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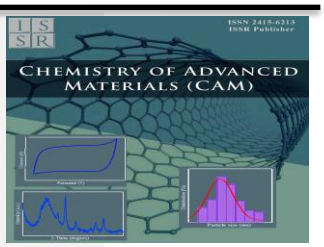
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Original paper

Antioxidant, Anti-inflammatory and Neuroprotective Activities of a Plant Extract Derived from Traditional Chinese Medicine: SuHeXiang Wan (AT000)

Samy Iskandar [a,b,*], Martial Senankpon Gnansounou [a,b,c], Maxime Robin [a,b], Jean Lorquin [d], Carole Di Giorgio [b], Philippe Piccerelle [a,b]

[a] Laboratoire de Pharmacie Galénique, Pharmacotechnie Industrielle, Biopharmacie et Cosmétologie, Faculté de Pharmacie, Aix Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille, France
[b] IMBE (Institut Méditerranéen de Biodiversité et d'Ecologie Marine et Continentale), UMR CNRS 7263, IRD 237, Faculté de Pharmacie, Aix Marseille Université, 27 Boulevard Jean Moulin, 13385 Marseille, France
[c] Laboratoire d'Etude et de Recherche en Chimie Appliquée, Ecole Polytechnique d'Abomey Calavi, Université d'Abomey Calavi, Bénin
[d] Mediterranean Institute of Oceanography (MIO), UM 110, CNRS/INSU, IRD, Aix Marseille Université, 13288 Marseille, France

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*Corresponding author:
samy.iskandar@hotmail.com

Abstract

In this paper, we present SuHeXiang Wan, a medicine used in traditional Chinese medicine for the treatment of epilepsy and convulsions. We investigated the antioxidant, anti-inflammatory and neuroprotective activities of the same treatment designated as AT000. The synergy of the two plants, *Dryobalanops aromatica* and *Saussurea lappa*, of which the use in alimentary supplements is considered controversial, was evaluated for the first time. The antioxidant activity of the extract was assessed by DPPH and ORAC tests while the anti-inflammatory activity was determined by measuring the capacity of macrophages to generate a strong inflammatory response when stimulated with antigens, inducing NO release. The extract efficacy on the attenuation of the $A\beta_{25-35}$-induced learning deficits (spatial working memory: spontaneous alternation in the Y-maze and contextual long-term memory: passive avoidance test) was evaluated *in vivo* in mice seven days after the peptide administration. The impact on lipid peroxidation in the hippocampus, an index of oxidative

stress, was also evaluated. AT000 extract showed a strong antioxidant activity at 2 mg/mL, 10 mg/mL and 301774 Trolox equivalents according to the DPPH and ORAC tests respectively. The 21-days AT000 treatment dose-dependently alleviated $A\beta_{25-35}$-induced deficits, with significant prevention at the highest dose tested (250 mg/Kg/day) on the spontaneous alternation, step-through latency and escape latency parameter. 21-days AT000 treatment dose-dependently attenuated also $A\beta_{25-35}$-induced increase lipid peroxidation, with a significant and complete blockade at the highest doses tested. Synergistic experiments showed that the presence of *Dryobalanops aromatica* and *Saussurea lappa* is crucial to obtain a neuroprotective effect. According to these results, AT000 could be a candidate compound in the development of therapeutic drugs for the prevention and treatment of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is an incurable neurodegenerative disease of cerebral tissue that leads to progressive and irreversible loss of mental functions and especially memory. It is the most common cause of dementia in humans [1]. AD is characterized by two primary lesions: senile plaques which are toxic extracellular aggregates of amyloid peptides and neurofibrillary tangles which are intracellular aggregates formed of pairs of helical filaments due to the abnormal formation of hyperphosphorylated tau protein masses [2]. The clinical symptoms of this disease include memory loss [3], especially recent events in the early phases of the disease and deterioration of other cognitive functions that interfere with mood, reasoning, judgment and language [4, 5].

From a histopathological point of view, abnormal cleavage by beta-secretase of amyloid precursor protein leads to the extracellular accumulation of neurotoxic peptide $A\beta_{42}$ generating senile plaques which compress neurons [6]. Oligomeric peptides Aβ play a significant role in mediating neurotoxicity and leading to AD [7]. They provoke strong alterations of plasticity mechanisms leading to a decline in memory [8, 9].

Oligomers of Aβ peptides vary in length between 40 and 43 amino acids. $A\beta_{40}$ and $A\beta_{42}$ are the most abundant species found in AD brains [10], however only the $A\beta_{42}$ fragment forms fibrillar deposits readily [11]. Another toxic minor fragment $A\beta_{25-35}$ has also been identified and used widely in research [12, 13]. Aβ oligomers interact with the intracellular organelles responsible for the regulation of calcium homeostasis leading to oxidative stress and causing neuronal apoptosis [14]. Aβ peptides can also provoke an inflammatory response in the brain and cytokine production by active astrocytes and microglia [15].

Several studies have shown the presence of lipids and proteins oxidation products in the tissues of Alzheimer's patients after death [16]. Further studies suggested the presence of a link between increased oxidative stress promoted by Aβ and the presence of senile plaques [17].

Despite scientific advances in this field, at present, there is no effective treatment to inhibit the progression of the disease and stop the cognitive decline. Since brain lesions (amyloid plaques and

neurofibrillary degeneration) trigger a decrease in the neurotransmitter acetylcholine that allows neurons to communicate. Drug treatment options are currently limited to acetylcholine esterase inhibitors. Anticholinesterasics include three different molecules that have been rigorously tested and have proven to be effective in mild-to-moderate forms of the disease: Donepezil, Galantamine and Rivastigmine [18]. These treatments help to stabilize the disease, however; they do not reverse it nor cure it. In addition, these molecules can interact with a wide range of drugs and their consumption is associated with numerous side effects [19].

In non-drug therapies, to alleviate the symptoms of AD and improve cognitive functions, plants were used. The advantage of medicinal plants is their richness in compounds, which can act synergistically with other compounds from the same or another plant. plant-derived molecules can also boost the activity of the constituents or neutralize the toxic effects of compounds from other plant species [20]. Today, traditional Chinese medicine is a potential alternative to drug treatments. By testing different combinations of plants having potential neuroprotective activities, several studies in this field have shown that plant extracts derived from traditional Chinese medicine help fight against the symptoms of neurodegenerative diseases such as AD [21-24].

SuXeHiang Wan, an extract of 9 plants that have been used in the treatment of epilepsy and convulsions, has demonstrated a sedative and anticonvulsant effect as well as inhibitory effects on the central nervous system following the inhalation of its Essential oil [24]. In mice, the essential oil, SuXeHiang Wan, was shown to attenuate the amyloid beta-induced alteration of memory by inhibiting the phosphorylation of tau protein [25]. This extract contains an unlisted plant in the French Pharmacopoeia (*Dryobalanops aromatica*) and another plant which its roots are declared in mutual recognition in the list B of French pharmacopoeia (*Saussurea lappa*). In the present study, the same extract designated as AT000 was evaluated for its antioxidant, anti-inflammatory and cytotoxic activities. We also investigated whether the oral administration of AT000 mixture can alleviate the pathology induced in mice injected intracerebroventricularly (i.c.v) with oligomeric amyloid-β_{25-35} peptide ($A\beta_{25-35}$). In addition, this study evaluated for the first time the synergistic effect of *Dryobalanops aromatica* and *Saussurea lappa in vivo*.

2. Materials and Methods

2.1 Materials

All medicinal plants were purchased from GIVAUDAN Shanghai-China. 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH), N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA), phosphate buffer, fluorescein, 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), dimethyl sulfoxide (DMSO), Propyl gallate, Ascorbic acid, Griess modified reagent, Dexamethasone, RAW 264.7 cells, and Lipopolysaccharides (LPS) of *E.coli*, were purchased from Sigma-Aldrich (St Louis USA). Trolox was obtained from Calbiochem (San Diego, United States), Dulbecco's modified Eagle Minimum Essential Medium (DMEM), Penicillin/streptomycin and inactivated calf serum were purchased from PAN BIOTECH. $A\beta_{25-35}$ and scrambled amyloid-β protein (25-35) Sc.Aβ were purchased from Polypeptides (France).

