RESEARCH ARTICLE

Anti-Alzhimer Activity of Bay Leaves in Scopolamine-induced Rat Model

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ABSTRACT

Indian spices always play a great role in Ayurveda and Indian medicine. So, analyzing these plants for their unknown and specialized activity is great. Nearly all the spices have some activity on the brain and CNS. Bay leaves are one of the most common culinary spices from day to day life of Indians. In this study, we have studied bay leaves for their anti-Alzheimer activity which depends on the inhibition of acetylcholine esterase and butyrylcholinesterase. For this study, firstly bay leaf oil was extracted by hydrodistillation. Further phytoconstituent like alpha-pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol were isolated and purified by using TLC, HPLC, and column chromatography followed by fractional distillation.

These isolated phytoconstituents were evaluated for their acetylcholinesterase and butyrylcholinesterase inhibition activity in scopolamine-induced rats. This study used galanthamine as a reference, and compared the anti-alzheimer activity of all isolated phytoconstituents.

Keywords: Acetylcholinesterase, Anti-alzheimer activity, Butyrylcholinesterase, Herbal, Hydrodistillation, Phytoconstituents, Scopolamine-induced rats.

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INTRODUCTION

It is indigenous to the Mediterranean area, an evergreen, aromatic shrub or tree that may reach heights of 10 to 18 m. It is either reddish blue or olive green in hue. Short lanceolate or lanceolate, oblong, acuminate stalks alternate with evergreen leaves. The leaves are approximately 3T4 cm broad and 5T8 cm long. The leaves are mostly utilized in cuisine, where their essential oils and dried leaves are used as flavoring and soup ingredients. The leaves are evergreen while they are fresh.[1] For many nations, the bay leaf holds significant cultural importance. For instance, it is seen on the Japanese ten yen note, and in ancient Greece, usage of bay leaves to crown the skulls of champion athletes was extremely significant. It was already noted, the bay leaf plays a crucial part in culinary preparations. Although it hasn't been said to be of great significance in conventional medicine, current scientific investigations have looked into its molecular components. Some experts, who practice complementary or alternative medicine, think that changing one's diet can be a fantastic way to avoid disease (CAM).[2] In past, hysteria and emmenagogues were treated with fruit and bay leaves. Bay fruit powder was infused to have diuretic and carminative (prevent or relieve flatulence) effects. Fatty oils from bay fruit were used externally to cure furuncles, sprains, bruising, and rheumatism as well as to repel insects. Several scholarly articles have demonstrated the analgesic, anticonvulsant and anti-inflammatory properties of bay leaf essential oils.[3-5]

We have also isolated and separated these phytoconstituents from plants by using some instrumental and conventional techniques and further this phytoconstituents studied for its acetylcholine esterase and buterylcholine esterase inhibition activity by using scopolamine-induced rats.

MATERIALS AND METHODS

Selection of Plant

The leaves of *L. nobilis* were collected from the medicinal garden of SGMSPM's Dnyanvilas College of Phamacy, Alandi, Pune, India. Plant leaves authenticated by Dr. D. L. Shirodkar, Botanical Survey of India, Koregaon Park, Pune on dated 21/11/2020.

Isolation of Essential Oil from Bay Leaves

Powder of laurel leaves are subjected to hydrodistillation, Clevenger apparatus was used.[6] For 5 minutes, distillation was carried out utilizing dry leaves and a 1:10 water-to-leaf ratio. Anhydrous sodium sulfate was used to dehydrate the

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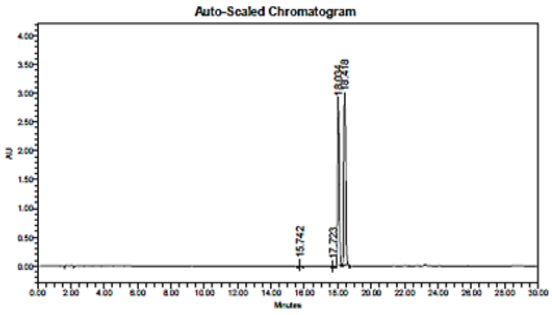

Figure 1: Auto-scaled chromatogram.

