Section A-Research paper

IN VITRO AND IN VIVO ANTI-ALZHEIMER'S ACTIVITIES OF ETHANOLIC LEAF EXTRACT OF *Persea americana* MILL. ON SCOPOLAMINE INDUCED DEMENTIA IN RAT MODELS

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ArticleHistory: Received: 15.05.23 Revised: 09.06.23 Accepted: 16.06.23

Abstract

Background: *Persea Americana* (family Lauraceae) is universally known as "Avocado" and has precious applications in traditional medicines of South Americans countries and to treat numerous disease. The *P. americana (Persea americana)* were heretofore reported that used for childhood convulsions, hypoglycemia, epilepsy, dysentery, diarrhea, and also acts as antihypertensive, anti-inflammatory, analgesic, antioxidant, anti-tussive and antimicrobial agents. *P. Americana* has been numerous compounds like phenolics (polyphenols), tannins, saponins, terpenoids, glycosides and amino acids. Yet, no report exists on the anti-Alzheimer properties of *P. Americana*. Objective: The objective of the research for evaluation of *in vitro* and *in vivo* anti-Alzheimer's activities of *P. Americana* leaf extract on scopolamine induced Alzheimer dementia in rats. Methods: Total phenolic content were estimated from *P. Americana* extract by Folin-Ciocalteu reagent. The neuroprotective effect of *P. Americana* leaf extract against Scopolamine induced Alzheimer dementia were evaluated. Alzheimer dementia was induced by 1 mg/kg of Scopolamine through intraperitoneally. Ethanolic leaf extract of *P. Americana* was subjected on rats in low dose (200 mg/ kg, p.o.) as well as high dose (400 mg/kg, p.o.) for investigation of learning and memory. Furthermore, enzymes inhibition activity assay *in vitro* in order to assess their anticholinesterase properties of *P. Americana*. Results: *P. Americana* leaves were found to encompass of Gallic acid equal of total phenolics in extract. *P. Americana* leaf extract (200 & 400 mg/kg, p.o.) produce expressively enhancement in learning and memory of rats and also significantly decreased acetylcholinestrase level in brain by dose dependent manner when compared with negative control group. Conclusion: The present pharmacological investigation indicated that *P. americana* leaf extract improve the Alzheimer's symptoms through the control of acetyl cholinesterase in brain, due to presence of phenolic compounds in *P. americana* leaf and exhibited adequate anti-Alzheimer's activity. This concluded that *P. Americana* leaves has profound of the safe neuroprotective, memory accompaniment mediator and can be used for initial management of Alzheimer's dementia.

Keywords: Acetylecholinesterase, Alzheimer dementia, Persea americana, Polyphenol.

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Introduction

Alzheimer's disease is an irreversible, chronic, neurodegenerative disorder with a multifaceted etiology and pathogenesis in the aging people, the common indication is dementia. It is described by liberal and perceptive destruction of neurons, leading to uncharacteristic changes of attitude, absent-mindedness, destabilize, mental decay and finally leads to death.[1-3] The World Health Organization has conveyed in 2010 totally 35.6 million people are troubled with Alzheimer's disease and it is projected to virtually twofold on every 20 years in worldwide. Alzheimer's disease is predominantly in developing countries like India, China and Latin America.[4] Alzheimer's disease is bringing about by disproportionate accumulation of β –amyloid plaques in the extra cellular, loss of neuron and intraneuronal fibrillary tangles in definite areas of the brain. Additionally, brain aging associated to excessive loss of neuron, decreasing of acetylcholine level, raise of neuronal inflammation and oxidative stress.[5] Acetylcholine (ACh) is significant neurotransmitter in recognition functions such

as memory, and it has acquired from particular neurons by choline Acetyl transferase enzyme from choline and Acetyl CoA. Since hydrolysis of Acetylcholine into choline and acetate by the enzyme acetyl cholinesterase (AChE). This progression, it encourages dementia by loss of acetylcholine in brain. When decreasing synthesis of ACh level in the brain, this lead to Alzheimer's dementia that appears to be critical status results in dementia.[6] Barely few drugs are available for management of Alzheimer's disease in the markets. Those are entitled as acetylcholinesterase inhibitor which increases the obtain ability of acetylcholine at cholinergic synapses. The term of the AChE inhibitors is Physostigmine, Galantamine, Tacrine, Rivastigmine and Donepezil. Memantine is a NMDA receptor antagonist, for managing of moderate to severe Alzheimer's dementia.

