPI on learning and memory in object recognition test.

Discussion

Cognitive functions are categorized into memory, attention, creativity and intelligence. It is subjective in nature and can be affected by number of factors including ageing, stress, hypertension, various pathological conditions such as dementia related to Parkinson's disease (PD), Alzheimer's disease (AD), schizophrenia, cancer and HIV. Cognitive enhancement may be defined as the amplification or extension of core capacities of the mind through improvement or augmentation of internal or external information processing systems. The enhancement aspects of cognition, such as learning and memory, now seems possible for people with normal age related decline and in healthy people, although so far the effects of these cognition enhancers are modest[1]. Nootropic drugs belong to the category of psychotropic agents with selective facilitatory action on intellectual performance, learning and memory. A number of drugs including piracetam have now been introduced in therapy to ameliorate cognitive deficits [16]. The present study indicated that BEPI containing flavonoids, alkaloid and phenolic compound possessed nootropic activity indicated by shortening of transfer latency (TL). Both piracetam and BEPI meet a major criterion for nootropic activity, namely improvement of memory in absence of cognitive deficit. Similarly, in object recognition test also, BEPI showed facilitatory effect on learning and memory.

Despite of extensive experimental and clinical studies, the neurochemical basis for learning and memory remains controversial. However, a predominant role of cholinergic mechanism has long been emphasized in learning and memory processes. The role of the central cholinergic system is fairly well established and its deficiency being implicated in memory deficit [17]. Cognitive deterioration occurring in patients with probable AD is associated with progressive loss of cholinergic neurons and consequent decline in levels of acetylcholine (ACh) in brain. Selective loss of cholinergic neurons and decrease in choline acetyl transferase activity was reported to be a characteristic feature of senile dementia of the AD [18].

One of the most distinctive actions of nootropic drugs is their ability to antagonize the cognitive deficits induced by anticholinergic agents. It is generally accepted that the behavioral effects exerted by scopolamine are brought about by a non selective, competitive and reversible blockade of the muscarinic receptor subtypes in the brain [19]. There are several models of amnesia; however scopolamine induced memory deficits have been proposed to have symptomalogical similarities with AD and related disorders [20]. Hence, in present study, we envisaged the nootropic effect of BEPI using scopolamine induced memory deficit model of amnesia on elevated plus maze in mice. Pretreatment with BEPI protected the animals from memory deficits produced by scopolamine. This suggests that nootropic effect of BEPI is

Fig 1. Calibration curve of standard gallic acid for determination of total phenolic content

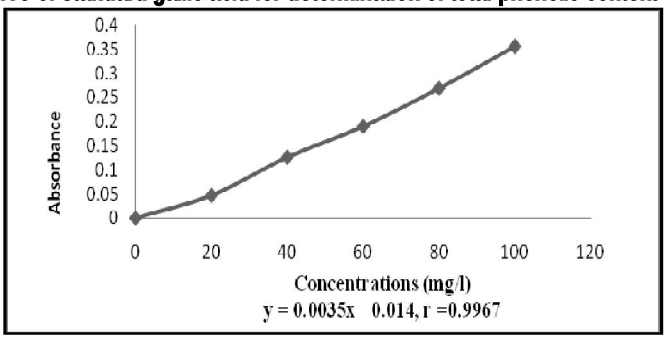


Fig 2. Calibration curve of standard chrysin for determination of total flavonoid content

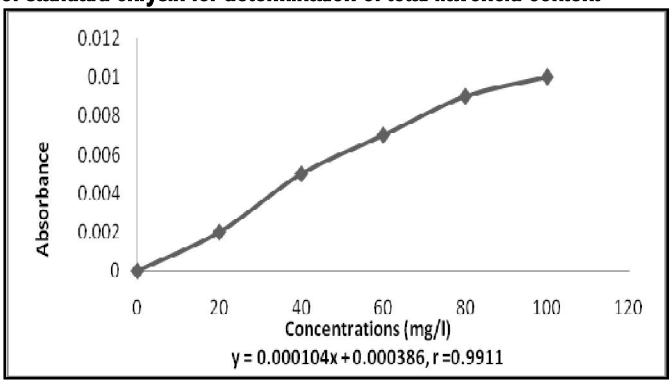
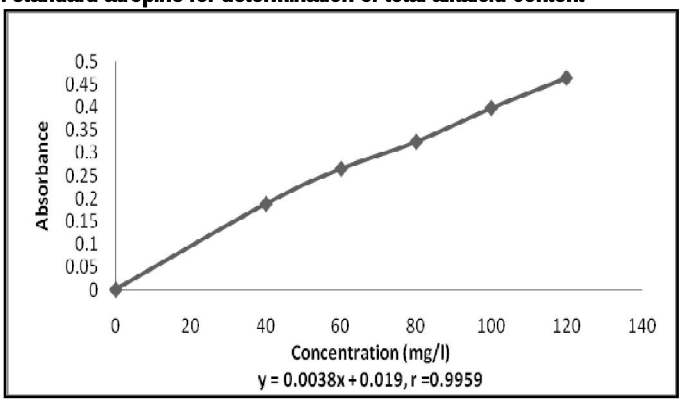