2.2 Preparation of plant extract

AT000 was prepared as described previously with some modifications [24]. A total of 175 g of the mixture of *Liquidambar orientalis* (20 g),

Saussurea lappa (20 g), *Aquilaria agallocha* (15 g), *Santalum album* (20 g), *Boswellia carteri* (20 g), *Eugenia caryophyllata* (20 g), *Cyperus rotundus* (20 g), *Styrax benzoin* (20 g), and *Dryobalanops aromatica* (20 g), was pulverized and extracted once with 10 vol. of water/ethanol mixture (70:30, v/v) at 80-85 °C with reflux condenser for 3 hours. The extract was then vacuum filtered using a Büchner flask and evaporated using a rotary evaporator at 60 °C to give a sticky brown oil. To evaluate the impact of *Dryobalanops aromatica* and *Saussurea lappa*, we made 4 different extracts designed as AT formulations in the following manner:

AT001: *Dryobalanops aromatica*

AT002: *Dryobalanops aromatica* + *Saussurea lappa*

AT007: AT000 without *Dryobalanops aromatica* and *Saussurea lappa*

AT008: AT000 without *Dryobalanops aromatica*

2.3 Determination of radical scavenging activity

The radical scavenging activity of the extract was determined by measuring its ability to trap the stable free radical, DPPH as described by Brand-Williams *et al.* with some modifications [26]. Briefly, 0.05 mM solution of DPPH˙ was prepared in methanol, and 2.9 mL of this solution was added to 0.1 mL extract solution in methanol at different concentrations (from 0.2 to 10 mg/mL). The reaction mixture was stirred at room temperature in a dark chamber for 30 minutes, and the absorbance was recorded at 517 nm using a UV-Vis spectrophotometer (Ultrospec 3000 pro). Control was prepared by adding 2.9 mL of the DPPH solution (0.05 mM) to 0.1 mL of methanol. IC_{50} values, which represent the concentration of the extract that causes neutralization of 50% of the DPPH radicals, were calculated from the percentage inhibition (PI, %) versus concentration curve as Eq. (1). The inhibition of free radicals by DPPH (%) was calculated using the following equation:

$$PI\ (\%) = [1–(A_1/A_0)] \times 100 \quad (1)$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample. Trolox, ascorbic acid and propyl gallate were used as positive controls. Measurements were performed in triplicate, and the corresponding standard deviation was calculated.

2.4 Oxygen Radical Absorbance Capacity Assay

The oxygen radical absorbance capacity (ORAC) assay was assessed in microplates as described by Ou *et al.* with some modifications [27]. The plant extract, AAPH, and fluorescein were diluted in 100 mM potassium phosphate buffer (pH 7.4). 25µL of each extract (0.94-7.5 µg/mL) or phosphate buffer (blank) were mixed with 150 µL of fluorescein solution (8.21×10^{-5} mM) and incubated for 15 min at 37 °C. A volume of 25 µL of AAPH solution (153 mM final concentration) was added, and fluorescence was immediately monitored using an Infinite M200 TECAN plate fluorimeter at 2-min intervals for 90 min. the excitation and emission wavelengths used are 485 nm and 530 nm respectively. A calibration curve was performed with Trolox (0.4–12.5 µM) as standard. The ORAC values were calculated using the neat AUCs and expressed as µmol of Trolox equivalent per 100g of extract (µmol TE/100g). Trolox was used as a control standard.

2.5 Cell culture

Mouse macrophages cells (RAW 264.7) were maintained in DMEM with stable L-glutamine supplemented with Penicillin 100 IU/mL, streptomycin 100 µg/mL and 10% of inactivated calf serum at pH 7.2, freshly prepared, stored no longer than 3 weeks.

2.6 Anti-inflammatory and cytotoxicity assays

In vitro anti-inflammatory assay was determined by measuring the capacity of macrophages to generate a robust inflammatory response when stimulated with antigens, inducing NO release [28]. Cells were seeded into 48-well tissue culture plates at a concentration of 1.10^5 cells/mL (200 µL/well) for 24 hours at 37 °C (5% CO_2). Then the culture medium was replaced by 200 µL of medium containing the appropriate concentrations of AT000 extract, and cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2/90% air for one hour. At the end of the incubation period, pro-inflammatory LPS from *E. coli* were added to cell cultures (1 µg/mL), and cells were incubated under the same conditions for 24 hours. NO release was evaluated indirectly by measuring the accumulation of nitrite/nitrate, the stable end-products of NO oxidation, in the culture supernatant by the Griess reaction. 100 µL of the supernatants were transferred into the wells of a 96-well tissue culture plate, and 100 µL of the Griess modified reagent were added to each well. After 15-min at room temperature, the Optical Density (OD) of each well was read at 540 nm by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The results obtained for wells treated with AT000 extract were compared to those of untreated control wells (DMSO, 100% viability) and converted to percentage values.

In parallel to the assessment of NO release, cell viability was measured to validate the assay. The WST-1 vital dye reagent was used to measure cell mitochondrial respiration. For this purpose, the culture medium was decanted, and 100 µL of WST-1 reagent (1/10 dilution) was added to each well. After a 30-min incubation period at 37 °C (5% CO_2), the Optical Density (OD) of each well was read at 450 nm by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The results obtained for wells treated with AT000 extract were compared to those of untreated control wells (DMSO, 100% viability) and converted to percentage values. Experiments were performed in duplicate and dexamethasone was used as a positive control at the concentrations of 1, 5, 10, 50 and 100 µM.

Inhibition of NO release and inhibition of cell viability were expressed as percentages as compared to the negative controls:

$$PI\ (\%) = 100 \times (OD_{test\ well} - OD_{blank})/(OD_{control} - OD_{blank}) \quad (2)$$

The concentrations of the extract causing respectively a 50% decrease of NO release ($IC_{50\text{-}NO\text{-}R}$) and a 50% decrease of cell viability ($IC_{50\text{-}cell\text{-}V}$) were calculated through non-linear regression analysis using software TableCurve Version 2.0. The anti-inflammatory ratio corresponded to the ratio between the anti-inflammatory activity and the toxicity. It was expressed as follows:

$$\text{Anti-inflammatory ratio} = IC_{50\text{-}cell\text{-}V}/IC_{50\text{-}NO\text{-}R} \quad (3)$$

2.7 Animal studies

Male Swiss mice, six weeks old, weighing 30-35 g, from JANVIER (Saint Berthevin, France), were kept for housing. Experiments took place within the animal facility building of the University of Montpellier 2 (CECEMA, Office of Veterinary Services agreement # B- 34-172-23) for experiments 1-2 and in Amylgen (Regional Directorate of Food, Agriculture and Forest of Languedoc-Roussillon, agreement #A 34-169-002 from May 02, 2014) for experiments 3. Animals were housed in groups with access to food and water *ad libitum*, except during behavioral experiments. They were kept in a temperature and humidity controlled animal facility on a 12 h/12 h light/dark cycle (lights off at 07:00 pm). Mice were numbered by marking their tail using permanent

markers. All animal procedures were conducted in strict adherence to the European Union directive of September 22, 2010 (2010/63/UE).

2.7.1 Drugs and administration procedures

The homogeneous oligomeric preparation of $A\beta_{25-35}$ peptide and scrambled $A\beta_{25-35}$ peptide (Sc.Aβ) was performed according to the AMYLGEN's owned procedure. The preparations were dissolved in distilled sterile water at a concentration of 3 mg/mL and stored at -20 °C until use. Before injection, peptides were aggregated by incubation at 37 °C for 4 days. Each mouse was anesthetized with isoflurane 2.5% and injected i.c.v. with $A\beta_{25-35}$ peptide (9 nmol/mouse) or Sc.Aβ peptide (9 nmol/mouse), in a final volume of 3 µL per mouse, according to the previously described method [29-33].

