

# In Vitro Anti-cholinesterase and Cognitive Enhancing Properties of Essential Oils from *Piper nigrum* L. and *Monodora myristica* (Gaertn) Dunal

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## Abstract

Aging in humans generally is associated with deterioration of cognitive abilities, particularly of learning and memory leading to dementia, including Alzheimer's disease. A number of herbal medicines are reported to improve brain function and intelligence. In the present study, the ameliorating effects of essential oil extracted from *Piper nigrum* and *Monodora myristica* on learning and memory in Scopalamine induced amnesic mice were determined using two cognitive behavioural paradigms: the Step-Through Passive Avoidance and Morris-Water Maze test. Essential oils were obtained by hydro-distillation using a Clavenger-type apparatus and their profiles analyzed by GC-MS. Inhibitory effects on AChE and BuChE were investigated by Ellman's method. The animals were assessed for performance by measuring the Step-Through Latency Time (SLT) and Escape Latency Time (ELT). Brain cholinesterase activities were assayed in brain tissues from the mice. The results showed that *M. myristica* and *P. nigrum* oils were characterized by 51 (94.76%) and 61 (90.65%) components, respectively. *M. myristica* is dominated by  $\alpha$ -phellandrene (18.13%), while *P. nigrum* is dominated by  $\beta$ -pinene (5.92%) and caryophyllene (4.55%). Both oils at 416  $\mu$ g/ml elicited significant inhibitory ( $p > 0.05$ ) activity with *M. myristica* exhibiting a stronger inhibition against AChE and BuChE than *P. nigrum*. This trend was also exhibited significantly ( $p > 0.05$ ) in both ELT and the SLT when compared to scopolamine-treated group. In conclusion, the seeds of *M. myristica* and *P. nigrum* are potential sources of active metabolites with anti-cholinesterase and cognition enhancing properties, but *M. myristica* exhibited a higher activity.

**Keywords:** Anti-Cholinesterase; Essential Oil; *Piper Nigrum*; *Monodora Myristica*; and GC-MS

## 1. Introduction

Alzheimer's disease (AD) is an age-associated neurodegenerative disorder characterized by progressive loss of memory and cognition (Figueiro et al., 2011), and by the presence of extracellular (Rauk, 2009) and intracellular (Knobloch et al., 2007; La Ferla et al., 2007) plaques of  $\beta$ -amyloid peptide ( $A\beta$ ), with intracellular tangles of hyper-phosphorylated tau protein (Ballatore et al., 2007). AD leads to loss of forebrain cholinergic neurons and a pronounced fall in acetylcholine levels (Auld et al., 2002), resulting in devastating cognitive impairment (Pimplikar, 2009). In fact, in animal models and humans alike, the association between plaques of  $A\beta$  densities and the degree of cognitive deficiency is not always a clear-cut (Caughey and Lansbury, 2003).

$A\beta$  is a toxic pro-inflammatory agent that causes neuro-inflammation in the brain (Tuppo and Arias, 2005) which are mediated by activation of microglial cells contributing to neurodegenerative processes such as it occurs in AD (Dheen et al., 2007; Kashaniet al., 2011). Inflammatory mediators (Strohmeier and Rogers, 2001), defect in cholinergic transmission (Lahiriet al., 2002) and glutamate-induced neurotoxicity (Bourin et al., 2003) have been demonstrated to be involved in AD. The clinical symptoms of AD is mainly memory impairment and loss of spatial memory (Burin et al., 2003; Keshena et al., 2011).

Memory function is likely to be formed by numerous discrete neural networks that can be disrupted by the pathophysiological processes in AD (Celone et al., 2006). The hippocampus, a well-known brain region involved in memory consolidation, is especially susceptible to AD. Early degenerative symptoms including significant deficiency in the performance of hippocampal-dependent cognitive abilities such as spatial learning and memory have been reported (Yamin, 2009).

Scopolamine, a muscarinic cholinergic receptor antagonist, serves as a useful pharmacological tool in producing a model of partial amnesia (Bartus, 2000; Sun et al., 2007; Wang et al., 2010). Muscarinic and nicotinic acetylcholine receptors mediate the action of acetylcholine (ACh). Clinical investigation of AD patients does not indicate changes in number and function of muscarinic acetylcholine receptors. However, a significant decrease in nicotinic acetylcholine receptors density has been reported for the cerebral cortex and hippocampus, two major areas that are involved in learning and memory processes (Hefco et al., 2003).

