Original Article

Ginkgo biloba extract potentiates Nootropics effects of piracetam against ethanol-induced dementia and brain damage

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ABSTRACT

Purpose: Alcohol-induced dementia is one of the common neurological problems associated with long-term alcohol drinking leads to structural and functional impairment of brain. There are several options investigated to enhance the memory and repair the degeneration. In this study, we used the approach of combining two memory enhancers such as piracetam and *Ginkgo biloba* extract (EGb) against alcohol-induced dementia in mice. **Methods:** We used two different types of animal model for the induction of dementia and brain damage. Dementia was induced by single i.p. injection of alcohol 12.6% whereas brain damage was induced by continuous drinking of 15% of ethanol. Pre-treatment of piracetam and EGb both alone at a higher dose and in combination was administered to mice for 7 days. On the past day of treatment, alcohol was injected 30 min before the memory testing. Memory was tested using elevated plus maze as well as plus-maze inhibitory avoidance discrimination task. Apart from this, brain acetylcholine esterase level was measured after necropsy. Furthermore, histopathological evaluation of brain was carried out for confirm structural changes. **Results:** Combination of piracetam and EGb at a lower dose has significantly improved memory in alcohol-induced dementia model in comparison with these agents administered individually at a higher dose. Furthermore, brain acetylcholine esterase level was significantly reduced in combination when compared with agents administered individually. All these results were also supported by histology of brain tissue where lipofuscin pigment was notably reduced in combination. **Conclusion:** Based on the results obtained in the study it can be concluded that the combination of piracetam and EGb has potentiation of Nootropics effect when compared with individual agents. In future, these agents can be tested in combination in alcohol-induced dementia to obtain a better outcome.

Keywords: Alcohol, dementia, *Ginkgo biloba* extract, piracetam

Introduction

The long-term or excessive alcohol consumption results in neurological damage and memory loss is also termed as alcoholic dementia.[1] Various studies have suggested the prevalence of alcohol-related dementia to be about 10% of all cases of dementia.[2] It is characterized by structural and functional damage in the brain.[3] There are several studies attempted to find out treatment option for alcohol-induced dementia and brain damage. In this paper, we have used a combination of two agents with an established Nootropics profile at low, medium, and high dose and compared with animals received the highest dose of these agents administered independently.

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We have used ethanol administration through the intraperitoneal route as well as in drinking water to induce neural damage and cognitive impairment which is well established and reproducible animal model.[4-6] *Ginkgo biloba* extract (EGb) and piracetam were used as a therapeutic intervention. This has been suggested in several reports about the effectiveness of EGb in several animal models as well as clinical conditions causing dementia such as aging, Alzheimer's disease, peripheral vascular diseases, and neurosensory problems (e.g. tinnitus).[7] There are several mechanisms proposed for the beneficial effects of EGb such as antioxidant effect due to the presence of flavonoids, protective effects on mitochondrial functions, anti-apoptotic, and anti-inflammatory effects. In addition to this, there are clinical studies investigated a combination of EGb and piracetam in combination for management of cognitive functions and working memory. In spite of several reports associated with beneficial effects of EGb, there is no single report combining the protective effects of EGb and piracetam. In the present study, we have tested multiple groups of

a combination of EGb and piracetam including low, intermediate, and high dose and compared with individual agent tested at a higher dose.

Material and Methods

Chemicals

Piracetam (Tablet Nootropil 400 mg, UCB India Pvt. Ltd, Vapi, Gujarat) and dried EGb (Tablet Bilovas, Zydus Healthcare, Sikkim) were purchased from local drug stores. EGb contains 40 mg of the dried extract representing 9.4 mg of Ginkgo flavone glycosides. All the other chemicals used in the study were of analytical grade and procured from SD fine Chemical, Mumbai; HiMedia Laboratories, Mumbai.