Two administration procedures were examined. In experiment 1, AT000 was administered *per os* (p.o.) by gavage twice-a-day (b.i.d.), starting on the same day as $A\beta_{25-35}$ and lasting until day 7 when the animals' behaviors were examined (7-days injection). In experiment 2, AT000 was administered per os by force-feeding twice-a-day (b.i.d.), starting 14 days before $A\beta_{25-35}$ injection and lasting until day 7 when the animals' behaviors were examined (21-days injection). Finally, a third experiment was performed to analyze the impact of the two controversial plants in the extract: *Dryobalanops aromatica* and *Saussurea lappa*. This last experiment was performed at the active dose of the 21-days injection procedure.

2.7.2 Animals and treatment groups

• Two hundred and forty (240) male Swiss mice (30-35 g) were used.

• Twenty (20) groups of animals were constituted in the following manner:

Experiment 1 (groups 1-5):

• On day 0, Sc.Aβ or $A\beta_{25-35}$-amyloid peptide was injected i.c.v. at 10:00 am.

• Between day 0 and day 9, AT000 or the vehicle 1 solution (DMSO 5% in water) was administered p.o. by force-feeding b.i.d., at 09:00 am and 05:00 pm. AT000 was administered only once at 09:00 am on day 9.

Experiment 2 (groups 6-10):

• Between day -14 and day 9, AT000 or the vehicle 1 solution (DMSO 5% in water) was administered p.o. by force-feeding b.i.d., at 09:00 am and 05:00 pm. AT000 was administered only once at 09:00 am on day 9.

• On day 0, Sc.Aβ or $A\beta_{25-35}$-amyloid peptide was injected i.c.v. at 10:00 am.

Experiment 3 (groups 11-20):

• On day 0, Sc.Aβ or $A\beta_{25-35}$-amyloid peptide was injected i.c.v. at 10:00 am.

• Between day -14 and day 9, AT000, AT001, AT002, AT007, AT008 or the vehicle solutions (vehicle 1 = DMSO 5% in water; vehicle 2 = DMSO 10% in grapeseed oil) was administered p.o. by force-feeding b.i.d., at 09:00 am and 05:00 pm.

Then, for all groups:

• On day 7, all animals were tested for the spontaneous alternation performance in the Y-maze test, an index of spatial working memory.

• On day 8 and 9, the contextual long-term memory of the animals was assessed using the step-through type passive avoidance procedure.

• On day 9, immediately after the retention session, animals were euthanized by decapitation and the hippocampus and cortex dissected out. Lipid peroxidation in the hippocampus was analyzed.

2.7.3 Formulation preparation

All solutions were freshly prepared twice a day before each injection as listed in Table 1. No

stock solution was prepared. Solutions were prepared from an initial concentrated solution in DMSO 100%. Then dilution was done in distilled water. Final DMSO concentration was 5%.

Groups 1, 2, 6, 7, 11, 12: vehicle 1 solution (DMSO 5% in water).

Groups 3, 8: AT000 6.2 mg/mL in vehicle 1 solution (corresponding to 37.5 mg/kg b.i.d. or 62.5 mg/kg/day).

Groups 4, 9: AT000 12.5 mg/mL in vehicle 1 solution (corresponding to 62.5 mg/kg b.i.d. or 125 mg/kg/day).

Groups 5, 10: AT000 25 mg/mL in vehicle 1 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

Groups 14, 15: vehicle 5 solution (DMSO 10% in grapeseed oil plus emulsifier).

Group 16: AT000 25 mg/mL in vehicle 2 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

Group 17: AT001 25 mg/mL in vehicle 2 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

Table 1. Groups of animals, treatment period and number of mice used for different experiments.

Experiment		**Groups of animals**	**Number of days**	**Number of mice**
Experiment 1	1	Sc.Aβ + vehicle 1	7	12
	2	$A\beta_{25-35}$ + vehicle 1	7	12
	3	$A\beta_{25-35}$ + AT000: 62.5 mg/kg/day	7	12
	4	$A\beta_{25-35}$ + AT000: 125 mg/kg/day	7	12
	5	$A\beta_{25-35}$ + AT000: 250 mg/kg/day	7	12
Experiment 2	6	Sc.Aβ + vehicle 1	21	12
	7	$A\beta_{25-35}$ + vehicle 1	21	12
	8	$A\beta_{25-35}$ + AT000: 62.5 mg/kg/day	21	12
	9	$A\beta_{25-35}$ + AT000: 125 mg/kg/day	21	12
	10	$A\beta_{25-35}$ + AT000: 250 mg/kg/day	21	12
Experiment 3	11	Sc.Aβ + vehicle 1	21	12
	12	$A\beta_{25-35}$ + vehicle 1	21	12
	13	$A\beta_{25-35}$ + AT000 (in vehicle 1): 250 mg/kg/day	21	12
	14	Sc.Aβ + vehicle 2	21	12
	15	$A\beta_{25-35}$ + vehicle 2	21	12
	16	$A\beta_{25-35}$ + AT000 (in vehicle 2): 250 mg/kg/day	21	12
	17	$A\beta_{25-35}$ + AT001: 250 mg/kg/day	21	12
	18	$A\beta_{25-35}$ + AT002: 250 mg/kg/day	21	12
	19	$A\beta_{25-35}$ + AT007: 250 mg/kg/day	21	12
	20	$A\beta_{25-35}$ + AT008: 250 mg/kg/day	21	12

Group 18: AT002 25 mg/mL in vehicle 2 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

Group 19: AT007 25 mg/mL in vehicle 2 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

Group 20: AT008 25 mg/mL in vehicle 2 solution (corresponding to 125 mg/kg b.i.d. or 250 mg/kg/day).

2.7.4 Euthanization

At the end of the passive avoidance retention session, on day 9, animals were euthanized by decapitation. The hippocampi and frontal cortex were removed. One hippocampus of n=6 animals per group was used to measure lipid peroxidation levels in tissue extracts. The other hippocampus and the cortex were kept at -80 °C awaiting further analysis.

2.7.5 Spontaneous alternation performances

On day 7, all animals were tested for spontaneous alternation performance in the Y-maze, an index of spatial working memory. The Y-maze was made of grey polyvinylchloride. Each arm was 40 cm long, 13 cm high, 3 cm wide at the bottom, 10 cm wide at the top, and converging at an equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries, including possible returns into the same arm, was checked visually. An entry was defined as the penetration of the hind paws of the animal at least 2 cm into the arm. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations was the total number of arm entries minus two and the percentage of alternation was calculated as: actual alternations/maximum alternations) x 100. Parameters included the percentage of alternation (memory index) and the total number of arm entries (exploration index) [29-33].

2.7.6 Passive avoidance test

The apparatus is a two-compartment (15 x 20 x 15 cm high) box, one illuminated with white polyvinylchloride walls and the other darkened with black polyvinylchloride walls and a grid floor. A guillotine door separates each compartment. A 60 W lamp positioned 40 cm above the apparatus lights up the white compartment during the experiment. Scrambled foot shocks (0.3 mA for 3 s) were delivered to the grid floor using a shock generator scrambler (Lafayette Instruments, Lafayette, USA). The guillotine door was initially closed during the training session. On day 8, during the training session, each mouse was placed in the white compartment. After 5 s, the door was raised. When the mouse entered the darkened compartment and placed all its paws on the grid floor, the door was closed and the foot shock delivered for 3 s. The step-through latency, that is, the latency spent to enter the darkened compartment, and the number of vocalizations was recorded. The retention test was carried out 24 h after training, on day 9. Each mouse was placed in the white compartment again. After 5 s, the door was raised. The step-through and escape latencies (corresponding to the re-exit from the darkened compartment) were recorded up to 300 s [31-33].

Animals that showed all latencies during the training and retention session lower than 10 s were considered as having failed to respond to the procedure and were discarded from the calculations. In this study, no animal was discarded accordingly.