Plant essential oils have been used to improve health and well-being of humans which is an age-long practice. Aromatherapy, non-pharmacological therapy, is currently used worldwide in the management of chronic pain, depression, anxiety as well as cognitive, sleep and stress-related disorders (Savelev et al., 2004; Anekonda and Reddy, 2005; Hritcu et al., 2012; Natarajan et al., 2013). In a recent study, Hritcu et al. (2012) reported that dementia patients of the AD type showed significant improvement in cognitive performance following aromatherapy consisting of rosemary and lemon essential

oils. Numerous studies have shown that essential oils from a variety of plant species such as *Pinus* spp (Ustun et al., 2012), *Geidiella acerosa* (Syad et al., 2012), *Hedychium gardnerianum* (Arruda et al., 2012) do possess both anticholinesterase and antioxidant properties. These properties have been demonstrated to be as a result of synergistic properties of constituents in the essential oils which were mainly monoterpenes and sesquiterpenes (Arruda et al., 2012).

In the southern part of Nigeria and perhaps in the southern part of the West African sub-region, the use of aromatic plants as spices for culinary purposes is quite popular. *Piper nigrum* (black pepper) and *Monodora myristica* (calabash nutmeg) are common spices used for the preparation of the traditional cuisine known as “pepper soup” which is often recommended, owing ostensibly for its health promoting benefits, for elderly persons, nursing mothers shortly after delivery and persons suffering from discomfort associated with cold-weather induced conditions. These benefits include: longevity, anti-aging, anti-fatigue, cognitive enhancement, anti-diabetic properties. However, this claim has not yet been subjected to rigorous experimental and clinical investigation for the purpose of validation. Thus, there is the need to carry out experimental studies with a view to give scientific backing and possibly elucidate the biochemical mechanism of action of the phyto-constituents in the seed essential oils. Hence, the aim of this study is to determine the chemical composition as well as evaluate the anti-cholinesterase and possible cognitive enhancing properties of the essential oils obtained from the seeds of *P. nigrum* and *M. myristica*.

## 2. Materials and methods

### 2.1. Materials

Electric eel acetylcholinesterase (AChE), horse serum butyrylcholinesterase (BuChE), acetylthiocholine iodide (ATChI), butyrylthiocholine iodide (BTChCl) and 5, 5'-dithio-bis-nitrobenzoic acid (DTNB), physostigmine hemisulfate (Eserine) and scopolamine hydrobromide were purchased from Sigma Chemical Company, St Louis, Missouri, USA. All other chemicals and solvents used, except otherwise stated, were of analytical grade.

The seeds of *P. nigrum* and *M. myristica* were purchased from the local market at Ile-Ife, Osun State, Nigeria and authenticated at the Herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife.

### 2.2. Experimental animals

Adult inbred albino mice of both sexes weighing between 20-33 g were obtained from the Animal House, College of Health Sciences, OAU, Ile-Ife. The animals were kept under ambient conditions in metal cages and acclimatized to laboratory conditions for two weeks before the commencement of the experiment. The animals were randomly selected into ten groups (five groups for the Passive Avoidance Test and five for the Morris Water-Maze Test) of five animals per group. Food was given in form of dry pellet (AJB Animal Feeds, Ile-Ife) and water was provided ad libitum.

### 2.3. Methods

Fifty grams of the dried seeds were powdered and mixed with 500 ml of doubly distilled water and the oil isolated by hydro-distillation using a Clevenger-type apparatus over 5 h. The obtained oil was separated from the water and dried over anhydrous sodium sulphate, with nitrogen gas bubbled through it. The oil was then stored in a freezer prior to further analysis. This process was repeated four times.

### 2.4. Gas chromatography and mass spectrometry (GC-MS) analysis

The analysis of the essential oil obtained was performed using Agilent GC-7890A series Gas Chromatograph equipped with a flame ionization detection (FID) and DB-1 fused silica capillary column (30 x 0.25 mm i.d, film thickness 0.25  $\mu$ m). The initial oven temperature was held at 80°C for 5 min, and increased at the rate of 15°C/min to 250°C. Helium was used as the carrier gas at a flow rate of 1 ml/min, and the sample size was 0.1  $\mu$ l, split ratio, 50:1. The percentage composition of the essential oil was determined with a Class-GC computer programme and the relative percentages of the oil constituents were expressed as percentages by peak area normalization. For the GC-MS detection a tandem Mass Spectrophotometer (Model 5975C VLMSD), using the injector (Model 7683B Agilent Technology) with an electron ionization system with ionization energy of 70 eV was used. The samples were diluted at a ratio 1:100, v/v in dichloromethane and 1.0  $\mu$ l were injected manually in the split-less mode. Volatile components were identified based on GC retention indexes calculated by using n-hydrocarbons and mass spectra by computerized matching of essential oil compounds with the National Institute of Standards and Technology (NIST), Timberland Regional Library (TRLIB) and Wiley libraries as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature.