Experimental animals

Male Swiss albino mice of age 3 months were obtained from the Departmental Animal House of Vidyabharati College of Pharmacy, Amravati, after Institutional Animal Ethics Committee approval (VBCOP/IAEC/2011-12/1). All the animals were kept under controlled temperature and humidity conditions. Animals were allowed to acclimatize for 3–4 days before the experiment. Food and water were given *ad libitum* to rats throughout the experimental period.

Induction of amnesia and brain damage

Two different types of animal models were used for evaluation of effects of EGb and piracetam against alcohol-induced amnesia and brain damage. For induction of amnesia, 12.6% (v/v) of ethanol solution was prepared from 95% (v/v) ethanol using normal saline whereas 15% (v/v) of ethanol solution was used for inducing brain damage. Ethanol (12.6% [v/v]) administered through intraperitoneal route at the dose of 1 g/kg as single dose 30 min before exploration to the elevated plus maze whereas 15% ethanol was given instead of drinking water for inducing brain damage.

Treatment schedule

All the animals were divided into seven groups as per the experimental design mentioned below ($n = 6$ animal each group). Pre-treatment of EGb and piracetam alone as well as in combination was started daily for 7 days and ethanol (strength 12.6%) 1g/kg dose was administered on past day of treatment through intraperitoneal route at the dose of 1 g/kg as single dose 30 min before exploration to the elevated plus maze. Both the drugs EGb and piracetam were powdered and triturated in normal saline 0.9% and administered through oral route at the dose volume of 10 ml/kg [Table 1: Experimental groups].

Table 1: Experimental design

Group	Treatment
Vehicle control	No treatment
Negative control	Normal saline 0.9% v/v was administered for 7 days
Piracetam 400 p.o.	Piracetam 400 mg/kg, oral route was administered for 7 days
EGb 400 p.o.	EGb 400 mg/kg, oral route was administered for 7 days
EGb+Piracetam (100+100) p.o.	EGb and piracetam both were administered individually at the dose of 100 mg/kg; oral route was administered for 7 days
EGb+Piracetam (200+200) p.o.	EGb and piracetam both were administered individually at the dose of 200 mg/kg; oral route was administered for 7 days
EGb+Piracetam (400+400) p.o.	EGb and piracetam both were administered individually at the dose of 400 mg/kg; oral route was administered for 7 days

EGb: *Ginkgo biloba* extract

Memory paradigm tests

Elevated plus maze method

The elevated plus maze for mice consisted of two open arms (35 × 5 cm^2) and two covered arms (35 × 5 × 15 cm^3) extended from a central platform (5 × 5 cm^2), and the maze was elevated to the height of 40 cm from floor. On the past day of treatment (experiment day 7), each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was defined as the time (in seconds) taken by the animal to move from one open arm into one of the covered arm with all its four legs. TL was recorded on the 1st day (training session) for each animal. The animal was allowed to explore the maze for another 2 min and then returned to its home cage. Retention of this learned task (memory) was examined 24 h after the 1st-day trial. TL was expressed as inflexion ratio.

Plus-maze inhibitory avoidance discrimination task

This protocol measures learning, anxiety, and locomotion. This process allows measurement of learning/memory (by percent time spent in aversive enclosed arms) and anxiety (by percent time spent in open arms). The test apparatus consists of a small chamber connected to a larger dark chamber (27 cm × 30 cm × 21 cm) through a guillotine door. The small chamber (10 cm × 30 cm × 21 cm) was illuminated with a 7W/12V bulb. The test animals were given an acquisition trial followed by a retention trial 24 h later. In the acquisition trial, the animal was placed in the illuminated compartment at a maximal distance from the guillotine door, and the latency to enter the dark compartment was measured. Animals that do not step through the door within a cutoff time, i.e., 90 s are not used. Immediately after the animal enters the dark compartment, the door was shut, and an unavoidable foot-shock (current 1 mA for 1 s) was delivered. The animal was then quickly removed (within 10 s) from the apparatus and put back into its home cage. The test procedure was repeated with or without the drug. The cutoff time on day 2 was 300 s. The drugs were coadministered and individually for seven successive days. Ethanol (1 g/kg i.p.) was administered 30 min before pre-test session in all groups except vehicle control and normal saline given to control animal. Each mouse was individually placed in the bright part of a two-chambered for pre-test session after the mouse enters the second darker chamber. Immediately thereafter the door was closed which prevents the mouse from escaping and a 1 mA, 1 s foot shock delivered through the grid floor. The mouse was then returned to the home cage. After 24 h of pre-test session, the testing was repeated by placing the mouse again in bright. The latency in entering the second darker chamber within 5 min test session is measured. The test drugs were administrated 90 min before training. A prolonged latency indicated that the animal remembered that it has been punished and therefore avoids entering the dark chamber.