Nonselective AChE inhibitors have limited efficacy, deplorable pharmacokinetic properties, peripheral cholinergic adverse effects, least therapeutic index and hepatotoxicity. For that intention it is beneficial to exploration the advantage of folklore medications for the treatment of certain intellectual illnesses.[7-8]

Persea Americana (avocado) is a tree being classified among the laurel family of Lauraceae. It is genus to middle and South America and likewise it is cultivated in the United States of America, part of European countries, tropical Africa and Asia.[9] Generally, *P. Americana* plant exhibit multifarious utilization. It is commonly used in traditional medicines as diarrhea, dysentery, toothache, childhood convulsions and pharmacotherapeutic agent against epilepsy, hyperglycemia. Moreover acts as antihypertensive, vasorelaxant, anti-inflammatory, antiviral, analgesic, hepatoprotective, antioxidant, anti-tussive, antiulcer, chondroprotective, antibacterial and wound healing. According to the research reveals that *P. americana* consist of multitudinous phytoconstituents like phenolics (polyphenols), tannins, saponins, terpenoids and glycosides.[10-11] Vitamin A, E, B complex and vitamin C has accounted in *P. americana*.[12-13] This present study, we assess the pharmacological activity of *P. americana* leaf extract against scopolamine induced Alzheimer's dementia in rat described the mechanism of action liable for their anti-Alzheimer's activity through the inhibition of acetyl cholinesterase.

Materials and Methods

Materials

Scopolamine was procured from Zydus Cadila Pharmaceuticals, Ahmedabad, India. Donepezil was obtained from Torrent pharmaceuticals limited, Ahmedabad, India. Normal saline were purchased from Baxter healthcare, Bangalore, India. Acetyl thiocholine iodide, electric eel acetyl cholinesterase and 5, 5-di thio-bis 2-nitrobenzioc acid was acquired from Sigma chemicals, sigma Aldrich, Bangalore, India.

P. Americana leaves were collected in the month of May 2016 from Valparai (Anamalai hills), Coimbatore, Tamil Nadu, India. The leaves were authenticated by the botanical scientist, C-I/C, TNAU, Coimbatore, India. The voucher number BSI/SRC/5/23/2016/Tec.173and has been deposited at department herbarium.

Preparation of plant extract

The leaves were dried (shade drying) for three weeks then grinded into coarse powder in mechanical mixer and the powder extracted in 95% Ethanol by Soxhlation method (continuous extraction) and reserved under the reflux for 5 days. The extract was concentrated by Rotary vacuum evaporator at 50-55 °C. *P. Americana* ethanolic crude extract (10.1% w/v of yield) was stored under 4°C.

Phytochemical Analysis

The preliminary phytochemical test investigation was carried out on the *P. Americana* extract to evaluation of presence of numerous phytocontituents such as glycosides, terpenoids, phenolics, saponins, tannins, steroids, alkaloids and flavonoids.[14]

Total phenolic content estimation

Quantitative analysis of phenolic content of *P. Americana* extract was estimated by microplate assay technique using FC (Folin Ciocalteu) reagent. The various quantities (50, 100, 150, 200 & 250 mg/ml) of gallic acid in ethanol were used for preparation of standard curve. The standard solutions (1 ml) of gallic acid, 5 ml FC reagent (1:10 ratio diluted in distilled H_2O) was added also 4 ml (7.5%) of $NaCO_3$ (Sodium carbonate) were added after 8 min.

These solutions were undergone for spectrophotometrically absorbance (765 nm) after incubated for 2 hours at room temperature.1 ml of *P. Americana* ethanolic extract (1 g in 10 ml) were added to 5 ml of FC reagent for the test solution. 4 ml (7.5%) of $NaCO_3$ (Sodium carbonate) were added after 8 min and incubated for 2 hours at room temperature then measured absorbance at 765 nm. The extract was estimated in triplicate then a calibration standard curve for obtaining of

gallic acid through five data points. The conclusion were correlated to calibration curve of gallic acid and the total phenolic content of *P. americana* ethanolic extract was show per gram of extract equivalents to milligram of gallic acid.[15]

Animal

Both sex of Wistar albino rats (270 - 300 g) were obtained from Nandha College of Pharmacy and Research Institute, Tamil Nadu, India. The rats were grouped into five, each group contains six rats and the rats were marked for identification. The rats were kept under conducive environmental conditions at 40 ± 10 ^{o}C (relative humidity) and 12 hours of dark-light cycle throughout the experiment. During acclimatization period, the rats were feed along with standard rat pellet diet as well as fresh drinking water. The entire study was conceded by CPCSEA and IAEC guidelines (Proposal No: NCP/IAEC/No: 10/2014 -15,688/02/C-CPCSEA).