### 2.5. In vitro anti-cholinesterase assays

Anti-cholinesterase, AChE and BuChE inhibiting activities of the essential oils were determined by using a modified method of Ellman et al. (1961) as described by Obuotor (2004). The oil extract was prepared in a stock solution of 2% Tween-20 in a buffer and was used for the cholinesterase inhibition assay. In a 96-well plate was added 240  $\mu$ l of buffer (50 mM Tris-HCl, pH 8.0), and 20  $\mu$ l of varying concentrations of the test samples (10, 5, 2.5 and 1.25 mg/ml) dissolved in 2% Tween-20, 20  $\mu$ l of the enzyme preparation (2.55 x 10<sup>-3</sup> units/ $\mu$ l), the reaction mixture was then incubated for 30 min at 37°C, after which 20  $\mu$ l of 10 mM DTNB was added. The reaction was initiated by the addition of 20  $\mu$ l of 25 mM ATChI or BTChCl. The rate of hydrolysis of ATChI or BTChCl was then determined spectrophotometrically by measuring the change in the absorbance per minute ( $\Delta A$ /min) due to the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm over a period of 4 min at 30 s interval. A solution of 2% Tween-20 was used as negative control and Eserine prepared in buffer was used as the positive control. All assays were carried out in triplicate.

The percentage inhibition (I%) of test sample was obtained using the formula:

$$I\% = [(V_0 - V_1) / V_0] \times 100$$

Where:

I% = percentage inhibition; V<sub>1</sub> = enzyme activity in the presence of test sample; V<sub>0</sub> = enzyme activity in the absence of test sample

### 2.6. Administration of test compounds and drug

The potentials of the essentials oils to enhance learning and memory in vivo in animal models using the mice was tested using the Passive Avoidance Test and the Morris Water-Maze Test as described by Sennuga (2007). For these tests, 0.2 ml of different concentration (50, 100 and 200 mg/kg bwt, o.p.) of oil extract (prepared as a solution of 2% Tween-20 was administered orally post training for 7 days in both the Morris Water-Maze and Passive Avoidance Test experiment. On the 7<sup>th</sup> day, amnesia was induced by Scopolamine hydrobromide (0.4 mg/kg bwt, i.p.) in these experiments (Das et al., 2002). Piracetam (10 mg/kg bwt, i.p.) was used as standard drug. The animals were subjected to acquisition test after the treatments.

### 2.7. Passive shock avoidance paradigm

Passive avoidance behavioral test based on negative reinforcement was used to examine the long-time memory (Dhingra et al., 2004). The apparatus consisted of acrylic box (24 cm x 32 cm x 18 cm)

with a copper grid floor and a wooden platform fixed in the centre of the box. Electric shocks (26 V) were delivered to the grid floor for 5 s with an isolated pulse stimulator. At the beginning of the training, mice were placed in the light chamber to habituate for 3 min. When the animal steps through from the light to dark chamber, electric foot shocks (26 V, 0.2 amp for 5 s, electric pulse simulator) were delivered to the grid floor.

The step-through latency time (SLT) from the light to dark compartment were monitored and recorded for 3 min as an index for learning, acquisition or memory retention for each of the animal in all the groups. The criteria/index for amnesic/cognitive activity was taken as an increase in SLT on the retention test in comparison with the acquisition test. After the training and administration of test sample (oil/piracetam), an acquisition test trial was carried out to verify the acquisition skills of the mice based on the training measured out to them, thereafter, a retrograde amnesia was induced by administration of Scopolamine (0.4 mg/kg i.p.) 5 min after this test in all extract treated group, Piracetam group as well as Scopolamine only-treated group except the control which was administered with the normal saline containing 2% Tween-20 (n=5). All animals were then exposed to a second test (retention test) 1 h after drug administration.

## 2.8. Morris water-maze test (MWT)

The MWT is a memory test paradigm used to test spatial memory on animal models. Thus the ability of the essential oils to reverse scopolamine-induced amnesia was evaluated in the MWT (Morris, 1984). In this study, the mice were pre-treated orally with different concentration (50, 100, 200 mg/kg, bwt) of the oil extract for 7 days. On the 7<sup>th</sup> day, amnesia was induced by administration of scopolamine (0.4 mg/kg bwt, i.p.). Ninety minutes after administration of Scopolamine, each animal was subjected to the acquisition test followed by a retention test 24 h after.

The test apparatus consisted of a large circular bowl (65 cm, i.d, and 40 cm, h) filled with water to three-quarter mark (55 litres) and maintained at ambient temperature. The water was made opaque by the addition of evaporated milk. The pool was divided into four equal quadrants, and a removable black plastic platform (10 cm x 5 cm). The platform which was to serve as an escape refuge from the water was submerged to a level of 1 cm below the water level at the centre of one quadrant. The platform was not visible to the mice and remained in one location for a trial. The training phase of the Water-Maze Testing consisted of two trials per day. At the start of testing on day 1, each mouse was placed in the water with its forepaws touching the tank wall, at one of four equally spaced points around the pool. Starting release points were counter-balanced alternately near or far from the platform across subjects and groups. The mouse was allowed 120 s to locate the hidden platform. A trial terminates when the animal reaches the platform and remains on the platform

for 20 s. If the animal does not locate the platform within this time frame, the animal will then be guided to the platform and made to remain there for at least 20 s. Thereafter the animal was removed from the platform, dried and returned to its cage for the next trial. The criteria/index for amnesic/cognitive activity was taken as increase/decrease in escape latency time (ELT) on retention test (second trial) in comparison with the acquisition test (first trial).