Retention of learned task (memory) was examined 24 h after the 1st-day trial. TL was expressed as inflexion ratio using formula mentioned below.

$IR=(L_1-L_o)/L_o$ where L_1 is TL on day 1 and L_0 is TL on day 2.

Measurement of acetylcholinesterase activity

This assay is spectrophotometric method, which involves two linked reaction to produce a colored compound. The production of the compound was monitored by measuring the absorbance of light by the reaction mixture over time. Acetylcholine is hydrolyzed enzymatically to give acetate and thiocholine. Thiocholine reacts with 5,5'-dithiobis-2-nitrobenzoic acid anion (TNB). TNB has absorbance maxima at wavelength of 412 nm. The extinction coefficient of the thionitrobenzoic acid is 1.36×10^4/molar/cm. After assessing the learning and memory paradigm in ethanol-induced amnesia, three mice from each group were euthanized by cervical dislocation carefully to avoid any injuries to the tissue. Then the whole brain was carefully removed from the skull, and the fresh whole brain was weighed and transferred to a glass teflon homogenizer and homogenized in an ice bath after adding 10 volumes of 0.1 M phosphate buffer, pH 8. The reaction mixture consisted of 2.6 ml of phosphate buffer (0.1 M, pH 8.0), 0.4 ml aliquot of homogenate, and 0.1 ml of 0.01M dithiobisnitrobenzoic acid. After the addition of the substrate, 0.02 ml of acetylthiocholine iodide (0.075 M), change in the absorbance was measured every 2 min for 10 min at 412 nm using a spectrophotometer. The activity was expressed as micromoles of substrate hydrolyzed per min per gram of tissue. The enzyme activity is calculated using the following formula:

$$R = 5.74\times10^{-4} A/Co$$

Where,

R - Rate in moles of substrate hydrolyzed minute/g tissue. A - Change in absorbance/min, Co - Original concentration of the tissue (mg/ml).

Histological parameters

The animals were killed after 24 h of completion of the treatment by cervical dislocation. Brain was excised and longitudinally cut into two equal halves and fixed in 10% neutral buffered formalin for 24 h at 4°C. The formalin-fixed halves of brain were washed under running tap water for 24 h, dehydrated through alcohol grades, cleared in xylene, and embedded in paraffin. 5 µ thick sections were cut on the rotary microtome and used for the histochemical demonstration of lipofuscin granules by Zeihl–Neelsen Carbol fuchsin method. Slides were examined under a light microscope.

Statistical analysis

Results are expressed as Mean ± SEM. Graphpad prism software was used for statistical analysis. The significance of difference between multiple groups was evaluated using one-way analysis of variance followed by *post hoc* Bonferroni test. Level of significance was set at $P < 0.05$.

Results

Effect of EGb and piracetam coadministration on inflexion ratio in ethanol-induced amnesia in elevated plus maze

Ethanol decreased inflexion ratio. EGb and piracetam 400 mg/kg independently significantly improved inflexion ratio in comparison with ethanol group (negative control). EGb and piracetam combination at the dose of 100, 200, and 400 mg/kg significantly increased inflexion ratio when compared with ethanol, piracetam, and EGb independent group [Table 2].