Induction of Alzheimer dementia by Scopolamine

The rats were divided into 5 groups of 6 rats each. On the 7th day of experimental study, Scopolamine (1 mg/kg, i.p) were used as inducing agent for Alzheimer dementia. Normal control rats (Group I) were received (1 ml/kg) normal saline (0.9 %) orally. Negative control (Group II) rats were received 1 mg/kg Scopolamine through intraperitoneally and normal saline (1 ml/kg) orally. Positive control rats (Group III) were received 1 mg/kg, i.p of Scopolamine and after 30 minutes Donepezil (5 mg/kg) were given orally. Test group rats (Group IV) received 1 mg/kg, i.p of Scopolamine and after 30 minutes *P. Americana* extract (200 mg/kg,) were administered orally. Test group rats (Group V) Scopolamine (1 mg/kg, i.p) and after 30 minutes *P. Americana* (400 mg/kg) orally administered.

Morris water maze test

The *in vivo* screening method was similar that earlier described in 1984 by Morris. The water maze apparatus consist of a circular tank, diameter about 120 cm and 45 cm height. The invisible platform (height in 35 cm and diameter in 15 cm) was placed on below (1.5 cm) the water surface. The temperature was well-maintained at 23 °C. The pool were placed in experimental room and several indicators outer to the maze was visible from the pool (lamps) which can be used for the spatial orientation of the rats. The indicators were placed at constant position for during the task.

Training trials

The trails were undergone before administering Scopolamine and other treated drugs. The rats were received two trials each day for seven consecutive days.

On the starting of a training trial, rats was placed randomly in one of 4 fixed point facing to the wall (East, West, North & South) then were allowed to swimming for 90 seconds, or until rats avoidance the task by find out the platform.

At constant position of the platform was placed during the test session in center of one-fourth, equidistant from the mid and end of the pool. The hidden platform was noted for latency to escape in every training period. If they find out the platform, the rats were allowed to continue there for 20 seconds and then back to the home cage. On the seventh day were excluded, if the rat that cannot reach within twenty seconds to the platform.

Test trials

On the seventh day, immediately after the 14th training trial, Scopolamine (1 mg/kg) injected intraperitoneally; thirty minutes later, *P. Americana* (200 & 400 mg/kg, p.o.) and Donepezile (5 mg/kg, p.o.) were administered for trained rats. 1 hour later the administration of *P. americana*, and Donepezile, rats were permitted to swim then recorded time spent and reach to the platform.[16] After assessing of learning and memory interpretation on scopolamine induce dementia, all the group of rats undergone euthanized by cervical decapitation and then entire brain was rapidly removed and placed in phosphate buffer (ice cold) for washing. The brain was homogenized in five ml of phosphate buffer on the transparent Teflon homogenizer to evaluation for enzymatic activity.

Acetyl cholinesterase enzyme assay by *Invitro* study

The modified technique described in 1961 by Ellman was used. *Electrophorus electricus* (Electric eel) acetylcholinesterase were used, while ATCI (Acetyl thiocholine iodide) act as a substrate for the reaction. AChE activity measured by (DTNB) (5, 5-dithiobis 2-nitrobenzioc) acid. *P. Americana* extract (10µl) diluted in 150 µl of sodium phosphate (0.1 M) buffer at pH 8.0, and then the enzyme solution (20 µl) were mixed and incubated at 25 °C for

15 minutes. 10 mM of DTNB (10 µl) was added and then the reaction was induced through addition of 10 µl of ATCI (14 mM) substrate solution. The coloured substance anion (5- thio-2-nitrobenzoate) was formed by thiocholine and DTNB reaction, which is formed by enzyme hydrolysis. This process could be used for measurement of ATCI hydrolysis.
After 10 minutes, the measurement of coloured substance was measured at 410 nm. Donepezil was used as AChE inhibitor for positive control and then that was dissolved in CH_3OH. The following formula was used for calculate the Acetylcholinesterase inhibition.