## 2.9. Statistical and data analysis

All data obtained were subjected to descriptive statistical calculation using GraphPad® Instat Statistical Package (Prism 5) and expressed as mean values  $\pm$  standard error of mean of multiple measurements (n = usually between 3 and 6). The IC<sub>50</sub>, K<sub>m</sub>, V<sub>max</sub> values were estimated from graphical linear and non-linear regression plots. The Kinetic parameters K<sub>i</sub> was calculated from the slope of the inhibition curve by the equation {slope = K<sub>m</sub>/V<sub>max</sub>(1 + [I<sub>0</sub>/K<sub>i</sub>)} . Data calculated for specific activity of AChE in the brain from the in vivo experiments, the SLT and ELT in the Passive Avoidance and MWT experiments were subjected to analysis of variance (ANOVA) followed by a Student's Neumann-Keuls post-test to ascertain differences between treatment groups. The level of significance was chosen as p < 0.05.

## 3. Results

### 3.1. Essential oils

The yield of essential oil obtained from *M. myristica* and *P. nigrum* were 3.64  $\pm$  0.86% and 0.33  $\pm$  0.04% respectively, based on the dry weight. A total of 51 components were detected in essential oils obtained from *M. myristica* while in *P. nigrum* only 61 compounds were detected. The oil from *M. myristica* contained a complex mixture consisting mainly of monoterpenes (52.52%); sesquiterpenes (3.33%); oxygenated monoterpenes (3.25%) and oxygenated sesquiterpenes (4.55%). However, a significant portion (26.13%) of total composition could not be identified. The major constituents detected in *M. myristica* were  $\alpha$ -phellandrene (18.13%),  $\alpha$ -pinene (7.16%), other components were present at less than 2.0% (Table 4.1). *P. nigrum* essential oil contained a complex mixture of monoterpenes (22.76%) and sesquiterpene hydrocarbons (12.41%) and oxygenated monoterpenes (0.66%) and oxygenated sesquiterpene (1.66%); with over 50% not identified. The major constituents detected in *P. nigrum* were  $\beta$ -pinene (5.92%), 2,4-quinolinediol (4.74), caryophyllene (4.55%), limonene (3.94%), (+)-4 carene (3.88%),  $\alpha$ -pinene (3.58%),  $\alpha$ -cubebene (3.25%), Copaene (2.23%) and Ylangene (2.11%) with the rest of the constituents less than 2.0 % (see Table 1).

**Table 1:** The Essential Oil Constituents of *Monodora Myristica*

SN	Compound	RT	% Peak Area
1	p-Xylene	4.123	0.22
2	$\alpha$ -Thujene	5.061	4.71
3	$\alpha$ -Pinene	5.187	7.16
4	1,3,5-trimethylbenzene	5.559	0.24
5	$\beta$ -Pinene	5.730	0.75
6	$\beta$ -Terpinene	6.068	5.05
7	$\alpha$ Phellandrene	6.291	18.13
8	Cymene	6.600	16.26
9	Trans- $\beta$ -Ocimene	6.743	0.22
10	$\gamma$ -Terpinene	6.858	0.21
11	Octanoic acid	7.396	3.15
12	Isopulegol	7.607	0.81
13	Terpinen-3-ol	7.808	0.47
14	Cis-Z- $\alpha$ -Bisabolene epoxide	7.865	0.45
15	2-Amino-4-methylbut-2-enenitrile	8.076	0.27
16	3,5-dimethyl-Cyclohexene	8.111	0.52
17	10-Dodecyn-1-ol	8.231	0.32
18	Alpha,alpha-3-Cyclohexene-1-methanol	8.380	1.36
19	Alpha-phenyl-Benzene ethanol	8.557	7.66
20	Benzenepropanal	8.849	1.18