Table 1: HPLC Data

	RT	*Area*	*%Area*
1	15.742	108873	0.25
2	17.723	45201	0.10
3	18.034	19556328	45.06
4	18.418	23690769	54.59

essential oil, which was then stored at 4°C. Phytoconstituents of oil isolated from the extracted oil. For this firstly, we have performed TLC and HPLC. In HPLC we have observed different phytoconstituent peaks, which are separable. Then the sample is subjected to column chromatography and Hexane: Ethyl Acetate (93:7) solvent system is used to collect elutions.

These collected elutions contain mixture of phytoconstituents with nearly same Rf values. So we have performed coloum chromatography and preparative HPLC to separate phytoconstituents again. The separated elutions separated from the solvent by performing fractional distillation.

Separation and Purification of Phytoconstituents

Separation of phytoconstituents has been performed by performing TLC followed by HPLC and fractional distillation.

Isolations

HPLC

(Table 1 and Figure 1 showing data).

Structural Elucidation of Phytoconstituent

For structural elucidation, we have performed the following spectroscopic methods (Figure 2).

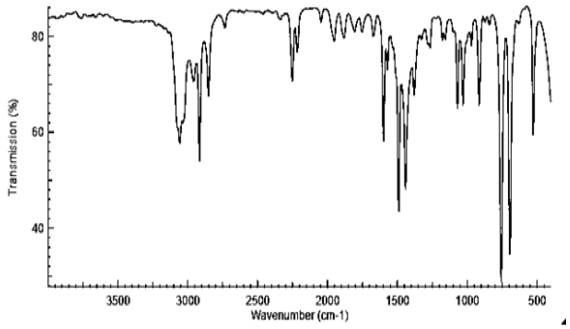

Figure 2: IR Spectra of 3-methoxy-1-methyl-5-(prop-2-en-1-yl) piperidine.

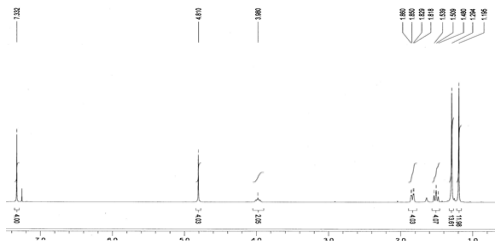

Figure 3: ^{1}H-NMR spectra of alpha-pinene.

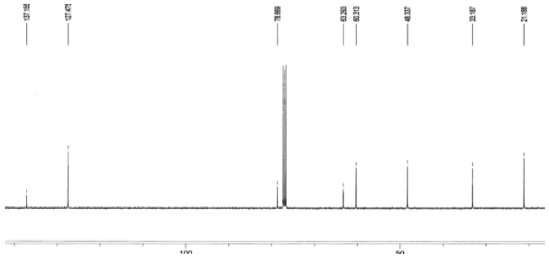

Figure 4: ^{13}C-NMR Spectra of alpha-pinene

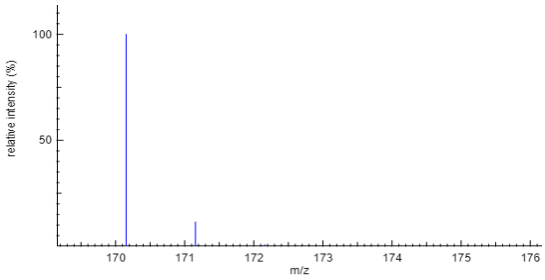

Figure 5: Mass spectra of of alpha-pinene.

IR

Interpretation of IR spectra: 3124.20 cm^{-1} (C-H str. alkene), 2925.48cm^{-1}, 2855.1 cm^{-1}(C-H str alkane), 1574.59 cm^{-1} (C=C bend alkene), 1101.1 cm^{-1} (C-O str.) (Figure 3).

