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Evaluation of Antiamnesic and Cholinesterase inhibitory effects of *Illicium verum* hook.f (Star anise) against Scopolamine induced memory impairment in Mice

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ABSTRACT

Objectives: The current study is carried out on the methanol extract of *Illicium verum* hook.f (MEIV) to evaluate and examine anti-amnesic effects of *Illicium verum* hook.f by using the radial arm maze model.

Study design and setting: This experimental observational study was performed in the Department of Pharmacology, Hamdard university from 20th November 2017 to 20th May 2018. after ethical approval from Board of Advanced studies and Research under agenda item NO.5.1 held on October 20, 2016.

Methodology: Total N=30 healthy albino mice were treated with graded doses of methanol extract of *Illicium verum* hook.f (MEIV) (300 and 500mg/kg p.o.) to verify its effectiveness regarding scopolamine induce memory impairment. Afterward, in-vitro studies were performed on isolated parts of brain mice by using an F2000 fluorescent spectrophotometer (Hitachi) to analyze the acetylcholinesterase level.

Results: Investigation reveals that methanol extracts of *Illicium verum* hook.f (MEIV) by oral administration reduces the incidence of working and reference memory errors additionally this investigation was proved that active avoidance responses in scopolamine-treated group decreased. The acetylcholine esterase level was significantly reduced at the dose of 300 mg/kg (102.05±1.93µmol/min/mg) as compared to the positive control group (161.33±2.347 µmol/min/mg).

Conclusion: Based on behavioral and biochemical investigations, we concluded that MEIV produces anti-amnesic effects by the inhibition of acetylcholinesterase, which might be due to the presence of various essential phytochemicals such as alkaloids, flavonoids, triterpenoid, polyphenols. Our finding reveals that *Illicium verum is* among the screened plants to be assessed further as an herbal substitute for the treatment of Alzheimer's disease.

Keywords: Antiamnesic effect, Working memory, Reference memory, *Illicium verum* hook. f, Alzheimer's disease.

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INTRODUCTION:

Memory is the complex process of the neurocognitive system including the accomplishment of surrounding knowledge also strengthening of the obtained information and then saving it for further prospects.[1,2] Dementia is loss of memory and mental infirmity severe enough to disturb daily life activities which usually occurs in older age.[3] Neurogenerative disorders such as Alzheimer's disease (AD) and amnesia occurred as a result of discrepancies in the cholinergic nervous system.[4] Acetylcholinesterase (AChE) is an enzyme responsible to terminate cholinergic neurotransmission by hydrolysis of Acetylcholine (ACh) into acetate and choline in the synaptic cleft.[5] Neurological and psychological disorders are treated by several herbal medicines since a long time ago[6] Many preclinical studies had proposed several agents from the natural origin which increased cholinergic activity by inhibiting the cholinesterase enzyme to reduce oxidative stress and generate positive effects on learning, concentration, and memory Investigation reveals that *Illicium verum* hook. f having the property to inhibit the cholinesterase enzyme.[7]

Illicium verum hook.f (*Illiciaceae*) usually recognized as star anise, a spice consumed traditionally in Chinese medicine for thousands of years.[8] It is used as a flavoring agent in cuisine, additionally numerous therapeutic and pharmacological applications along with pharmaceutical and cosmetics employment.[9] This spice contains many major phytoconstituents including anethole (85%--90%), trans-anethole responsible for cholinesterase inhibiting activity.[10] Additional constituents including astragalin, cinnamaldehyde, citronellol, caffeic acid, kaempferol, quercetin and their derivatives p-coumaric acid, neurotropic sesquiterpenoids,

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veranisatins A, B and C, phenylpropanoids, Limone, safrol, lignans, and several others were reported as neuroprotective, cholinergic activating, and as an antioxidant agent.[11-13] Star anise has been numerous remedial and therapeutic uses such as colic, spasmodic pain, and flatulence in China and Japan. Additionally consumed as a flavoring agent for cuisine furthermore a chief part of cosmetics and pharmaceutical preparations. Keeping this view in mind that star anise has an action on the central nervous system the current investigation is designed to evaluate the anti-amnesic activity of this spice.