Fig 3. Calibration curve of standard atropine for determination of total alkaloid content



mediated through facilitation of brain cholinergic neurotransmission. However, further studies are necessitated to identify the exact mechanism of action.

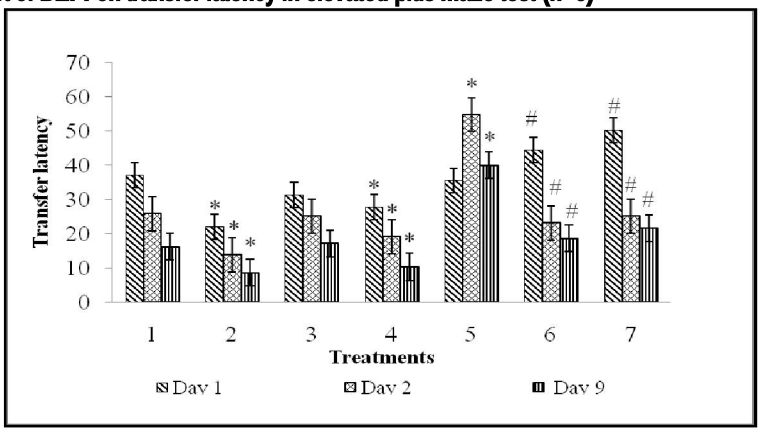
The review of various herbal medicines with significant nootropic activity has revealed that Phytoconstituent like saponins, glycosides, flavonoids and alkaloids are responsible of their nootropic potential [21]. The preliminary phytochemical investigation and quantitative estimation of BEPI showed presence of significant amount of flavonoids, alkaloids and phenolic compounds. Thus, the observed nootropic effect in this study was probably associated with these major constituents detected in BEPI. Furthermore, these components may act synergistically or other minor constituents may also contribute to the observed activity.

Conclusion

Altogether, the present study results confirmed that BEPI posses significant nootropic activity. Furthermore, pretreatment with BEPI protected the animals from memory deficits produced by

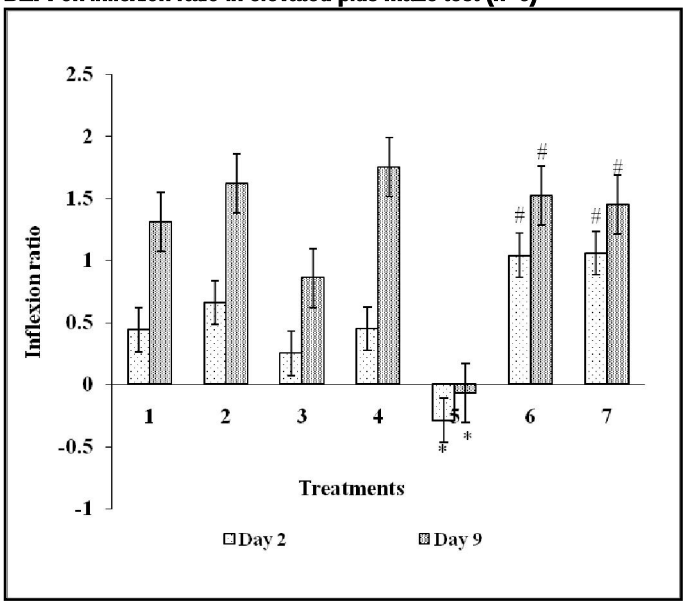
scopolamine which suggested that nootropic effect of BEPI is mediated through facilitation of brain cholinergic neurotransmission and is attributed to major constituents detected in BEPI. However, further studies are necessitated to identify the exact mechanism of action.