^{1}H-NMR

Interpretation: δ 1.419-1.539 (5H, 1.54 (dt, *J* = 11.3, 3.3 Hz), 1.72 (ttdd, *J* = 10.2, 3.9, 3.3, 2.6 Hz), 1.82 (dd, *J* = 7.6, 3.9 Hz), 1.59 (dt, *J* = 11.3, 10.2 Hz), 2.28-2.51 (6H, 2.35 (dd, *J* = 10.3, 5.1 Hz), 1.82 (dd, *J* = 7.6, 3.9 Hz), 2.35 (s), 2.44 (dd, *J* = 10.3, 4.6 Hz), 2.39 (dd, *J* = 4.6, 2.6 Hz), 3.21-3.41 (4H, 3.26 (s), 4.81 (dd, *J* = 17.0, 2.5 Hz), 4.81 3.34 (tdd, *J* = 10.2, 3.3, 2.6 Hz), 2.63 (1H, dd, *J* = 5.1, 2.6 Hz),4.74-4.89 (2H, (dd, *J* = 10.5, 2.5 Hz) (Figure 4).

^{13}C-NMR

Interpretation: δ 21.188 (1C, s), 33.147 (1C, s), 35.6 (1C, s), 48.7 (1C, s), 53.3 (1C, s), 57.9-57.9 (2C, 60.31 (s), 63.293 (s)), 78.66 (1C, s), 127.47 (1C, s), 137.9 (1C, s).

Mass (Figure 5)

Molcular Ion Peak=169.

BIOLOGICAL ACTIVITY

Animals

Between 100 and 150 g, male Sprague Dawley rats were purchased from the National Institute of Biosciences in

Table 2: Chemical used

Scopolamine	Vital Laboratories
Acetylthiocholine iodide, Butyrylthiocholine iodide, DTNB Reagent	SRL- Chemicals

Table 3: Scopolamine Data

Chemical Names	*(-)-hyoscine, Skopolamin; Oscine*
Source	Hyoscyamusniger, Atropa belladonna (family: Solanaceae)
Formula	C17H21NO4
MW	303.353 g/mol
CAS No.	138-12-5
IUPAC	(1R,2R,4S,5S,7R)-9-methyl-3-oxa-9-azatricyclo [3.3.1.0^{2},^{4}] nonan-7-yl (2S)-3-hydroxy-2-phenylpropanoate
Metabolism	Liver
Half life	4.5 hrs
Excretion	Kidney

Dhangawadi, Pune, 412205, and kept in clean atmosphere with 12:12 hr light or dark cycle at a temperature between 230 and 20°C and 30% relative humidity. The animals were provided food before and after the experiment, and filtered water was supplied. The rats were acclimated for a period of 7 days in typical ambient conditions and temperature as stated after randomization into separate groups and before the experiment began.[6,7]

Chemicals

Fresh medication solutions were made on day of the experiment as pharmacological agents (Table 2).

Scopolamine Model of Alzheimer's

A competitive, nonselective muscarinic receptor blocker, scopolamine. The cholinergic theory of aging and AD is where the scopolamine model got its start. Scopolamine was employed in obstetrics from the turn of the 20th century until the middle of the 1950s to cause a twilight state and amnesia during delivery. In 1974, healthy young volunteers were administered scopolamine, and later, they had memory profiles that were strikingly comparable to those of elderly people. The scopolamine model was used in cognitive research to examine clinical aspects of ACh insufficiency. The anticholinergic scopolamine decreases acetylcholine's ability to operate at a given concentration at the synapses without altering concentration. The efficacy of acetylcholine is decreased by scopolamine because it binds to part of receptor sites on the post-synaptic membrane without causing depolarization. Scopolamine's interaction with muscarinic acetylcholine receptor in CNS was described as a two-step process that involved first attaching the ligand to the receptor and then isomerizing the receptor-ligand complex into a form with a greater affinity. The regional cerebral blood flow that is assessed while doing memory tasks may be impacted by scopolamine's interference with other neurotransmitter systems. Scopolamine, a model drug, has been administered parenterally in dosages ranging from 0.25 to 1 mg in human investigations. Similar to atropine, after intramuscular injection, the maximum plasma concentration (C_{max}) and the time it takes to attain C_{max} (t_{max}) are very variable. Single dosages of scopolamine only have transient effects because of the drug's relatively quick excretion. After 1 to 3 hours, central pharmacodynamic effects reach their peak and last for 5 to 6 hours.[7] Table 3 shows scopolamine data.