METHODOLOGY:

This experimental observational study was carried out in the department of pharmacology, Hamdard university from Aug 2019 to March 2020 after ethical approval by institutional review Board of Advanced Studies and Research (BASR) of Hamdard university with reference no:HU/DRA/2018/977.

The study was performed on thirty 30 healthy albino mice of either sex weighing between 25-30 grams of age (6-8 weeks). The apparent physical condition of these animals was monitored during this particular period. The laboratory environment was checked for a week, before administration of the drug. Specific changes were observed in animals like aggressive behavior, loss of hair, loss of activity, hematuria, and diarrhea, etc. Animals were housed in separate cages under standard conditions, humidity (55-60%), and temperature (23 ± 2°C) and circadian cycle of 12 hours light/dark was maintained. Mice were fed with a standard diet and water regularly. The entire experimental procedures, including the handling of animals, were operated according to the National Institute of Health guidelines and research protocol approved by the ICCBS Animal Care and Use Committee.

Fruits of Star Anise (*Illicium verum* Hook. f) were purchased from the general store in Karachi. Later on recognizing and authenticated by Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan. The voucher specimen number (IV-01-17) was issued and deposited in the Pharmacognosy Department, University of Karachi, Pakistan.

For the prepration of plant extract the dried fruits of Star anise (*Illicium verum* hook.f) in the form of raw material were washed, air-dried, crushed and the extract was prepared via the addition of 100 ml methanol in approximately 100 grams of star anise dried sample in the coarse powder form having a particle size <0.890mm. Soak it for 24 h at room temperature with occasional shaking. The saturated material was then clarified and filtrated by Whatman filter paper (150mm) and the filtrate was accumulated separately. Afterward, methanol extract was evaporated under reduced pressure in a rotary evaporator at 40 °C.[14] The final extract yield (17.5% w/w) in the dried powder form was kept at -20°C until practice.

All chemicals and drugs were utilized of analytical standard, including (Scopolamine hydrobromide, APP Pharmaceuticals, LLC, Piractim, Global Pharmaceutical, Pakistan), which was used as a standard. Ellman's reagent, Acetylcholine chloride, 5,5-dithio-bis2-nitrobenzoic acid, acetyl thiocholine iodide, trichloroacetic acid, thiobarbituric acid (TBA) purchase from Martin Dow marker specialties (Pvt) Ltd Pakistan.

According to the OECD 423 guidelines, an acute toxicity test was performed.[15] The methanol extract of *Illicium verum* (MEIV) at different doses of (100-2000mg/kg body weight) was administered orally to the normal healthy mice. After administration of the drug animals were observed for the first 4 hours. Primarily symptoms such as convulsion, tremors, salivation, diarrhea, weight loss, hematuria, loss of activity, lethargy were noticed. Similarly, as per guideline animals were observed for a further 14 days for any changes in behavior pattern and mortality. Investigation reveals that there was no toxicity up to the dose of 2000mg/kg of methanol extract of *Illicium verum* hook.f.

The several consecutive methanol extracts of *Illicium verum* hook. f. (Star anise) was directed to a preliminary phytochemical screening by employing various qualitative assays for confirming the presence of phytoconstituents. The presence of these phytoconstituents (alkaloids, carbohydrates, phenols, flavonoids, and glycosides) in *Illicium verum* was verified by thin-layer chromatography (TLC).

The animal was distributed into five groups, each group containing six animals. The resulting groups were the following.

Group 1: Vehicle control; only purified water is given to mice.

Group 2: Positive control; only vehicle was given to mice against scopolamine (1mg/kg, i.p.) induced amnesia.

Group 3: Standard drug (piracetam 200mg/kg i.p) was given to mice against scopolamine induced amnesia.

Group 4: Test drug 1 (MEIV 300 mg/kg, p.o) treated mice against scopolamine-induced amnesia.