Effect of EGb and piracetam on the increase in percentage latency alone and combination in ethanol-induced amnesia on one trial inhibitory avoidance paradigm

Ethanol decreased inflexion ratio. EGb, piracetam 400 mg/kg independently and low dose combination of EGb and piracetam significantly improved inflexion ratio in comparison with ethanol group. EGb and piracetam combination at the dose of 200 and 400 mg/kg significantly increased inflexion ratio when compared with ethanol, piracetam, and EGb independent group [Table 3].

Effect of EGb and piracetam on inhibition of acetylcholinesterase activity alone and in combination in ethanol-induced amnesia

Ethanol significantly increased acetylcholinesterase activity. EGb, piracetam 400 mg/kg independently and low dose combination of EGb and piracetam significantly reduced acetylcholinesterase activity ratio in comparison with ethanol group. EGb and piracetam combination at the dose of 200 and 400 mg/kg significantly reduced acetylcholinesterase activity when compared with ethanol, piracetam, and EGb independent group [Table 4].

Table 2: Effect of EGb and piracetam on inflexion ratio alone and in combination in ethanol-induced amnesia

Group	TL on day 7 in (s)	TL on day 8 in (s)	Inflexion ratio
Vehicle control	130.67±3.52	83.17±10.98	0.79±0.33
Negative control	146.33±4.14	142±5.43	0.03±0.02[b]
Piracetam 400p.o.	135.17±4.90	58±2.77	1.72±0.25**
EGb 400p.o.	147.67±4.43	51±2.76	1.58±0.16**
EGb+Piracetam (100+100) p.o.	120.33±7.65	28±1.37	3.32±0.26***$^{\#\#}$^^
EGb+Piracetam (200+200) p.o.	131.67±8.22	28±1.24	3.78±0.46***$^{\#\#\#}$^^^
EGb+Piracetam (400+400) p.o.	123±6.34	30±0.68	3.14±0.32***$^{\#}$^^

Data were expressed as mean±SEM (n=6); [b]P<0.01 as compared to vehicle control (student t-test); **P<0.01 and ***P<0.001 as compared to negative control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 and $^{\#\#\#}P$<0.001 as compared to piracetam 400 p.o.; ^ ^P<0.01 and ^ ^ ^P<0.001 as compared to EGb 400 p.o. (one-way ANOVA followed by Bonferroni test). ANOVA: Analysis of variance, EGb: *Ginkgo biloba* extract, TL: Transfer latency

Effect of EGb and piracetam on fluid and food intake alone and in combination in ethanol-induced amnesia

Ethanol significantly increased fluid and food intake whereas EGb and piracetam combination at all doses low, medium, and high improved fluid intake in comparison with negative control whereas food intake was not improved in any of the treatment group [Table 5].

Histopathological observation of brain tissue on ethanol-induced brain damage

Hippocampus of chronic alcohol drinking animals showed very high accumulation of lipofuscin granules in comparison with vehicle control animals. Hippocampus of chronic alcohol drinking treated with only piracetam 400 mg/kg orally showed few numbers of accumulations of lipofuscin granules. Similar to piracetam, the hippocampus of chronic alcohol drinking treated with EGb 400 mg/kg orally showed few numbers of accumulations of lipofuscin granules. When EGb and piracetam were administered in combination, we observed very few numbers of lipofuscin granules in comparison with hippocampus of animals administered piracetam and EGb individually [Figure 1].