2.7.7 Lipid peroxidation measures

At day 9, 6 mice from each group were euthanized by decapitation, and both hippocampi were rapidly removed, weighed and kept in liquid nitrogen until assayed. After thawing, one hippocampus per mice was homogenized in cold methanol (1/10 w/v), centrifuged at 1,000 g for 5 min and the supernatant placed in Eppendorf tube. The reaction volume of each homogenate was added to $FeSO_4$ 1 mM, H_2SO_4 0.25 M, xylenol

orange 1 mM and incubated for 30 min at room temperature. After reading the absorbance at 580 nm ($A_{580}1$), 10 µL of cumene hydroperoxide (CHP) 1 mM was added to the sample and incubated for 30 min at room temperature, to determine the maximal oxidation level. The absorbance was measured at 580 nm ($A_{580}2$). The level of lipid peroxidation was determined as CHP equivalents according to CHPE = $A_{580}1/A_{580}2$ x [CHP (nmol)] and expressed as CHP equivalents per mg of tissue and as a percentage of control group data (V-treated Sc.Aβ-administered mice).

2.7.8 Statistical analyses

All values, except passive avoidance latencies, were expressed as mean ± S.E.M. Statistic analyses were performed on the different conditions using one-way ANOVA (*F* value), followed by the Dunnett's post-hoc multiple comparison tests. Passive avoidance latencies do not follow a Gaussian distribution since upper cut-off times were set. Therefore, they were analyzed using a Kruskal-Wallis non-parametric ANOVA (*H* value), followed by a Dunn's multiple comparison tests. $p < 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1 Antioxidant activity

To determine the antioxidant potential of our extract, we evaluated the reducing power by the widely used DPPH test, and the ability to delay the oxidation of fluorescein by the ORAC assay. $DPPH^{\cdot}$ is a stable radical, nitrogen-centered and colored due to the delocalization of electron with a visible absorption maximum at 517 nm in an alcoholic solution. The reduction of the DPPH radical by the antioxidant compound is manifested by a decrease in the absorbance of the DPPH radical and by its discoloration (from violet to yellow) [34]. The ORAC assay is currently considered the most relevant chemical test for measuring antioxidant activity since it is a dynamic test based on stress induced by peroxyl radicals mimicking the cellular mechanisms induced by them [35, 36]. The DPPH scavenging effect of AT000 extract increased with increasing concentration of extract (Fig. 1), which demonstrated strong antioxidant ability at 2 mg/mL and 10 mg/mL (82.08 and 93.39%) respectively. Table 2 shows the antioxidative potency of AT000 extract compared with foods known to have substantial antioxidant activity (USDA Database for ORAC): Nutrient Data Laboratory, Agriculture Research Service, United States Department of Agriculture). AT000 extract exhibited a powerful antioxidant activity with a value of 301774 Trolox equivalents compared to the reference compounds.

This strong antioxidant activity is due to the richness of the plants constituting AT000 extract in phenolic compounds having the capacity to trap free radicals. Eugenol, the main component isolated from *Eugenia caryophyllata* possesses potent antioxidant properties [37, 38]. The ethanolic extract of *Liquidambar orientalis* leaves also showed a high antioxidant activity [39]. Ethyl acetate extract of *Aquilaria agallocha* have been reported to have free radical scavenging activity [40], and antioxidant properties *in vitro* at different concentrations by inhibition of nitrite-induced oxidation of hemoglobin in human blood hemolysate [41].

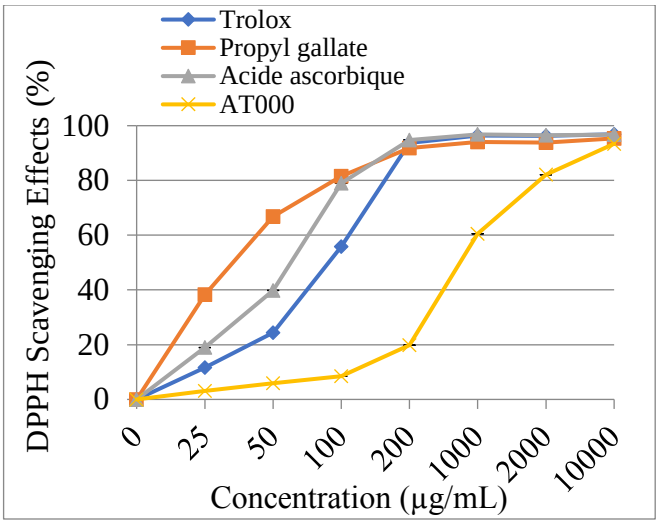


Figure 1. Free radical scavenging activity of methanol extract of AT000, trolox, propyl gallate, and ascorbic acid by 2,2-diphenyl-1-picrylhydrazyl radicals.

Table 2. ORAC values of AT000 and foods expressed as µmol Trolox Eq. (µmol/100g)

Sample	**Trolox Eq. (µmol/100 g)**
AT000	301774
Ground cloves spice	290283
Indian gooseberry, dried	261500
Oregano spice, dried	175295
Rosemary spice, dried	165280
Cinnamon spice, ground	131420

Coumarins, tannins, and flavonoids isolated from *Cyperus rotundus* tuber extracts exhibited antibacterial, antioxidant, antimutagenic, cytotoxic and apoptotic properties [42, 43]. Cyanidin-3-glucoside, the primary pigment identified in *Santalum album* berries extract possessed antioxidant potentials and high scavenging activity [44]. This combination of molecules acts synergistically to boost the overall antioxidant activity of the extract.

3.2 Anti-inflammatory and cytotoxicity assays

Anti-inflammatory activity and cytotoxicity of AT000 extract were tested *in vitro* and compared to dexamethasone as a positive control. The results are summarized in Table 3. AT000 showed the release of NO with IC_{50} value < 20 µM on macrophages RAW 264.7 and exhibited cytotoxicity with IC_{50} value > 50 µM.

Aging is characterized by inflammation involved in the pathogenesis of all age-related diseases such as AD along with increased expression of inflammatory mediators [45]. this chronic inflammation is stimulated by Aβ plaques and tangles [46]. The hallmark of the inflammatory state is the increase of serum levels of inflammatory mediators such as cytokines [47, 48], free radicals as reactive oxygen species and nitric oxide [49, 50]. AT000 extract showed an effective anti-inflammatory activity compared to dexamethasone, by inhibiting NO release from LPS-stimulated murine mouse macrophages RAW 264.7 cells and reduced LPS-induced cytotoxicity (Table 3). The plants constituting AT000 extract are rich in molecules with high anti-inflammatory properties; Eugenol has shown an anti-inflammatory activity by suppressing the expression of cyclooxygenase-2 in mouse macrophage cells stimulated with lipopolysaccharide (LPS) [51]. *Aquilaria agallocha* oil demonstrated anti-inflammatory activity, thus significantly reducing edema in carrageenan-induced paw edema model [52]. Triterpene acids isolated from *Boswellia carteri* resin demonstrated potent anti-inflammatory activity in the mouse ear edema assay [53]. Delivery of Boswellia resin extract resulted in a dose-dependent inhibition of TH1 cytokines