Group 5: Test drug 2 (MEIV 500 mg/kg, p.o) treated mice against scopolamine-induced amnesia.

Active avoidance test is used to assess the associative learning about the animal. A significant increase in the active avoidance test is the principal determinant for improved cognitive activity.[16] A radial arm maze model was modified to assess the memory function of animals. The radial arm maze consists of eight (8) arms extended to an octagonal-shaped and 30 cm diameter of a central hub. The platform of the radial maze is 40 cm above the floor. At the end of each arm contains a small black metal cup which is 3cm in diameter and 2 cm deep that serves as a receptacle for reinforces food.

Memory was assessed on pre-selected animals in one day

exercising track. An experimental trial was started by placing a food pellet on one receptacle. All night fasted rats were retained in the central hub of arm maze and permitted them to select the arm freely to pick up the food. A trail was completed vigilantly when mice visited all eight arms. Each entry into the arm was noted as the correct response, in which mice were not entered previously; however, re-entries were recorded as an "Error". A "successful" trial (animals made no or only one error out of eight choices) was recorded. 60 minutes after administration of the last dose at eight days, animals of corresponding groups were exposed to drug scopolamine (1mg/kg i.p) for the induction of amnesia. Subsequently, after 30 minutes each animal one by one were placed in the central hub and tested for another successful trial. Afterward trained rats were chosen for analysis. Animals were dosed once a day for 8 days with their respective drugs. On the 8th-day scopolamine 1, mg/kg was given 45 minutes before the treatment and after one hour all animals were tested on the behavior model (radial arm maze). Each successful trial was recorded by a single investigator.[16,17]

After 24 hours of concluding behavioral test, animals were euthanized by cervical displacement with care and the whole brain were removed. Afterward, the brain were homogenized with ice-cold phosphate buffer having pH 8. The homogenates (10% w/v) were then centrifuged at 10,000 rpm in a centrifuge machine (Biofuge) for 15 min and the supernatant liquid was used for the biochemical estimations.

Acetylcholinesterase (AChE) (enzyme considers as a marker for loss of cholinergic neurons in the forebrain) activity was measured by Ellman method.[18] The assay mixture consists of supernatant (0.4 ml), phosphate buffer of pH 8 (2.4ml), 20 ul of acetylcholine iodide and 100ul of DTNB (5,5-dithionitrobenzoic acid) (Ellamn reagent). The biochemical reaction of the choline with dithiobinitobenzote ion was producing a yellow color end product. The optical density of the yellow color compound was measured by an F2000 fluorescent spectrophotometer (Hitachi) at 412nm for 10 min at 2 min intervals. AChE activity was specified in 1mol/min/mg of protein.

SPSS version 23.0. was used for data analysis. Statistical analysis was performed by applying one- way ANOVA follow by Bonferroni's test. All value was presented as mean ± Standard error mean (SEM) and P value <0.05 was considered as a significant statistically.

RESULTS:

The mice treated with MEIV dose from 100-2000mg/kg, p.o., showed normal behavior. All animals were alert with normal touch and pain response. There was no sign of apathy and inactiveness was noticed at all doses. However, slight convulsions were observed at higher doses. The other effects like loss of hair, aggressive behavior, and diarrhea, blood in urine, tremors, salivation and lethargy were not observed. The motor activity of all animals was normal and exhibits no sign and symptoms of depression. Attentiveness, muscle tone and grip strength of limbs along with the walking pattern of the animal were typical. The toxicity study reveals that the extract was safe up to the doses of 2000 mg/kg in mice.