Experimental Protocol

Animals were split into four groups of five rats each, and as follows, treatments were administered to each group:

- **Group I (Control):** Distill water (4 mL/kg/ p.o.) + Saline (1 mL/kg, i.p.) after 45 m vehicle administration.
- **Group II (Disease control):** Distilled water (4 mL/kg, p.o.) + scopolamine (3 mg/kg, i.p.) after 45 minutes administration of vehicle.
- **Group III (Standard treatment group):** Galanthamine (1.978 µmol/100 g body weight i.e. 3.921 mg/gm.bw) + Scopolamine (3 mg/kg, i.p.) after 45 minutes of drug administration.
- **Group IV (Drug treatment group):** 3-methoxy-1-methyl-5-(prop-2-en-1-yl)piperidine (5 mg/kg, p.o.) + scopolamine (3 mg/kg, i.p.) after 45 minutes of drug injected.

Following the injection of scopolamine for 30 minutes, the animals underwent behavioral assessments. Rats were slaughtered that day, and the brain tissues were separated to assess biochemical estimates.[7,8]

Behavioral Parameters

Before administering medication, all animals underwent a two-day training period.

Tests of Learning and Memory Functions

These were studied with an elevated plus maze, Y- and radial arm maze.

Elevated Plus Maze

Procedure

Raised plus maze testing of memory and learning has a long history.[1] Device projects from a central platform with two open arms (50–10 cm) and two closed arms (50-10-40 cm) (10–10 cm). The labyrinth is raised 40 centimeters above the surface of earth. Two steps were taken to complete experiment. Each rat was positioned at end of an open arm on day 1 distant from center. The major parameter for transfer latency was time to enter any one of closed arms (TL). The four legs that can all fit within the closed arm are each considered a separate entrance. A cutoff time of 180 seconds was provided to every rat. Animals who failed to enter the closing arms in the specified period were taken out. Animals that did not enter closed arms within allotted time were removed from the experiment. 24 hours following the first trial (day 2), retention testing was undertaken, and transfer latency was collected in the same way as previously. Reduced transfer latency was seen as a measure of memory enhancement (Figure 6).[9]

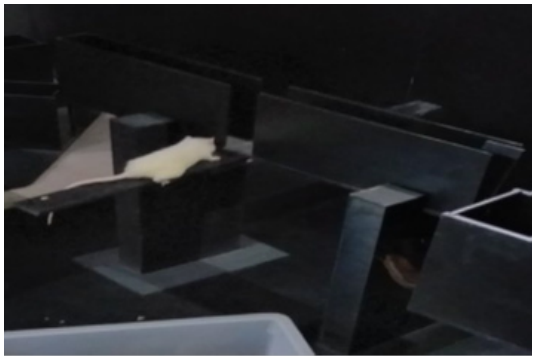

Figure 6: Elevated plus maze.