coupled with a dose-dependent potentiation of TH2 cytokines indicating that purified mixture of BAs from *Boswellia carteri* plant resin exhibits carrier-dependent immunomodulatory properties *in vitro* [54]. Extract of rhizomes of *Cyperus rotundus* increased HO-1 expression in a concentration-dependent manner, which was correlated with significant inhibition of iNOS/NO production in LPS-activated RAW 264.7 cells [55]. α-Cyperone, the primary compound isolated from the rhizomes of *Cyperus rotundus*, inhibits LPS-induced COX-2 expression and PGE2 production through the negative regulation of NFκB signalling in RAW 264.7 cells [56]. The (9R,10E)-9-hydroxy- α - santalal, a sesquiterpene isolated from *Santalum album* exhibited tumor-selective cytotoxicity against HL-60 cells [57]. Cyclosaplin, A new cyclic octapeptide purified from somatic seedlings of *Santalum album L.* exhibited cytotoxic activity against cultured human breast cancer (MDA-MB-231) cell line, by inducing apoptotic cell death by caspase 3 activation [58]. *Saussurea lappa* exhibited inhibitory effects on IL-8 Induction in Lipopolysaccharide-Activated Rat Macrophages [59]. Ethanolic extract of *Saussurea lappa* affects acute and chronic inflammation induced in mice and rats by inhibiting carrageenan-induced paw edema and preventing accumulation of inflammatory cells in carrageenan-induced peritonitis *in vivo* [60]. 'Dehydrocostus lactone', a sesquiterpene lactone from *Saussurea lappa* suppressed LPS-induced nitric oxide production [61] and inhibited release of TNF-α [62]. Another sesquiterpene lactone 'cynaropicrin' from *Saussurea lappa* inhibited TNF-α murine macrophage cell line and dose-dependently suppressed the proliferation of lymphocytes stimulated by Con-A [63]. Santamarin, a sesquiterpene lactone isolated from *Saussurea lappa*, represses LPS-induced inflammatory responses via expression of heme oxygenase-1 in murine macrophage cells [64]. Borneol, the main component isolated from *Dryobalanops aromatica* inhibit nicotinic receptor-mediated catecholamine secretion in bovine adrenal chromaffin cells [65, 66]. All these properties add up to demonstrate the anti-inflammatory activity of AT000 extract.

3.3 AT000 extract recovers cognitive functions in $A\beta_{25-35}$-treated mice

To determine the effective dose and the optimal injection time of the extract, the neuroprotective effect of AT000 was examined on the attenuation of the $A\beta_{25-35}$-induced learning deficits according to two administration procedures (7-days and 21-days injection).

3.3.1 Spontaneous alternation performances

The spatial working memory was first examined by evaluating spontaneous alternation in the Y-maze test. As shown in Fig. 2a and 2c, the $A\beta_{25-35}$ treatment induced highly significant spontaneous alternation deficits as compared to Sc.Aβ/V-injected mice. The 7-days AT000 treatment tended to attenuate the $A\beta_{25-35}$-induced deficits, but no significant effect vs. $A\beta_{25-35}$-treated animals was noted (Fig. 2a). No effect was noted on locomotion (Fig. 2b). On the other hand, the 21-days AT000 treatment prevented the $A\beta_{25-35}$-induced deficits at the highest dose tested (250 mg/Kg/day) with a highly significant effect vs. the $A\beta_{25-35}$ treated group (Fig. 2c). A mild effect was noted on locomotion since the ANOVA was significant, but no group effect was observed (Fig. 2d). Compared to 21-days pre-treatment, the daily injection of AT000 extract starting on the same day as $A\beta_{25-35}$ injection and lasting 7 days after, reduced $A\beta_{25-35}$-induced deficits at 250 mg/kg/day but without having a significant effect on spontaneous alteration in the Y-maze (Fig. 2a, b). This result

indicates that the extract acts according to a preventive way and not the curative way. In the case of a preventive treatment, the optimal effect was observed with increased treatment duration. The hypothesis is that the extract administered as a prolonged treatment induces a modification in the neurons, which makes the nervous system more resistant and less sensitive to aggression by $A\beta_{25-35}$-induced toxicity. Treatment for 21 days produces a more significant effect than 7 days treatment because it has more time to induce these modifications which are still poorly understood.

Table 3. Anti-inflammatory, cytotoxic activity and the anti-inflammatory ratio of AT000 and Dexamethasone.

	NO release IC_{50} (µM)	Toxicity IC_{50} (µM)	Anti-inflammatory ratio
AT000 (10 mg/mL)	1.06	> 50.00	> 47.00
Dexamethasone	4.31 ± 1.45 µM	163.22 ± 74.96 µM	37.87

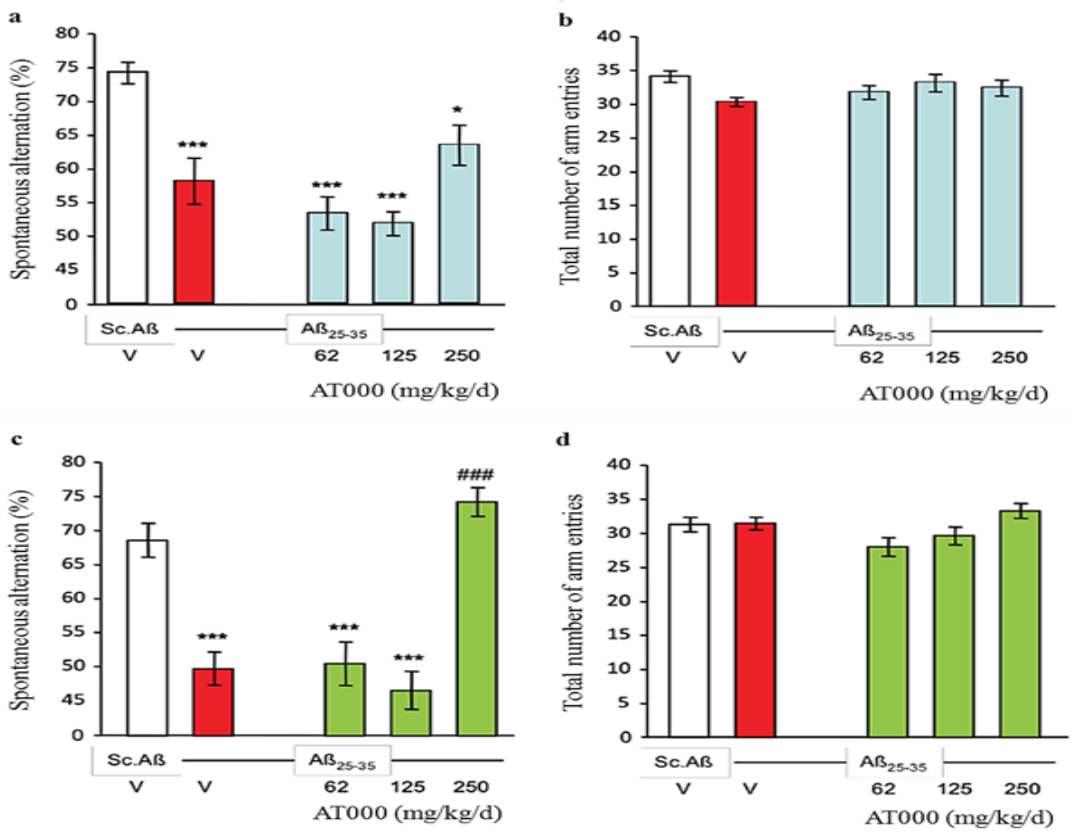


Figure 2. Effects of AT000 p.o. administration during 7 days (a, b) and 21 days (c, d) on $A\beta_{25-35}$-induced spontaneous alternation deficits in mice. V, vehicle solution. *** $p < 0.001$, * $p < 0.05$ vs. the V-treated Sc.Aβ group; ### $p < 0.001$ vs. the $A\beta_{25-35}$ treated group; Dunnett's test.

3.3.2 Step-through passive avoidance test

The contextual long-term memory was evaluated using the step-through passive avoidance test. The $A\beta_{25-35}$ treatment led to highly significant passive avoidance deficits as compared to Sc.Aβ/V-injected mice, both regarding step-through latency (Fig. 3a, c) and escape latency (Fig. 3b, d) during the retention session. The 7-days AT000 treatment alleviated the $A\beta_{25-35}$-induced deficits, with important prevention at the highest dose tested (250 mg/Kg/day) on the step-through latency parameter (Fig. 3a). However, the 21-days AT000 treatment dose-dependently alleviated the $A\beta_{25-35}$-induced deficits, with a significant prevention at the highest dose tested (250 mg/Kg/day) on both the step-through latency (Fig. 3c) and escape latency parameter (Fig. 3d). Note that the treatments did not affect the step-through latency or shock sensitivity during the training session.