Calculation

R = 5.74 x 10-4 x A/CO
R= Rate in moles of substrate hydrolysed / mint/g tissue
A = Absorbance change / min
CO = Original concentration of the tissue (mg / ml). [17- 18]

Statistical analysis

The results were representing the means ± SEM calculated by Graphpad prism v 5. All the data were obtained and evaluated using one- way analysis of variance (ANOVA) followed by multiple comparison (Dunnett' s post- hoc) test and with SPSS, *P*-values ≤ 0.05 were expressed as statistically significant.

Results

Determination of total phenolic content of *P. Americana* extract

The total phenolic content was determined by spectroscopic method. Total content of phenolic in *P. Americana* ethanolic leaf extract was carried out by using Folin Ciocalteau reagent. The linear equation were used for estimation of total phenolic contents and calculated based on the standardization curve of gallic acid; Y= 0.0017X+ 0.362, R2 = 0.9995, where Y indicates is the absorbance and X indicates the amount of gallic acid in µg.

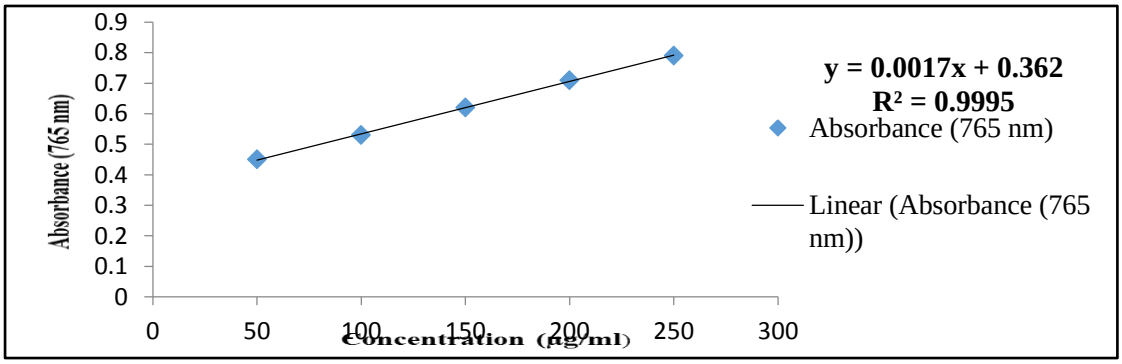

Figure 1: Standard curve of gallic acid at 765 nm.

Effect of *P. Americana* on Moris water maze test

The effect of *P. Americana* extract on Moris water maze model that indicates time latency by scopolamine induced Alzheimer dementia in rats. After the administration of scopolamine by intraperitoneally , rats showed loss of spatial memory, that compared to the group II (negative control), in that exist no changes in the latency of finding hidden platform, which indicates time latency significantly ($p<0.001$) increased in group II (negative control) as compared to the group I (normal control). In group IV and V were treated with *P. Americana* (200 & 400 mg/kg) and rats in group III were received Donepezil (5 mg/kg) orally, after administration of scopolamine (1 mg/kg) by intraperitoneally, which indicates significantly ($P \leq 0.01$) decrease of time latency, that are compared with negative control (group II), this result observed the reduction of Alzheimer dementia.