21	2-Cyclohexen-1-one	8.906	0.39
22	3-(2-Hydroxy-cyclopentylidene)-2-methyl-propionic acid	9.078	0.26
23	Thymol	9.501	2.89
24	Fumaric acid	9.673	0.47
25	Copaene	9.827	0.34
26	$\alpha$ -Farnesene	10.010	0.27
27	$\alpha$ -Cubebene	10.073	0.60
28	$\gamma$ -Murolene	10.216	0.56
29	Caryophyllene	10.474	0.64
30	$\alpha$ Caryophyllene	10.766	0.84
31	(+)-Epibicyclosesquiphellandrene	10.846	0.23
32	$\beta$ -Cadinene	10.949	0.46
33	Bergamotol	11.000	0.36
34	$\alpha$ Muurolene	11.149	1.58
35	$\alpha$ -Cadinene	11.281	1.68
36	1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-(1S-cis)- naphthalene	11.355	2.95
37	Bornyl bromide	11.790	2.02
38	Tau-Cadinol	12.299	0.69
39	Tumerone	12.465	2.41
40	Curlone	12.717	1.00
41	n-Hexadecanoic acid	15.601	1.75
42	Cis-Vaccenic acid	17.810	5.43
43	Octadecanoic acid	17.975	0.73
44	9-Oxabicyclo(6.1.0)nonane	20.213	0.61
45	Phthalic acid	20.739	0.59
46	Cis,cis-7,10-Hexadecadienal	25.460	0.01
47	(Z)-13-Octadecenal	26.061	0.09
48	Oleic Acid	26.530	0.84
49	2,3-dihydroxypropyl ester	26.633	0.10
50	2-hydroxy-1-(hydroxymethyl)ethyl ester	26.679	0.05
51	9-octadecenoic acid	26.724	0.02
	Total		
	Monoterpenes hydrocarbons		52.52
	Sesquiterpenes hydrocarbons		3.33
	Oxygenated monoterpenes		3.25
	Oxygenated sesquiterpenes		4.55
	Fatty acid ester		10.22
	Others		26.13

**Table 2:** The Essential Oil Constituents of Piper Nigrum

SN	Compound	RT	% Peak Area
1	1,2-dimethyl-cyclohexane	3.419	0.11
2	2,6-dimethylheptane	3.459	0.14
3	Octyl-cyclohexane	3.510	0.73
4	1,1,3-trimethyl-cyclohexane	3.567	0.83
5	Dihydro-5,5-dimethyl-2(3H)-furanone	3.768	0.14
6	1,2,4-trimethyl-cyclohexane	3.808	0.31
7	Piperidine	3.916	0.17
8	Ethylbenzene	4.002	0.66
9	3-methyl-4-penten-1-ol	4.071	0.43
10	p-Xylene	4.157	2.65
11	1-ethyl-2-methyl-cyclohexane	4.409	0.73
12	1,3-dimethyl-benzene	4.517	0.94
13	2,4-dimethylhexane	4.655	0.83
14	1-ethyl-2-methyl-cyclohexane	4.706	0.19
15	Cyclohexane ethanol	4.883	0.52
16	Propyl-cyclohexane	5.061	0.58
17	$\alpha$ -Pinene	5.147	3.58
18	3-ethyl-2-methylheptane	5.250	0.41
19	Camphene	5.330	0.31
20	N-Benzyl-2-phenethylamine	5.427	0.60
21	1,2,3-trimethylbenzene	5.570	1.32
22	$\beta$ -Pinene	5.788	5.92
23	1-ethyl-2-methyl-cyclohexane	5.868	0.34
24	1,2,3-trimethylbenzene	5.994	1.79
25	6-ethyl-2-methyl-decane	6.097	0.62
26	$\alpha$ -Phellandrene	6.131	0.53
27	3-Carene	6.205	1.62
28	1-ethyl-2,4-dimethylbenzene	6.394	0.86
29	Limonene	6.480	3.94
30	3,7-dimethyl-1,3,6-Octatriene	6.589	1.22
31	3-isopropenyl-5,5-dimethyl-cyclopentene	6.703	0.98
32	2-ethyl-1,4-dimethyl-benzene	6.823	0.37
33	(+)-4-Carene	7.195	3.88
34	3,7-dimethyl-1,6-octadien-3-ol	7.413	7.08
35	1,3,5,5-tetramethyl-1,3-cyclohexadiene	7.659	0.31
36	7-methyl-3-methylene	8.191	0.20
37	1-methyl-4-(1-methylethylidene)-cyclohexene	8.357	1.30
38	2,5,5-trimethyl-heptatriene	9.724	0.54
39	$\alpha$ -Cubebene	9.833	0.65

40	Copaene	10.096	3.25
41	1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)cyclohexane	10.251	3.86
42	1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-1H-cycloprop[azulene	10.417	2.98
43	Caryophyllene	10.531	4.55
44	4,11,11-trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene	10.548	1.36
45	1-methyl-5-methylene-8-(1-methylethyl)-1,6-cyclodecadiene	10.640	2.23
46	Ylangene	10.708	2.11
47	7,11-dimethyl-3-methylene-1,6,10-dodecatriene	10.811	4.29
48	2,4-Quinolinediol	11.057	4.74
49	2,6-dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene	11.166	4.02
50	Cis-(-)-2,4a,5,6,9a-hexahydro-3,5,5,9-tetramethyl(1H)benzocycloheptene	11.275	1.76
51	7,11-dimethyl-3-methylene-1,6,10-dodecatriene	11.515	8.32
52	Humulene	11.618	0.27
53	3,7,11-trimethyl-1,6,10-dodecatrien-3-ol	11.681	0.68
54	Caryophyllene oxide	11.899	1.66
55	1H-cyclopropa[a]naphthalene	11.973	0.20
56	Octahydro-1H-inden-1-one	12.082	0.88
57	2,6,6,9-tetramethyl-tricyclo[5.4.0.0(2,8)]undec-9-ene	12.236	1.07
58	Aromadendrene	12.293	0.50
59	Methyl methylphosphonofluoridate	12.505	1.07
60	2,3,3-trimethyl-2-(3-methyl-1,3-butadienyl)cyclohexanone	12.648	1.24
61	9,12-Octadecadienoic acid	17.746	0.60
TOTAL			
Monoterpenes hydrocarbons			22.76
Sesquiterpenes hydrocarbons			12.41
Oxygenated monoterpenes			0.66
Oxygenated sesquiterpenes			1.66
Fatty acid ester			8.75
Others			53.76