3.3.3 Lipid peroxidation measure

Lipid peroxidation, one of the essential biochemical parameters of amyloid toxicity, was also analyzed in the hippocampus to validate the neuroprotective activity of the extract. According to the 7-days AT000 injection procedure, $A\beta_{25-35}$ treatment induced highly significant increase (+87%) in lipid peroxidation in the hippocampus, as compared to Sc.Aβ/V-injected mice (Fig. 4a). The 7-days AT000 treatment dose-dependently attenuated the $A\beta_{25-35}$-induced increase, with a significant effect at the two highest doses tested (125 and 250 mg/Kg/day) (Fig. 4a). At 250 mg/kg/day, the lipid peroxidation level remained significantly higher than Sc.Aβ/V-injected mice (+29%). According to the 21-days AT000 injection procedure, $A\beta_{25-35}$ treatment induced highly significant increase (+33%) in the level of peroxidized lipids in the hippocampus, as compared to Sc.Aβ/V-injected mice (Fig. 4b). The 21-days AT000 treatment dose-dependently attenuated the $A\beta_{25-35}$-induced increase, with a significant and complete blockage at the highest doses tested (Fig. 4b).

The pre-treatment by oral administration of AT000 extract for 21 days, alleviated the pathology induced intracerebroventricularly in mice and showed significant activity on the attenuation of $A\beta_{25-35}$-induced learning deficits, with a highly significant effect at 250 mg/kg/day (Fig. 2, 3, 4). This neurotoxic peptide leads to cognitive and behavioral disorders including spatial working memory and contextual long-term memory, as well as biochemical modifications such as lipid peroxidation. The effect inducing concentration of AT000 extract was significantly higher than the previously reported concentration for Amino-tetrahydrofuran derivative ANAVEX1-41 [10 μg/kg; [32]. However, ANAVEX1-41 was administered intraperitoneally suggesting that the mode of administration plays a role in determining the effective dose. Koo *et al.* and Jeon *et al.* studied the effect of SuHeXiang essential oil on attenuation of amyloid-beta-induced alteration of memory. However, their studies did not report an effective dose [24, 25]. On the other hand, oral administration in the present study showed an advantage over inhalation for the determination of an active dose of this extract *in vivo* for the first time.

3.4 Experiment 3 (Evaluation of synergy)

In this experiment, we evaluated the impact of *Dryobalanops aromatica and Saussurea lappa* in the composition to demonstrate synergy between the different plants constituting the AT000 extract. For this, we prepared 4 extracts by eliminating these two plants. We evaluated the neuroprotective effect of these 4 extracts on the attenuation of $A\beta_{25-35}$-induced learning deficits first with spatial working memory: spontaneous alternation in the Y-

maze and contextual long-term memory: passive avoidance test. The impact of these extracts on lipid peroxidation in the hippocampus was also evaluated. This last experiment was performed at the active dose of the 21-days injection procedure.

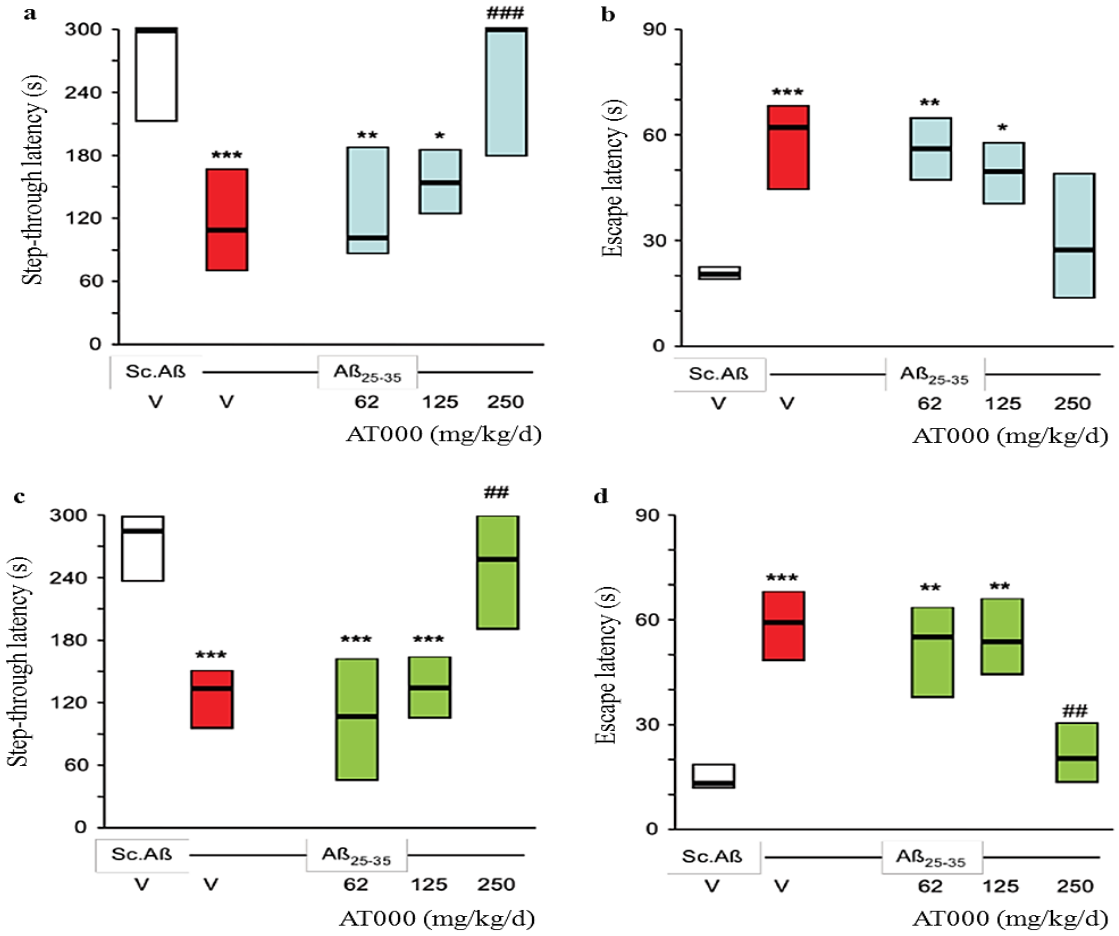


Figure 3. Effects of AT000 administration during 7 days (a, b) and 21 days (c, d) on $A\beta_{25-35}$-induced passive avoidance deficits in mice: (a, c) step-through latency and (b, d) escape latency measured during the retention session. V, vehicle solution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the V-treated Sc.Aβ group; ## $p < 0.01$, ### $p < 0.001$ vs. the $A\beta_{25-35}$-treated group; Dunn's test.

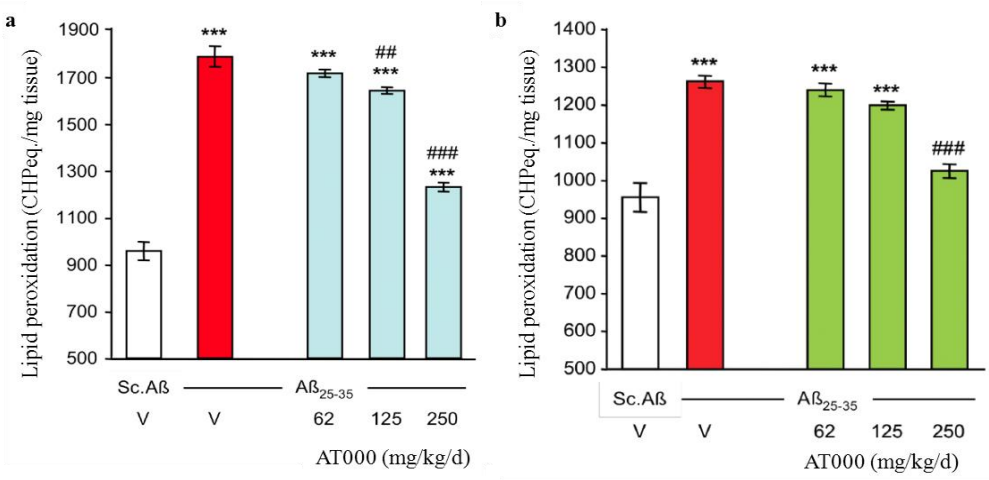


Figure 4. Effects of AT000 administration during 7 days (a) and 21 days (b) on $A\beta_{25-35}$-induced increase in lipid peroxidation in the mouse hippocampus. V, vehicle solution. *** $p < 0.001$ vs. the V-treated Sc.Aβ group; ## $p < 0.01$, ### $p < 0.001$ vs. the $A\beta_{25-35}$-treated group; Dunn's test.