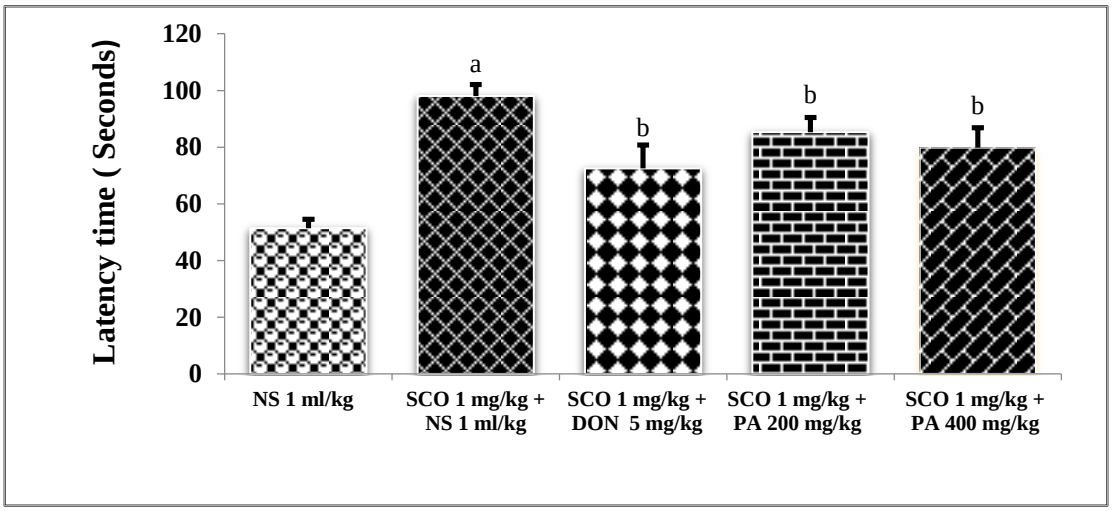

Figure 2: Effect of *P. Americana* on Moris water maze test

The mean ± SEM (n=6/ group). $P \leq 0.001^a$ when compared with vehicle treated group. $P \leq 0.01^b$ compared with negative control group.

Effect of *P. Americana* extract on *invitro* acetylcholinesterase activity

From this result group II shows very significantly ($P \leq 0.001$) escalation in AChE activity is compared to group I. The rats (group III) were treated with Donepezil 5 mg/kg shows significantly ($P \leq 0.01$) reduction in AChE activity as compared to group II, where as a significant ($P \leq 0.01$) reduction in AChE action is also perceived in the groups IV and V treating with *P. americana* extract (200 & 400 mg/kg) as compared with group II ($P \leq 0.001$). The result shows *P. Americana* extract has good neuroprotective effects.

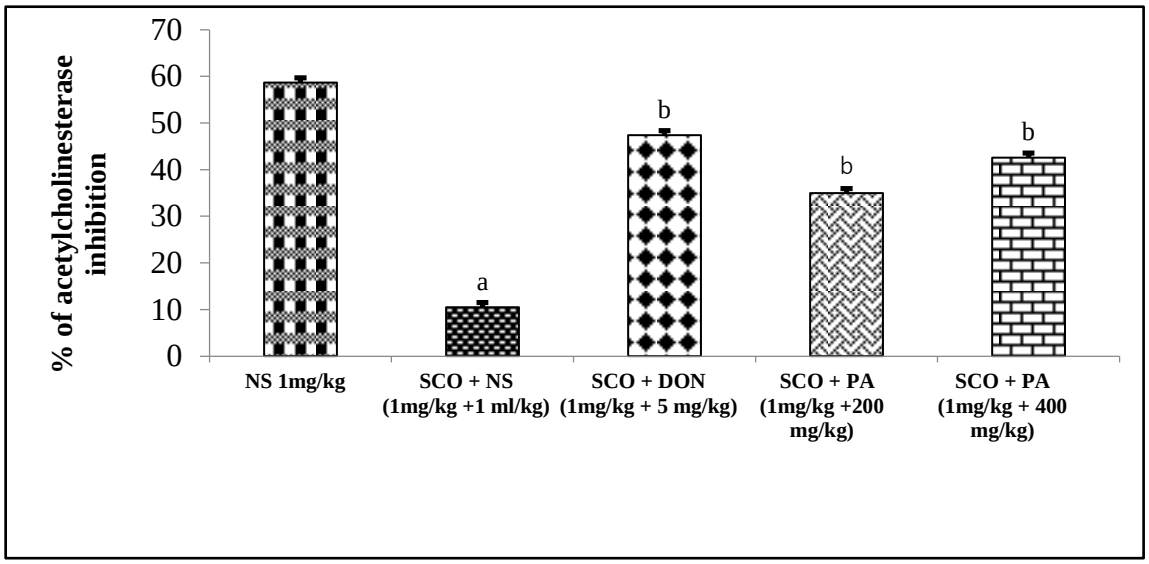

Figure 3: Effect of *P. Americana* extract on *invitro* acetylcholinesterase activity

Values are mean ± SEM (n=6), $P \leq 0.001^a$ when compared with control. $P \leq 0.01^b$ when compared with negative control.