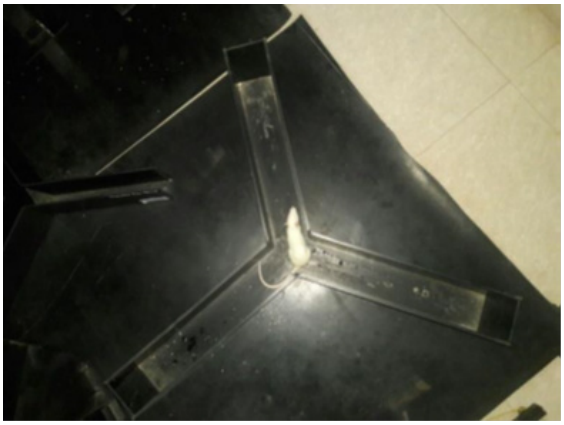

Figure 7: Y-maze.

Spontaneous Change Behavior y-maze Test

Procedure

A three-armed labyrinth with equal angles between each arm, Y-maze. It had walls that measured 5 cm wide, 30 cm in length, and 12 cm high. The walls and floor of maze were made of dark grey polyvinyl resin. The initial step was to insert mice into one arm. During course of eight minutes, time and number of arm admissions for each mouse were manually tallied. To gauge short-term memory, researchers looked at proportion of triads in which mice entered all three arms, i.e. ABC, CAB, or BCA but not ABB. Mice received CGA (0, 3, 6, or 9 mg/kg, p.o.) one hour prior to each test. After 30 minutes, scopolamine (0.5 mg/kg, i.p.) was given to reduce memory. Instead of CGA, control group got distilled water. Between trials, Y-maze arms were cleaned with 10% diluted ethanol to get rid of smells and contaminants. The method below, which multiplies the difference between actual and possible alternations (defined as total number of arm entries less two) by 100, was used to determine the alternation score (%) for each mouse:

$$\%\text{Alternation} = [(\text{Number of alternations})/(\text{Total arm entries}-2)] \times 100$$

A measure of locomotor activity was number of arm entries each trial (Figure 7).[10]

Radial Arm Maze

Procedure

Device is an elevated 8-arm radial labyrinth made of wood that extends from a central platform that is 26 cm diameter. Individual arm 5 cm broad, 56 cm long and has rails 2 cm high all the way down its length. There are various indications and the maze is well-lit. At the tips of the arms are food pellets that serve as rewards. Rats are fed once daily during the test, and to encourage rats to explore mazes, their body weights are maintained 85% of their able eating weight. Every day, animals are instructed to gather food pellets in maze. The rat must earn the most prizes with the fewest blunders before the session, which features eight possibilities, ends. All arms are often rewarded while studying working memory, and the animal is instructed to visit each arm once. The initial entry into a baited arm was the definition of a valid answer. Only a few of the arms provide rewards when employed to test reference memory, and the animal should only visit those arms that have been baited. Two different mistakes were noted: First entry to an unbelted arm was treated as a reference memory mistake; subsequent trips to a baited arm were seen as working memory errors (Figure 8).[10]

Biochemical Estimations

Collection of Brain Sample

Following behavioral testing, neck dislocation was used to kill the animals. The entire brain was painstakingly taken out of the skull and weighed. The brain was then homogenized in an ice-chilled phosphate buffer to create a 10% w/v brain homogenate (pH 8, 0.1 M). The homogenate was then spun in a chilled centrifuge for ten minutes at a speed of 3000 rpm to get the supernatant, which was then utilized to calculate the biochemical values.

Reagent: After DTNB 0.01 M, 15 mg of $NaCo_3$ was administered. 39.6 mg were mixed in 10 mL of phosphate buffer at pH of 7.0. (0.1 M). The reagent was made in pH 7 buffer because it was more stable there than it was in pH 8 buffer.

Assay Procedure

Ellman *et al.* developed a method to measure acetylcholinesterase in brain (1961). In test tube, 0.4 mL of brain homogenate and 2.6 mL of phosphate buffer were combined (0.1 M, pH 8). The aforementioned mixture was then given additions of 100 l DTNB reagent and 20 l acetylthiocholine iodide solution. The change in absorbance per minute was estimated after five minutes of monitoring absorbance at 412 nm. For such run, blank is made up of buffer, substrate, and DTNB solutions. The following equation was used to determine how many moles of substrate were hydrolyzed each min per gm of tissue:[7]

$$R = 5.74 \times 10 - 4\ (\Delta A/C0)$$

Where;

C0= Original conc. of tissue (mg/mL)

R= Rate, in moles of substrate hydrolyzed per min per gram of tissue.