3.4.1 Spontaneous alternation performances

Spontaneous alternation in the Y-maze was evaluated first, as shown in Fig. 5a and 5b. $A\beta_{25-35}$ treatment induced highly significant spontaneous alternation deficits as compared to Sc.Aβ/V-injected mice. The AT000 treatment blocked the $A\beta_{25-35}$-induced deficit, when solubilized in Vehicle 1 solution (Fig. 5a) as well as in-vehicle 2 solution (Fig. 5b). Neither the 1 or 2 plants formulations (AT001 or AT002) or the remaining 7 or 8 plants formulations (AT007, AT008) affected the $A\beta_{25-35}$-induced alternation deficit (Fig. 5b). No effect was noted on locomotion.

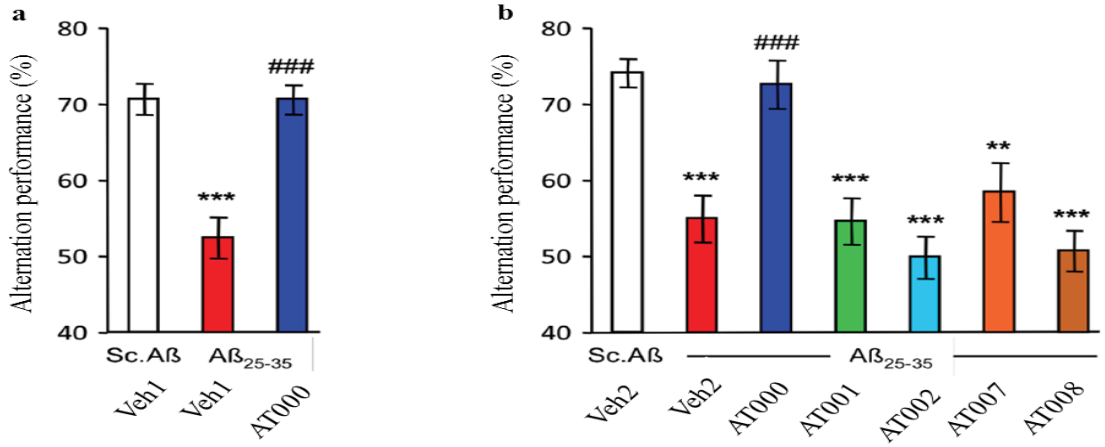


Figure 5. Comparative effects of *Dryobalanops aromatica*, *Saussurea lappa* and AT formulations p.o. administration during 21 days on $A\beta_{25-35}$-induced spontaneous alternation deficits in mice. Veh1, vehicle 1 solution (DMSO 5% in water); Veh2, vehicle 2 solution (DMSO 10% in grapeseed oil). *** $p < 0.001$, ** $p < 0.01$ vs. the V-treated Sc.Aβ group; ### $p < 0.001$ vs. the V-treated $A\beta_{25-35}$ group; Dunnett's test.

3.4.2 Step-through passive avoidance test

The contextual long-term memory was evaluated using the step-through passive avoidance test. $A\beta_{25-35}$ treatment induced highly significant passive avoidance deficits as compared to Sc.Aβ/V-injected mice, both regarding step-through latency (Fig. 6a, c) or escape latency (Fig. 6b, d). AT000 treatment blocked the $A\beta_{25-35}$-induced deficits when solubilized in Vehicle 1 solution (Fig. 6a, c) as well as in-vehicle 2 solution (Fig. 6b, d). Neither the 1 or 2 plants formulations (AT001 or AT002) or the remaining 7 or 8 plants formulations (AT007, AT008) affected the $A\beta_{25-35}$-induced passive avoidance deficits, both in terms of step-through latency (Fig. 6a, c) or escape latency (Fig. 6b, d).

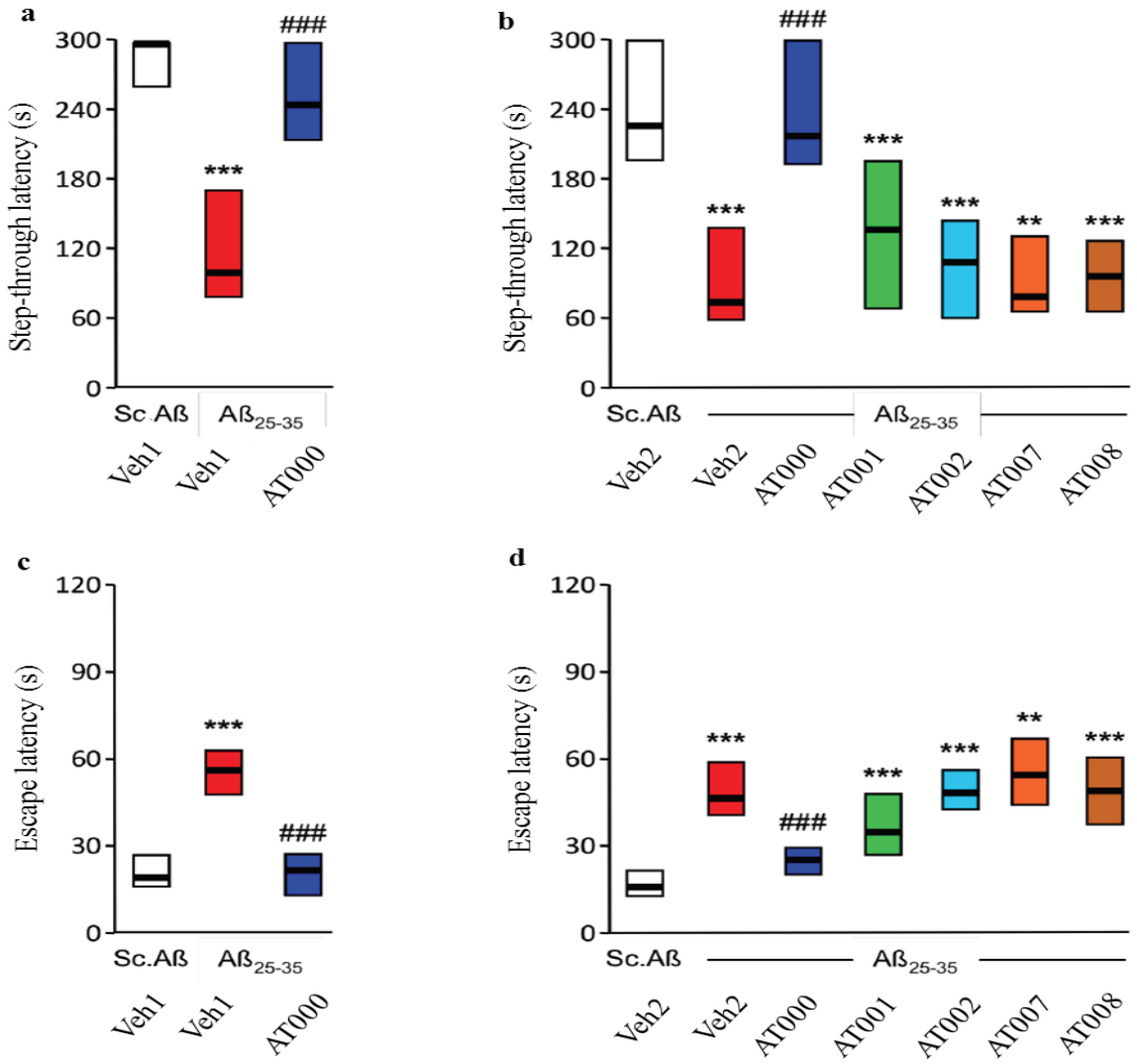


Figure 6. Comparative effects of *Dryobalanops aromatica*, *Saussurea lappa* and AT formulations on $A\beta_{25-35}$-induced passive avoidance deficits in mice: (a) step-through latency and (b) escape latency measured during the retention session. Veh1, vehicle 1 solution (DMSO 5% in water); Veh2, vehicle 2 solution (DMSO 10% in grapeseed oil). ** $p < 0.01$, *** $p < 0.001$ vs. the V-treated Sc.Aβ group; ### $p < 0.001$ vs. the $A\beta_{25-35}$-treated group; Dunn's test.