Fig 4: Effect of BEPI on transfer latency in elevated plus maze test (n=6)



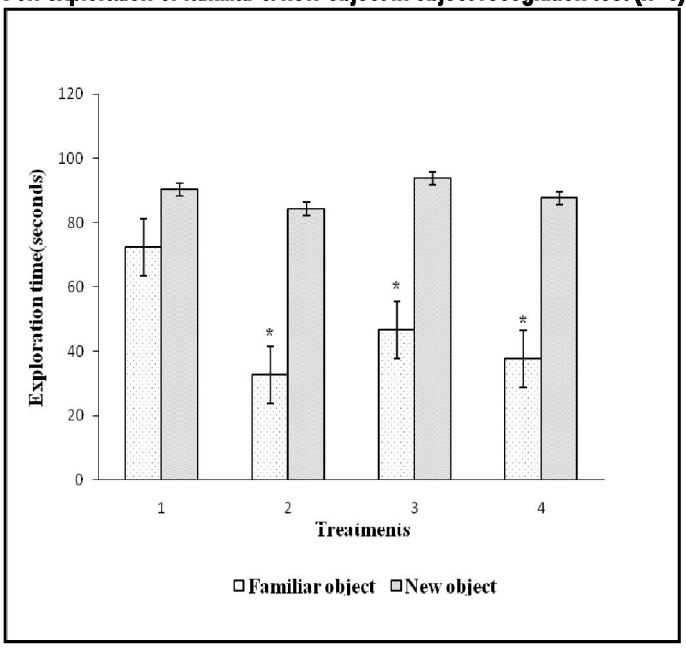
*** P≤0.001, (One way ANOVA followed by Dunnett's test) compared to vehicle group**
 # P≤0.001, (One way ANOVA followed by Dunnett's test) compared to scopolamine group
1: Vehicle (10 ml/kg), 2: Piracetem (100 mg/kg), 3: BEPI (150 mg/kg), 4: BEPI (300 mg/kg), 5: Scopolamine (0.3 mg/kg), 6: Scopolamine+ Piracetam (0.3+100), 7: Scopolamine+ BEPI (0.3+300)

Fig 5: Effect of BEPI on inflexion ratio in elevated plus maze test (n=6)



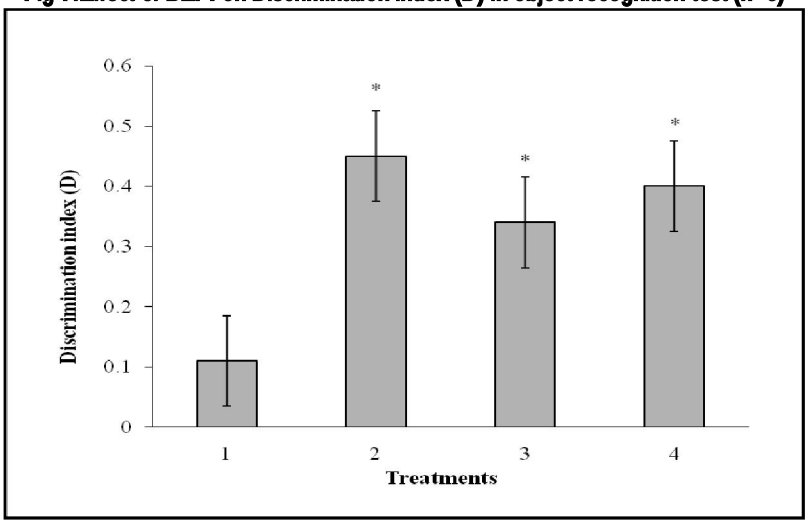
*** P≤0.001, (One way ANOVA followed by Dunnett's test) compared to vehicle group**
 # P≤0.001, (One way ANOVA followed by Dunnett's test) compared to scopolamine group
1: Vehicle (10 ml/kg), 2: Piracetem(100 mg/kg), 3: BEPI(150 mg/kg), 4: BEPI (300N mg/kg), 5: Scopolamine(0.3 mg/kg), 6: Scopolamine+ Piracetam (0.3+100), 7: Scopolamine+ BEPI (0.3+300)

Fig 6: Effect of BEPI on exploration of familiar & new object in object recognition test (n=6)



*** P≤0.001, (One way ANOVA followed by Dunnett's test) compared to vehicle group 1: Vehicle (10 ml/kg), 2: Piracetem(100 mg/kg), 3: BEPI(150 mg/kg), 4: BEPI (300mg/kg)**

Fig 7:Effect of BEPI on Discrimination index (D) in object recognition test (n=6)



*** P≤0.001, (One way ANOVA followed by Dunnett's test) compared to vehicle group 1: Vehicle (10 ml/kg), 2: Piracetem(100 mg/kg), 3: BEPI(150 mg/kg), 4: BEPI (300mg/kg)**

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Conflict of Interest

There is no conflict of interest

Abbreviations

ACh: acetylcholine
BEPI: *n*- butanol extract of *P. incarnata* leaves
CE: chrysin equivalents
TL: transfer latency
PD: Parkinson's disease

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