ΔA= Change in absorbance/min.

Reagent: Following addition of 0.01 M dithiobisnitrobenzoic acid, 15 mg of sodium bicarbonate 39.6 mg were added in 10 mL of phosphate buffer at pH of 7.0. (0.1 M). The reagent was

Figure 8: Radial arm maze.

prepared in a buffer with a pH of 7, where it was more stable than in a buffer with a pH of 8.

Assay Procedure

Ellman *et al.* developed a method to measure acetylcholinesterase in brain (1961). In test tube, 0.4 mL of brain homogenate and 2.6 mL of phosphate buffer were combined (0.1 M, pH 8). The aforementioned combination received 100 l of DTNB reagent, then 20 L of butyrylthiocholine iodide solution was mixed. Change in absorbance per minute was then determined after recording absorbance at 412 nm for 5 minutes. For such a run, the blank is made up of buffer, substrate, and DTNB solutions. The following equation was used to determine how many moles of substrate were hydrolyzed each min/gm of tissue:[7]

$$R = 5.74 \times 10\text{-}4\ (\Delta A/C0)$$

Where;

ΔA= Change in absorbance per min.

R= Rate, in moles of substrate hydrolyzed per minutes per gram of tissue.

C0= Original concentration of tissue (mg/mL)

STATISTICAL ANALYSIS OF DATA

Every piece of information is displayed as the mean SEM of measurements taken on 6 animals from the individual group. One-way analysis of variance and Dunnett's multiple test were used in analysis, which was carried out with the aid of Graph Pad Instat (version 3) and p values < 0.05 were regarded statistically significant when compared to the pertinent control in Graph Pad Prism 7.

RESULTS AND DISCUSSION

Antialzhimer Activity on Rat (Biological Activity)

Behavioral Parameters

Elevated Plus Maze

Transfer delay was substantially longer in scopolamine-induced dementia in the elevated plus maze ($p < 0.01$) associated to the normal control group. Galanthamine pretreatment resulted in shorter transfer delay ($p < 0.01$) equated to scopolamine control. In comparison to scopolamine control group, simultaneous administration with alpha-pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol significantly reduced transfer delay (p 0.05).

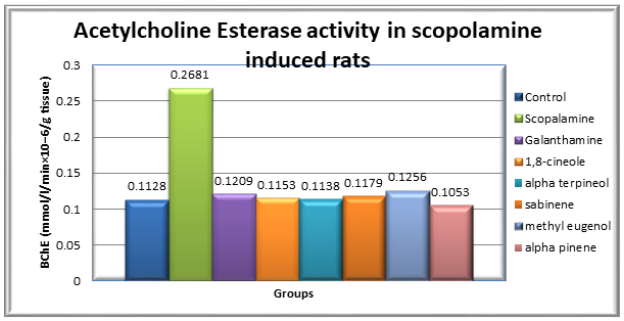

Figure 9: Effect of Galanthamine and phytoconstituents on AChE activity in scopolamine induced rats.

Spontaneous Alteration Y-Maze Test

As compared to the healthy control group, scopolamine dramatically lowered SAP% (p 0.05). As compared to the scopolamine control group, pretreatment with galantamine significantly increased SAP% (p 0.05). In compared to the scopolamine control group, the combined pretreatment with alpha-pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol significantly increased SAP% value (p 0.05).

Radial Arm Maze

Scopolamine significantly enhanced the number of working memory mistakes and reference memory errors when compared to the healthy control group (p 0.05 and p 0.01, respectively). When compared to the scopolamine control group, pretreatment with galanthamine significantly reduced working and reference memory error (p 0.05). Additionally, compared to the scopolamine control group, amount of reduced working and reference memory error was considerably decreased ($p < 0.05$). When alpha-pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol and scopolamine were given to rats simultaneously.