3.4.3 Lipid peroxidation measure

Finally, we evaluated lipid peroxidation, an index of oxidative stress. The $A\beta_{25-35}$ treatment induced highly significant increase (+44%) in lipid peroxidation in the hippocampus, as compared to Sc.Aβ/V-injected mice in Veh1 conditions (Fig. 7a) and a highly significant increase (+63%) in lipid peroxidation in the hippocampus, as compared to Sc.Aβ/V-injected mice in Veh2 conditions (Fig. 7a). AT000 treatment blocked the $A\beta_{25-35}$-induced increase highly significantly as compared to $A\beta_{25-35}$/V-treated mice in both vehicle conditions (Fig. 7a, b). Neither the 1 nor 2 plants formulations (AT001 or AT002) or the remaining 7 or 8 plants formulations (AT007, AT008) affected the $A\beta_{25-35}$-induced increase in lipid peroxidation (Fig. 7b).

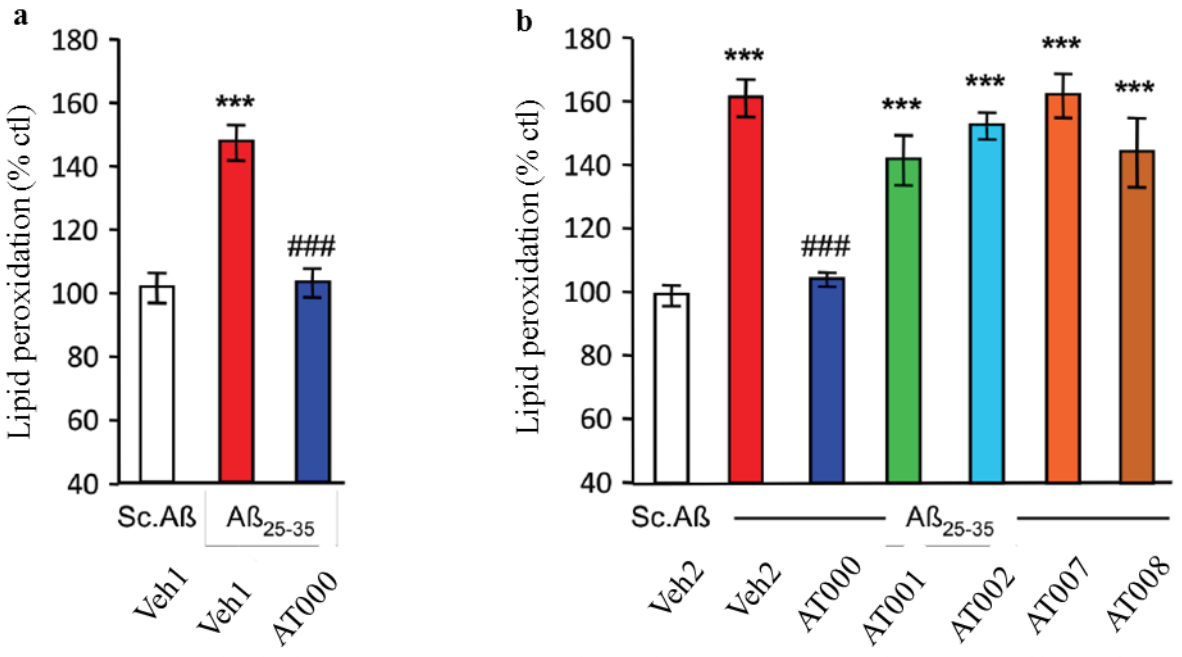


Figure 7. Comparative effects of *Dryobalanops aromatica, Saussurea lappa,* and AT formulations p.o. administration during 21 days on $A\beta_{25-35}$-induced increase in lipid peroxidation in the mouse hippocampus. Veh1, vehicle 1 solution (DMSO 5% in water); Veh2, vehicle 2 solution (DMSO 10% in grapeseed oil). *** $p < 0.001$; ### $p < 0.001$ vs. the V-treated $A\beta_{25-35}$ group; Dunnett's test.

The synergistic effect of *Dryobalanops aromatica* and *Saussurea lappa* was evaluated. For this purpose, 4 different extracts were prepared to study the impact on the overall activity of the extract. The 4 extracts were tested on spatial working memory, contextual long-term memory and lipid peroxidation at the active dose (250 mg/kg/day) and 21-days treatment. The results obtained showed that the elimination of these plants from the extract led to the total disappearance of the neuroprotective activity (Fig. 5, 6, 7). The results also showed that *Dryobalanops aromatica* and *Saussurea lappa* alone do not show any neuroprotective activity. The activity is due to the combination of these 9 plants which, by extracting them together, will interact according to the potentiation and the effect will be boosted. Additionally, the two controversial plants were

found to be essential for the neuroprotective activity of the extract.

The mechanisms of action of the plants constituting AT000 extract have been previously studied. Essential oil of *Eugenia caryophyllata* possesses anticonvulsant activity against maximal electroshock (MES)-induced tonic seizures in male mice [67]. *Liquidambar orientalis* has also been reported to have anticonvulsant and sedative properties by substantially delaying the appearance of PTZ-induced convulsion [68]. Aqueous extract of *Aquilaria agallocha* stems showed inhibitory effects on histamine release from rat peritoneal mast cells leading to inhibition of immediate hypersensitivity reactions [69]. Benzylacetone, α-gurjunene and calarene, principles volatile obtained from Agarwood oil showed sedative activity in mice using a system of spontaneous vapor administration [70]. Alcoholic extract of *Aquilaria agallocha* (AEAA) has anticonvulsant activity using PTZ (Pentylenetetrazole) to induced convulsion. At a higher dose, an AEAA showed a significant anticonvulsant effect by increasing latency of clonus, an onset of tonic seizures and declined mortality of mice [71]. *Cyperus rotundus* tubers extract attenuated significantly learning and memory disturbance in passive avoidance paradigm and spatial cognitive deficit in Morris water maze produced by lesioning of the NBM in rats [72]. *Cyperus rotundus* tubers extract treatment showed therapeutic potential in aging and age-related neurodegenerative disorders by preventing the cognitive impairments significantly following NBM lesion [72]. Pre-treatment of neurons with *Cyperus rotundus* extract ameliorates the mitochondrial and plasma membrane damage induced by SIN-1, restores the cellular morphology and improves the antioxidant status by regulating the oxidative stress biomarkers [73]. Total oligomeric flavonoids (TOFs) extracted from *Cyperus rotundus* demonstrated neuroprotective effect against the ischemic–reperfusion, induced neurodegeneration in the rat model by reducing oxidative stress, excitotoxicity, neurological and behavioral alterations [74]. Pre-treatment with an alcoholic extract of *Saussurea lappa* roots increased latency and also reduced mortality rate which indicates anticonvulsant property against Pentylenetetrazole and picrotoxin-induced convulsions in mice [75].

4. Conclusions

Our results demonstrated that AT000 extract has a significant neuroprotective effect on the attenuation of the $A\beta_{25-35}$-induced learning deficits and lipid peroxidation in the hippocampus. The extract also showed an effective antioxidant and anti-inflammatory activities, which indicates that AT000 extract has potential as a potent therapeutic agent for neuroprotection and prevention of AD. We are continuing studies on the AT000 extract to better understand the synergy mechanisms between the different plants in the mixture as well as chemical composition of AT000 extract. For that the analytical study part including the chemical composition of the plants is crucial.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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