Table 3: Effect of EGb and piracetam on the increase in percentage latency alone and in combination in ethanol-induced amnesia on one trial inhibitory avoidance paradigm

Group	TL on day 7 in (s)	TL on day 8 in (s)	Increase in latency (%)
Vehicle control	32.33±4.48	37.33±5	13.01±2.49
Negative control	37.83±3.96	40±4.12	4.45±0.66^{b}
Piracetam 400 p.o.	42.17±3.57	59±4.37	28.46±3.03***
EGb 400 p.o.	26±2.76	34.5±3.25	24.79±2.4***
EGb+Piracetam (100+100) p.o.	36.17±2.9	58.67±2.22	38.85±3.04***
EGb+Piracetam (200+200) p.o.	37.33±1.45	73.5±5.8	46.95±5.8***$^{\#\wedge\wedge}$
EGb+Piracetam (400+400) p.o.	35±2.58	71.5±4.6	49.07±6.52***$^{\#\#\wedge\wedge}$

Data were expressed as mean±SEM. (n=6); ^{b}P<0.01 as compared to vehicle control (student t-test); ***P<0.001 as compared to negative control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 as compared to piracetam 400 p.o.; $\wedge\wedge P$<0.01 as compared to EGb 400 p.o. (one-way ANOVA followed by Bonferroni test). ANOVA: Analysis of variance, EGb: *Ginkgo biloba* extract, TL: Transfer latency

Table 4: Effect of EGb and piracetam on inhibition of acetylcholinesterase activity in alone and in combination in ethanol-induced amnesia

Group	Micromole hydrolyzed/min/g of tissue
Vehicle control	1.39±0.20
Negative control	2.88±0.06^{bb}
Piracetam 400p.o.	2.24±0.05**
EGb 400p.o.	2.33±0.04*
EGb+Piracetam (100+100) p.o.	2.33±0.07*
EGb+Piracetam (200+200) p.o.	1.67±0.09***$^{\#\wedge\wedge}$
EGb+Piracetam (400+400) p.o.	1.70±0.09***#$^{\wedge\wedge}$

Data were expressed as mean±SEM. (n=6); ^{bb}P<0.01 as compared to vehicle control (student t-test); *P<0.05, **P<0.01 and ***P<0.001 as compared to negative control; $^{\#}P$<0.05 as compared to piracetam 400 p.o., $\wedge\wedge P$<0.01 as compared to EGb 40 p.o. (one-way ANOVA followed by Bonferroni test). ANOVA: Analysis of variance, EGb: *Ginkgo biloba* extract, TL: Transfer latency

Discussion

In the present study, we elucidated the potentiation of Nootropics effects of piracetam with the addition of EGb in alcohol-induced memory loss and structural damage in mice. In our preliminary study conducted on EGb for determining the maximum tolerated dose using OECD guideline 425, we observed there was no adverse sign observed in mice in 2000 mg/k single dose (data not shown). We have used alcohol 1 g/kg dose (12.6%) intraperitoneal dose for the induction of loss of memory. We observed a decrease in inflexion ratio which corresponds to memory in the ethanol-induced negative control group. Our results are similar to previous studies using the same dosage of alcohol.[8,9] These results showed that ethanol consumption leads to impairment of task acquisition, formation of long-term memory or memory recall.

Pre-treatment of mice with Nootropics agents, i.e., piracetam and EGb alone or in combination in our study reversed damaging effects of alcohol on memory. Furthermore, a combination of piracetam and EGb has much better effects at a lower dose in comparison with higher dose tested individually. There is evidence which suggest a combination of these agent can be used for enhancing the pharmacological effect of each other.[10]

We have also measured the level of brain acetylcholine esterase level. Acetylcholine esterase enzyme is attached to the intercellular matrix of the synaptic cleft, causes rapid hydrolysis of acetylcholine to acetate and choline setting the pace for rapid responses by enabling reuptake and recycling. This synthesis is catalyzed by choline acetyl-transferase, which is a marker of the cholinergic synapse. Hippocampus, the center of learning and memory receives a strong cholinergic input and which has involvement in short-term memory that may account for the loss of memory. We observed a significantly higher level of acetylcholine esterase which corresponds low level of acetylcholine. Low acetylcholine levels produced by alcohol intake caused long- and