Biochemical Estimations

Acetylcholinesterase Activity

In comparison to the healthy control group, scopolamine treatment significantly enhanced brain AchE activity ($p < 0.01$). Galanthamine pretreatment significantly reduced AchE activity when equated to the scopolamine control group ($p < 0.05$). Additionally, when alpha-pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol was pretreated simultaneously with scopolamine, the level of AchE activity significantly decreased ($p < 0.05$) in compare to scopolamine control group (Table 4 and Figure 9).

Butyrylcholinesterase Activity

In comparison to healthy control group, scopolamine treatment substantially elevated brain BuchE activity ($p< 0.01$). In association to scopolamine control group, pretreatment with galanthamine resulted in substantial reduction in BuchE activity ($p<0.05$). Additionally, when galantamine and alpha-

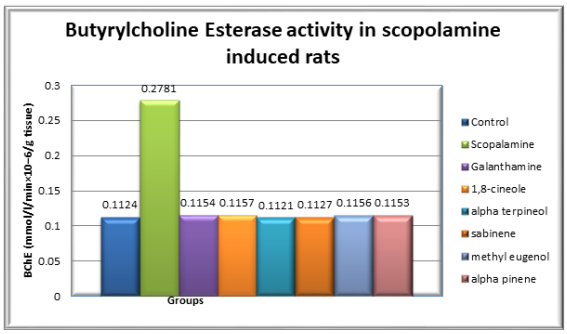

Figure 10: Effect of Galanthamine and phytoconstituents on BuChE activity in scopolamine-induced rats.

Table 4: Effect of Galanthamine and AChE activity in scopolamine-induced rats.

Group and Dose (mg/kg oral)	*AChE (mmol/l/min×10−6/g tissue) Mean ± SEM*
Control	0.1128 ± 0.0044
Scopolamine	0.5781 ± 0.1225
Galanthamine	0.1154 ± 0.0034
1,8-cineole	0.1153 ± 0.0143
alpha terpineol	0.1138 ± 0.0028
sabinene	0.1179 ± 0.0126
methyl eugenol	0.1256 ± 0.0132
alpha pinene	0.1182 ± 0.0045

Information is presented as mean S.E.M. of 6 animals. Non-parametric One-way and Dunn's multiple comparisons test were used for analysis; results showed that $p < 0.01$ vs. control and $p < 0.05$ vs. scopolamine.

pinene, terpineol, 1,8-cineol, sabinene, and methyl eugenol were administered together as a pretreatment to scopolamine-induced rats, the level of BuchE activity was significantly decreased ($p < 0.05$) compared to scopolamine control group. (Table 5 and Figure 10).

CONCLUSION

In this study all phytoconstituents are investigated first time for its anti-Alzheimer's activity.

Among all these compounds, alpha-pinene shows best activities while 1,8-cineole and alpha terpineol show better activity, and other phytoconstituents like sabinene and methyl eugenol also show good inhibition activity.

Table 5: Effect of Galanthamine and phytoconstituents on BuChE activity in scopolamine induced rats

Group and Dose (mg/kg oral)	*BuChE (mmol/L/min×10-6/g tissue) Mean ± SEM*
Control	0.1124 ± 0.0038
Scopolamine	0.2781 ± 0.0416
Galanthamine	0.1154 ± 0.0034
1,8-cineole	0.1157 ± 0.0063
alpha terpineol	0.1121 ± 0.0013
Sabinene	0.1127± 0.0034
methyl eugenol	0.1156± 0.0039
alpha pinene	0.1153± 0.0049

Information is presented as mean S.E.M. of 6 animals. Non-parametric One-way and Dunn's multiple comparisons test were used for analysis; results showed that $p < 0.01$ vs. control and $p < 0.05$ vs scopolamine.

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