**Table 3:** In Vitro Cholinesterase Inhibitory Activity

	IC <sub>50</sub> (mg/ml)	
	AChE	BuChE
M. Myristica	0.205 ± 0.060	0.178 ± 0.016
P. Nigrum	0.272 ± 0.018	0.223 ± 0.002

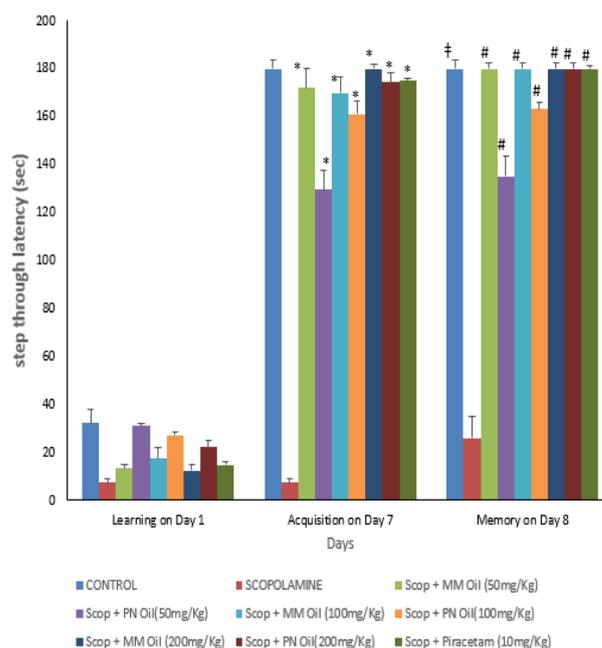
### 3.2. In vitro anti-cholinesterase assay

M. myristica exhibited a higher cholinesterase inhibition in both AChE and BuChE in a dose dependent manner, than P. nigrum. M. myristica essential oil at 416µg/ml gave 78.72% while P. nigrum gave 74.93% AChE inhibition with IC<sub>50</sub> values of 0.205 ± 0.06 and 0.272 ± 0.18 mg/ml, respectively. Similarly, at the same concentration M. myristica essential oil inhibited BuChE 89.19% while P. nigrum essential oil gave 84.96% and IC<sub>50</sub> values of 0.178 ± 0.016 and 0.223 ± 0.002 mg/ml, respectively.

### 3.3. Cognitive behavioral test

#### 3.4. Morris water-maze

The Escape Latency Time (ELT) of the first and the seventh day of treatment reflected the learning and acquisition behavior of animals, whereas, the ELT of the eighth day reflected retention of information or memory. Administration of Scopolamine hydrochloride (0.4 mg/kg, i.p), significantly prevented the reduction in the day seven ELT of the group administered scopolamine only when compared to the control group ((-)scopolamine), indicating memory impairment. However, the animal in the group treated with 10 mg Piracetam, 50, 100, and 200 mg/kg of the M. myristica and P. nigrum oil extracts showed a remarkable reduction (p<0.05) in ELT of the seventh day as well as eighth day in a dose dependent manner when compared with Scopolamine treated group, indicating significant improvement in learning and memory (Figure 1).

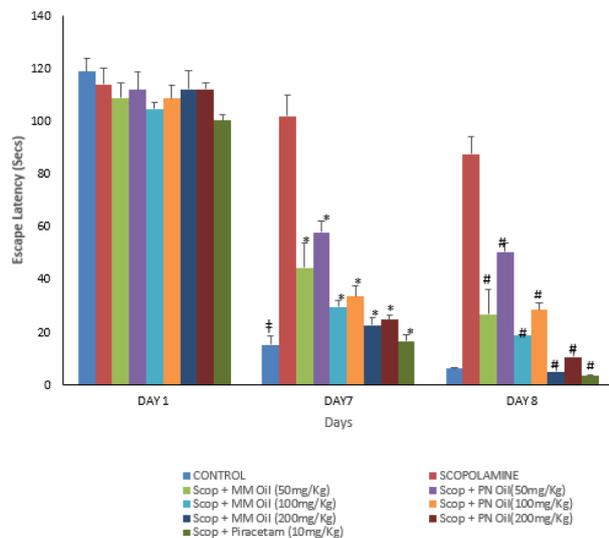


**Fig. 1:** Effect of M. Myristica and P. Nigrum Oil Extracts on Spatial Memory Performance In Mice Using the Morris Water-Maze Test. Values Are Expressed as Mean ± S.E.M (N=5 in Each Group). Value with # P < 0.05, \* P < 0.05, † P < 0.05 and ‡ P < 0.05 Are Statistically Significant Compared to the Scopolamine Group.