Numbers of avoidance were found to be significant (P<0.05) reduce in the group treated with scoplamine as compared with control group (Table 1). Conversely piracetam, which is a standard drug showed significant results. Similarly, test drug MIEV at the dose of (300 and 500 mg/kg, p.o.) appeared to indicate the protective effects against memory impairment by scopolamine induction thru inhibiting the less number of avoidances. However, MEIV at the dose of 500mg) 10.16 ± 0.41s showed more noticeable results contrary the dose of 300 mg/kg by the cumulative increase in the avoidance reactions 8.69 ± 0.33s as compared to positive control group 6.13 ± 0.45s. Working memory errors were significantly (P<0.05) raised in animals treated with scoploamine as compared to control group. While animals group pre-treated with (300 and 500 mg/kg, p.o.) with MEIV and (200mg/kg, i.p.) treated by standard drug (piracetam) showing a significant reduction of working memory errors when contrasted with the positive control group 19.7 ± 0.11s as presented in (Table-2). Lower dose of MEIV i.e. is 300mg dose has revealed more clear and noticeable outcomes 12. 7 ± 0.15s to reduce the errors of working memory. The incidence of reference memory errors was less appearing in the group receiving the standard drug (piracetam) and the animal group treated with MEIV (300 and 500 mg/kg, p.o.). Each group is comparatively observed in the positive control group as displayed in (Table-3). *Illicium verum* improving memory by inhibiting reference memory errors. Low dose (300 mg/kg p.o.) of MEIV revealed more noticeable results 9.1 ± 1.19s in decreasing the incidence of reference memory errors as compared positive control group 19.6 ± 1.30s and standard drug group 9.4 ± 1.55s. Brain actylcholine-estrase level was decreased at the dose of 500mg of MEIV (P<0.05) as shown in (Table-4) highly significant result 102.05±1.93 and 300mg of MEIV show significant 122.98±2.15 result as compared to the positive control group. However positive control group that (scopolamine treated) raised the level acetylcholinestrase 161.33±2.347 in brain as compared to control group treated mice.

DISCUSSION:

Alzheimer's disease (AD) is an irreversible neurodegenerative disease with the manifestation of many neuropsychiatric and cognitive impairment that leads to progressive disability and incapacitation in old age.[4] The neurotransmitter of the cholinergic system plays a major role in the regulation of cognitive function. According to the researcher's loss of cholinergic neurons in the area of the brain, the cortex reduces the synthesis of acetylcholine neurotransmitters, which is the main featuring of AD.[19] This process can be inhibited by inactivating acetylcholinesterase (AChE), an

Figure 1: Radial arm maze Model for Sample recording

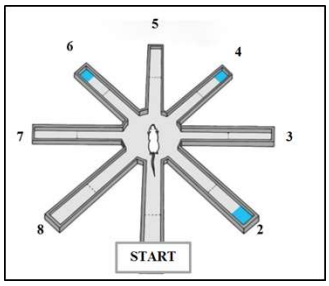

Table 1: Effects of *Illicium verum* hook. f. (Star anise) on active avoidance response against scopolamine induce amnesia

Groups (n = 6)	Treatment	Response of Active avoidance (s)
1	Control (Vehicle)	13.10 ± 0.22
2	Positive (Scoplamine 1mg/kg, i.p)	6.13 ± 0.45*
3	Standard (Piracetum 200mg/kg,i.p)	12.16 ± 0.13#
4	Test 1 (MEIV 300 mg/kg, p.o)	8.69 ± 0.33#
5	Test 2 (MEIV 500mg/kg, p.o)	10.16 ± 0.41#

N = 6, All values are presented as mean ± S.E.M.
P < 0.05 considered as statistically significant.*P < 0.05 compared with the normal control group.
#P < 0.05 compared with the scopolamine treated group. Statistical analysis was done by using one way ANOVA followed by Boneferroni test.

Table 2: Effects of *Illicium verum* hook. f on working memory against scopolamine induced impairment

Groups (n = 6)	Treatment	Working memory error (s)
1	Control (Vehicle)	7.9± 0.13
2	Positive (Scoplamine 1mg/kg, i.p)	19.7 ± 0.11*
3	Standard (Piracetum 200mg/kg,i.p)	13.0 ± 0.23#
4	Test 1 (MEIV 300 mg/kg, p.o)	14.3 ± 0.31#
5	Test 2 (MEIV 500mg/kg, p.o)	12.7 ± 0.15#

N = 6, All values were presented as the mean ± S.E.M.
P < 0.05 considered as statistically significant.*P < 0.05 compared with the normal control group.#P < 0.05 compared with scopolamine.Statistical analysis was done by one way ANOVA followed by Boneferroni test.