Discussion

Alzheimer's disease is developing community healthcare with demoralizing impacts. These

results dispute to the essentially new therapeutic potential of selected plants with activity against acetylcholinesterase (AChE) associated with Alzheimer's dementia. *P. Americana* extract concomitant in the present investigation with both a neuroprotective effect and inhibition in acetylcholinesterase enzyme. In this anti-Alzheimer's studies, we used Scopolamine to induce Alzheimer's dementia. Scopolamine blocks the muscarnic receptor; this leads to in the modulation of memories which result to reduction the responsiveness and learning aptitudes. Scopolamine induced memory loss assessment is extensively used as primary screening investigation for anti-Alzheimer's activities.[19]

Acetylcholine is considered as an utmost vital neurotransmitter in ruling of intellectual task. Cholinergic neuron acting significant characters in cognitive insufficiency associated with Alzheimer's disease. Throughout neurotransmission, Acetylcholine is released after the presynaptic neuron into the synaptic cleft and bind to cholinergic receptors on the postsynaptic membrane and communicating the signals from the neuron. Acetylcholinesterase enzyme respectively found in post-synaptic membrane and terminates the signals communication by hydrolyses of Acetylcholine. The choline is occupied up over by the pre-synaptic neuron and Acetylcholine is produced by merging with Acetyl coenzyme - A over by choline acetyltransferase. A cholinomimetic agent interrupts this progression by substitute as a parasympathetic neurotransmitter, which is resistant to acetylcholinesterase's lysing action.[21]

The anti-Alzheimer's activities of ethanolic leaves extract of *P. Americana* was assessed in rats with two models such as Moris water maze test and *in vitro* acetylcholinesterase activity. The Moris water maze test to support as an *in vivo* model to investigate learning and memory and latency time in rat. As scopolamine improved the latency time in Moris water maze test as compared to respective normal control rats, The rats were treated with *P. americana* extract (200 and 400 mg/kg) significantly decreased in scopolamine induced Alzheimer's dementia as compared to the negative control rats in dose-dependent manner and shows the reduction on the latency time and escalate the memory enhancement. This clearly specified that *P. Americana* extract expressively improved the learning memory performance. Acetylcholinesterase activity was inhibited by *P. Americana* extract (200 and 400 mg/kg) on dose depended manner shows promising effect as Acetylcholinesterase inhibitor.

The results of phytochemical analysis confirmed the existence of various category of secondary metabolite in *P. Americana* leaf extract including phenols and tannins (polyphenols). The active elements like as polyphenol is present in *P. Americana* leaves may responsible for anticholinesterase action. It was earlier found that the expression of polyphenols were accountable for the anti-Alzheimer's activities. Epidemiological reviews have confirmed a link between the depletion of polyphenols rich foods or infusions and the prevention of neurological illnesses such as Alzheimer's disease. Polyphenols have capability to reach the intestinal wall of mammals and easily to cross the BBB (Blood brain barrier) and produce their biological tasks. Many research reveals that, *in vitro* and *in vivo* pharmacological investigation have shown the capability of certain plant extracts and its biophenols (polyphenol) to inhibit Acetylcholinesterase, maybe formation of strong hydrogen bonds and multiple hydrophobic interactions with AChE.[22] These results support the indication of anti-Alzheimer activity of *P. americana* extract concluded its characters as cholinesterase inhibitor.

Conclusion

Subsequently the above, it was established that the leaf extract of *P. Americana* on Alzheimer's dementia ideal could be credited to its properties similar on anticholinesterase. As a result, *P. Americana* affords a durable biochemical balanced to stay an effective and safe neuroprotective, memory accompaniment mediator and can be used for inhibition or initial management of Alzheimer's dementia. However, anticholinesterase inhibitors have been recognized to be the greatest in effect for the pharmacological management of Alzheimer's dementia, right now. These interpretations point out that, the obtainable biodiversity of natural sources and the isolated bioactive phytoconstituents can act as prospective leads for the advance of clinically useful phytopharmaceuticals.

Conflict of Interest: No conflicts of interest for author's declaration.

ABBRIVATIONS: NS: Normal saline, SCO: Scopolamine, DON: Donepezil, PA: *Persea Americana*

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