### 3.5. Step-through passive avoidance task

The Step-Through Latency (SLT) of seventh and eighth day of the essential oil administered reflected the long-term memory of animals. The effect of Piracetam, M. myristica and P. nigrum oil extract on the performance of mice in PAV test is depicted in Figure 2. In the Scopolamine treated group, there was a significant decrease (p<0.05) in the SLT when compared to the control group

(saline) and that of the acquisition test of all groups, indicating improvement of memory. In the oil treated group, there was a significant ( $P < 0.05$ ) reversal of the Scopolamine induced amnesic effect at all doses of the extract in a dose dependent manner, including the 10 mg Piracetam.



**Fig. 2:** Effect of *M. Myristica* and *P. Nigrum* Oil Extracts on Long Term Memory Performance in Mice Using Passive Avoidance Task. Values Are Expressed as Mean  $\pm$  S.E.M (N=five in Each Group). Value with #  $P < 0.05$ , \*  $P < 0.05$ , †  $P < 0.05$  Are Statistically Significant Compared to the Scopolamine Group.

#### 4. Discussion

Enhancement of cholinergic activity through the inhibition of AChE is one of the major therapeutic strategies for AD. Most of the USDA approved drugs such as tacrine have been used for mild cognitive disorders. These cholinesterase inhibitors attenuate the cholinergic deficit underlying the cognitive and neuropsychiatric dysfunction in AD patients (Lane, 2005). Particularly, therapeutic agents with dual inhibition of neuronal AChE and BuChE, rather than agents that target selectively AChE only, have been speculated to bring about improved clinical outcomes (Andersen, 2004).

Results obtained clearly showed that the seeds of *M. myristica* and *P. nigrum* do possess volatile compounds that exhibits both anti-cholinesterase and antioxidant activity to an extent, which renders them potentially useful as an adjuvant in the management of cognitive disorders such as in Alzheimer's disease. The GC-MS analysis of both essential oils from *M. myristica* and *P. nigrum* indicate the presence of terpenes, mainly monoterpenes hydrocarbons and oxygenated monoterpenes such as  $\alpha$ -Pinene,  $\beta$ -Pinene,  $\alpha$ -Phellandrene,  $\alpha$ -Caryophyllene, Bergamotol, Piperidine,  $\alpha$ -Cubebene, and Copaene as the major compounds.

Observations made in this study are consistent with that of a number of other studies of essential oils isolated by hydro-distillation from plants. Owokotomo and Ekundayo (2012) reported that the essential oil from seeds of *M. myristica* contained germacrene D-4-ol (25.48%), tricyclo[5.2.1]1,5dec-2-ene (13.35%),  $\delta$ -cadinene (11.09%) and linalool (15.10%) as major constituents while the dominant constituents of the stem-bark oil were  $\gamma$ -cadinene (31.31%),  $\alpha$ -elemene (17.98%),  $\alpha$ -cubebene (6.70%) and  $\gamma$ -murolene (5.94%). Koudou et al. (2007) reported that the essential oil from the seeds of *M. myristica* contains mainly monoterpenoids (93.2%) out of which 77.4% are monoterpene hydrocarbons and some sesquiterpenoids (5.8%). The major constituents were  $\alpha$ -phellandrene (34.4%) and p-cymene (22.2%).

Tchoumboungang et al. (2009) studied the chemical composition of the essential oils obtained by hydro-distillation of different tissues of *Piper capense*, *Piper guineense*, *Piper nigrum* and *Piper umbellatum* grown in Cameroon and reported that oils from the fruits were rich in  $\alpha$ -pinene (5.6 - 12.3%) and  $\beta$ -pinene (6.7-59.3%). The