Table 3: Effects of Methanolic extract of *Illicium verum* hook. f on impairment of reference memory

Groups (n = 6)	Treatment	Reference memory error (s)
1	Control (Vehicle10 ml/kg))	7.3 ± 1.21
2	Positive (Scoplamine 1mg/kg.ip)	19.6 ± 1.30*
3	Standard (Piracetum 200mg/kg.i.p)	9.4 ± 1.55#
4	Test 1 (MEIV 300mg/kg.p.o)	9.1 ± 1.19#
5	Test 2 (MEIV 500mg/kg.p.o)	13.3 ± 1.40#

Abberivation: MEIV (methanol extract of *Illicium verum*), p.o per oral,i.p intraperitonial
N = 6, All values were presented as the mean ± S.E.M. P < 0.05 was considered a significant.*P <0.05 compared with the normal control group.# P< compared with the scopolamine treated group. Statistical analysis was performed by applying one- way ANOVA follow by Boneferroni test.

Table 4: Effects of Methanol Extract of *Illicium verum* on acetylcholinestrase level in scopolamine

Groups (n = 6)	Treatment	AchE(µmol/ min/mg protein
1	Control (Vehicle)	85.04±1.760
2	Positive control (Scopolamine1mg/ kg,i.p)	161.33±2.347*
3	Standard Drug (Piracetum 200mg /kg, p.o)+(Scopolamine 1mg/kg,i.p)	97.55±1.36#
4	Test drug 1 (MEIV 300mg/kg, p.o) + Scopolamine	122.98±2.15#
5	Test drug 2 (MEIV 500mg/kg, p.o) + Scopolamine	102.05±1.93#

enzyme responsible to cleave acetylcholine and terminates neuronal signaling. Centrally acting cholinergic drug Scopolamine causing cognitive impairment.[20] Therefore such drug treatment is required which reduces cognitive impairment by increasing cholinergic neurotransmission in AD patients. Cognitive deterioration associated with age progression as a result of oxygen-free radical develops Alzheimer's disease in older people.[21] It has been proved from previous studies that *Illicium verum* possesses antioxidant activities as well. Previous investigations revealed that antioxidant properties of *Illicium verum* produce neuroprotective effects by decreasing oxidative stress in brain cells as a result of which reduction in brain damage along with improvement of neuronal function.[22] Dementia symptoms develop due to impaired neurotransmission and deterioration of neuronal circuits in the brain areas. In the brain of AD patient progressive loss of cholinergic neurons occur leading to the reduction of acetylcholinesterase level and cognitive deterioration.[23]

Previous studies reported that anethole is the major constituent abundantly present in *Illicium verum* responsible for enhancing memory and cognition by inhibiting the cholinesterase enzyme. Previous studies reported that some other active constituents of this plant such as flavonoids, quercetin, and Kaempherol also generating CNS effects.[24,25]

Previous pharmacological studies with *Illicium verum* showed that this spice possessed antioxidant and anxiolytic properties. In the current study, we examined that pre-treatment with MEIV at the dose of (300 and 500mg/kg) decreases enzyme acetylcholinesterase level and improved cognitive memory in mice on the behavioral model on a radial arm maze model which is based on appetite motivated task.

CONCLUSION:

Based on a current investigation we concluded that *Illicium verum* possesses antiamnesic effects which might be may the existence of major phytoconstituents such as flavonoids, sesquiterpenes, and polyphenol having the ability to inhibits acetylcholinesterase.

Authors Contribution:
Hafiza Tuseef Sayyar: Perceived the idea, conducted research, write-up of manuscript data analysis.
Muhammad Liaquat Raza: Supervised the wholeproject, critically examined the manuscript.
Syeda Rida Baqir: Bibliography and help in data analysis

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