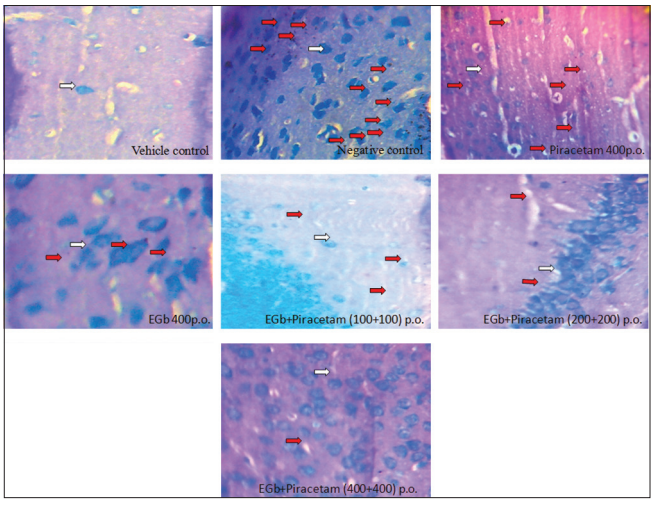

Figure 1: Effect of *Ginkgo biloba* extract and piracetam on histology alone and in combination in ethanol-induced amnesia

Table 5: Effect of EGb and piracetam on fluid and food intake alone and in combination in ethanol-induced amnesia

Group	Fluid intake (ml/day/mice)	Food intake (g/day/mice)
Vehicle control	3.60±0.06	3.73±0.01
Negative control	3.93±0.11^{b}	3.75±0.09^{bb}
Piracetam 400p.o.	3.74±0.01	3.60±0.06
EGb 400p.o.	3.72±0.01	3.70±0.09
EGb+Piracetam (100+100) p.o.	3.60±0.00**	3.73±0.03
EGb+Piracetam (200+200) p.o.	3.65±0.07*	3.65±0.05
EGb+Piracetam (400+400) p.o.	3.61±0.04**	3.59±0.04

Data were expressed as mean±SEM. (n=6); $^{b}P<0.05$, $^{bb}P<0.01$ as compared to vehicle control (student t-test); $^{*}P<0.05$, $^{**}P<0.01$ as compared to negative control (one-way ANOVA followed by Bonferroni test). ANOVA: Analysis of variance, EGb: *Ginkgo biloba* extract, TL: Transfer latency

short-term memory deficits, trouble focusing, concentrating, as well as Stage 4 (REM) sleep deprivation. Pre-treatment of EGb and piracetam significantly reduced acetylcholine esterase level in comparison with alcohol-induced negative control group. Improvement in acetylcholine esterase level by EGb was correlated with previous studies.[11,12]

Combination of EGb and piracetam has much better effects at a moderate and higher dose in comparison with agents tested individually. Combination of EGb and piracetam almost restored the level of acetylcholine esterase level to normal animals which are positively correlated with the protective capability and cognitive enhancing properties of these agents.

We also investigated the effect of alcohol drinking on lipofuscin pigment deposition on hippocampus of mice brain. We observed increased deposition in rat hippocampus in an alcohol-induced negative control group which demonstrates aging like changes in brain. Accumulation of this pigment in nerve cells is one of the most consistent changes observed during aging of the cells.[13] Pre-treatment of EGb and piracetam individually reduced lipofuscin pigment accumulation which represents protective effects of these agents on an intraneuronal membranous system which is similar to previous studies.[14] We also observed very few lipofuscin pigments in the hippocampus of animals treated with a combination of EGb and piracetam.

In summary, we observed potentiation of Nootropics effects of piracetam when it was administered with EGb extract. In the future both of these agents can be tested clinically against alcohol-induced dementia to expect a better outcome than tested individually.

On the past day of treatment all the groups except vehicle control, ethanol (strength 12.6%) 1 g/kg dose was administered through intraperitoneal route at the dose of 1 g/kg as a single dose 30 min before exploration to the memory paradigm tests.

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