other major constituents were sabinene (14.7%) for *P. capense*, limonene (15.8%) and  $\beta$ -caryophyllene (20.8%) for *P. guineense*. Oil from the fruits of *P. nigrum* contained sabinene (11.2%),  $\delta$ -3-carene (18.5%), limonene (14.7%) and  $\beta$ -caryophyllene (12.8%) while that of *P. umbellatum* contained linalool (14.4%) and (E)-nerolidol (10.0%) as major constituents. Jirovetzet al. (2002) also reported the presence of germacrene D (11.01%) and  $\beta$ -pinene (10.02%) in *P. nigrum* seed oil. Arruda et al. (2012) reported that the essential oils obtained from the leaf of *Hedychium gardnerianum* contained monoterpenes, sesquiterpenes hydrocarbons which were responsible for both anti-cholinesterase and antioxidant properties observed. Sharififar et al. (2012) investigated the antioxidant and anti-cholinesterase effect of essential oil and methanolic extract of *Zataria multiflora* Boiss and observed that the oil exhibited a high anti-cholinesterase activity ( $IC_{50} = 0.97 \pm 0.12 \mu\text{g/ml}$ ) which was comparable to physostigmine ( $IC_{50} = 0.13 \pm 0.02 \mu\text{g/ml}$ ). Ali et al. (2012) also investigated the composition, antimicrobial, antioxidant and anti-cholinesterase properties of leaf essential oil of *Pulicaria stephanocarpa* from Soqotra and observed that the oil elicited a 47% AChE inhibitory activity at a concentration of 200  $\mu\text{g/ml}$ . Caryophyllene (13.4%) a monoterpene hydrocarbon and nerolidol (8.5%),  $\alpha$ -cadinol (8.2%), spathulenol (6.8%) which were oxygenated monoterpenes were the major components of the oil. The anti-cholinesterase activities of the essential oil of *Salvia chionantha* with  $\beta$ -caryophyllene (8.71%), Germacrene (25.03%), spathulenol (5.86%) and humulene (4.82%) as the major compounds have also been demonstrated. At a concentration of 500  $\mu\text{g/ml}$  the oil elicited a moderate AChE ( $56.7 \pm 1.9\%$ ) and BuChE ( $41.7 \pm 2.9\%$ ) inhibitory activity.

The in vitro studies showed that the essential oil of *M. myristica* and *P. nigrum* inhibited AChE and BuChE potently and reversibly, a phenomenon known as "dual cholinesterase inhibition". Studies have also shown that both AChE and BuChE hydrolyze ACh in vitro, however, ACh shows higher affinity for AChE than BuChE (Soreq and Seidman, 2001; Perry et al., 2003), BuChE has a preference for higher choline esters, such as butyrylcholine (BuCh) and other neuroactive peptides. Until recently, inhibition of AChE was the major focus of most researchers as the therapeutic strategy in the attenuation of diseases associated with cognitive decline. However, recent studies have evaluated the importance of BuChE and the key role it plays in regulating cognitive functions. Thus, the dual inhibition towards AChE and BuChE by both oils may be considered as a positive outcome that could boost the potentials of the plants as a candidate in the management of cognitive disorders associated with Alzheimer's disease.

The anti-amnesic and memory enhancing properties of the oil extract evaluated using two learning and memory paradigms, the Morris Water-Maze and the passive avoidance task shows that both essential oils reversed the scopolamine induced amnesia as indicated by the increase in the ELT in the Morris Water-Maze Test when compared to the scopolamine administered group and decrease in SLT in the passive avoidance task, indicating memory enhancing properties of the two oils. Several studies have shown the cognitive enhancing properties of natural product (plants) using various learning and memory paradigms.

The anti-amnesic properties of essential oils have also been demonstrated by a number of investigators. Majlessi et al. (2012) in a study to demonstrate new neuro-protective plants for the management of AD reported that the essential oil of *Zataria multiflora*, a member of the Lamiaceae family, at a dose of 200  $\mu\text{l/kg}$  bwt was able to ameliorate the Amyloid-protein fragment-induced cognitive deficit in a Morris Water-Maze task. The investigators added the effect to the antioxidant, anti-inflammatory and anticholinesterase constituents of the plant.

Hritcu et al. (2012) investigated the anti-amnesic effects of two different lavender essential oils from *Lavandula angustifolia* sp. *angustifolia* Mill. (Lamiaceae) and *Lavandula hybrida* Rev. (Lamiaceae) on male Wistar rats subjected to scopolamine (0.7 mg/kg)-induced dementia. It was observed that chronic exposures to lavender essential oils (daily, for 7 continuous days) significantly improved the spatial memory performance in Y-Maze and Radial

Arm-Maze tasks thus suggesting positive effects on memory formation. It was inferred from the study that multiple exposures to lavender essential oils could effectively reverse spatial memory deficits induced by dysfunction of the cholinergic system in the rat brain and might provide an opportunity for management of neurological abnormalities in dementia conditions.

## 5. Conclusion

We have shown that the *M. myristica* and *P. nigrum* essential oil contains terpenes and terpenoid such as  $\alpha$ -Pinene,  $\beta$ -Pinene,  $\alpha$ -Phellandrene, Caryophyllene and Copaene, among others with potent anti-cholinesterase and antioxidant activities. *M. Myristica* exhibited higher activity in all assays when compared to *P. nigrum*, and that these constituents could be acting alone or synergistically with others to bring about the anti-amnesic outcomes observed. In all, it can be suggested that the *M. myristica* and *P. nigrum* oils have health-promoting properties and as a dietary spice its use is advocated, especially by the elderly who are more prone to cognitive disorders. It could also be employed in the treatment and management of cognitive dysfunctions such as Alzheimer's and Parkinson's diseases. However, further studies on these essential oils using animal models of AD would be desirable.

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