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Phyto-chemical analysis and protective potential of novel herbal formulation against neurotoxicity induced by $A\beta_{1-42}$ peptide, inflammation and oxidative stress

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ABSTRACT

Alzheimer's disease is a neurodegenerative disorder that causes a progressive deterioration of memory and intellectual capacities. It causes irreversible lesions in the brain and leads to a decline in cognitive functions. New non-drug approaches should, therefore, be explored to slow the progression of the disease. We previously demonstrated the biological activities of a plant composition, designated as AT000. This herbal extract contains a plant heretofore unlisted in the European Pharmacopoeia. In this study, we substituted this plant with camphor leaves and studied the antioxidant, anti-inflammatory and neuroprotective activities in vitro of this new formulation, designated as AT00X. The chemical composition of the AT00X extract using gas chromatography was also evaluated. Results showed significant antioxidant and anti-inflammatory activity with 224010 equivalents of Trolox, an increase in the resistance of control blood to free radical attack up to 54.21% by KRL assay and an anti-inflammatory ratio of 99.13. The extract significantly improves neuronal survival and decreases ROS production at 5 µg/ml. It also stimulates neurite network reconstitution at 10 µg/ml. It can, therefore, be concluded that AT00X extract is an effective therapeutic agent for cognitive deficits caused by Alzheimer's disease.

Keywords: Alzheimer's disease, medicinal plants, amyloid beta, oxidative stress, inflammation.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of cerebral tissue that leads to irreversible loss of mental functions and difficulties in household handling routine in the elderly. It is considered the primary cause of dementia in humans (Selkoe et al., 1982). Two types of lesions characterize Alzheimer's disease. The senile plaques, which correspond to extracellular deposits of beta-amyloid peptide and the neurofibrillary tangles, which correspond to intraneuronal fibrils of abnormal Tau protein (Allanbutterfield et al., 2002; Cummings, 2004).

Amyloid $A\beta_{1-42}$ peptide plays a central role in the pathogenesis of Alzheimer's disease. Its abnormal brain accumulation triggers the following pathophysiological

processes: oxidative stress, lipid peroxidation of cell membranes, formation of reactive oxygen species (ROS), inflammation, hyperphosphorylation of tau protein, and increased glutamatergic excitotoxicity (Butterfield and Lauderback, 2002; Markesbery, 1997). These neurotoxic processes lead to the activation of the apoptotic cell death cascade (Hardy and Selkoe, 2002).

Lipid peroxidation is a key element in neurodegeneration (Butterfield, 1997; Varadarajan et al., 2000). Indeed, it is induced in brain cell membranes by Aβ peptide in a free radical process (Butterfield and Lauderback, 2002; Lauderback et al., 2001). The resulting reactive aldehydes (alkenals) alter the conformation of membrane proteins (Pocernich et al., 2001; Subramaniam et al., 2002) and are toxic to neurons (Lovell et al., 2001; Mark et al., 2002; Subramaniam et al., 2002).

The Food and Drug Administration has currently approved Aducanumab. It is a monoclonal antibody targeting the amyloid beta peptide (Cummings et al., 2021). It is the first treatment to target the underlying disease process in Alzheimer's disease and help slow cognitive decline (Rabinovici, 2021).

Other current treatments for Alzheimer's disease, however, are limited to cholinesterase inhibitors (Donepezil, Galantamine and Rivastigmine) (Misik et al., 2015) and NMDA receptor antagonists (Memantine), although doubts remain about the therapeutic efficacy of these drugs. In addition, these molecules can interact with a wide range of drugs and their consumption is associated with numerous side effects (Cacabelos, 2007).

Traditional Chinese Medicine (TCM) has a 3000-year history of human use (Lin et al., 2012). The plants are used by all ethnic groups in the traditional practices of Chinese medicine, with the hope of promoting health and managing various diseases such as pain, colds, inflammation, heart disease and neurodegenerative diseases (Adams et al., 2007). The characteristic of medicinal plants is their ability to generate many secondary metabolites, aromatic substances and microorganisms.

An ethnopharmacological approach has made it possible to identify potential new drugs from plant sources, indicating a potential for therapeutic use thanks to their wealth of assets (Sahoo et al., 2010). Herbal products are of interest to many patients and health practitioners as about 70% of the world's population uses herbal medicines for some of their primary health care (Wills et al., 2000). To date, there are more than 11,000 species of medicinal plants in use around the world and about 300 species are commonly used in Asian countries and other countries (Chen et al., 2012).

Many prescriptions in Chinese medicine contain a combination of medicinal plants to form preparation with a therapeutic effect. The interactions of plants are based on the notions of complementation or antagonism. It has been found that the presence of one plant can alter the effect of the other when they are co-administered. The combined, complementary or antagonistic effects are based on six basic modes of interaction between herbs; namely strengthening, potentiation, restraint, detoxification, neutralization and toxicity (Szeto et al., 2011; Thompson et al., 2012; Wang et al., 2012; Zhang et al., 2013).

We previously studied the antioxidant, anti-inflammatory and neuroprotective effects in vivo of a 9-plant extract from traditional Chinese medicine designated as AT000. Our results demonstrated that AT000 extract has a significant neuroprotective effect on the attenuation of learning deficits induced by the toxic peptide $A\beta_{25-35}$ and on the lipid peroxidation in the hippocampus at a dose of 250 mg/kg/day (Iskandar et al., 2018). The extract also showed strong antioxidant and anti-inflammatory activity. This indicates that the AT000 extract may be a potent therapeutic agent for neuroprotection and prevention of Alzheimer's disease.

AT000 extract contains an unlisted plant in the French Pharmacopoeia (*Dryobalanops aromatica*). Although this plant is exploitable in China, thanks to its therapeutic properties, its use in Europe in food supplements is considered controversial (Pharmanager, 2016). For this, an alimentary study of the 9 plants led us to substitute *D. aromatica* with *Cinnamomum camphora* that contains camphor, the major compound of *D. aromatica* (Soepadmo et al., 1995), while keeping at first the equimassic composition of the extract. This second composition designated by AT00X is therefore the first alimentary version of AT000 extract. In the present study, AT00X extract was evaluated for its biological activities *in vitro*: antioxidant, anti-inflammatory and neuroprotective activity *in vitro*. The chemical characterization of the extract was also evaluated using gas chromatography coupled to mass spectrometry (GC-MS).

MATERIALS AND METHODS

Materials

All medicinal plants were purchased from "Acegem Biological Technology Wuhu - 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, ABTS, potassium persulfate, 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, Leibovitz medium, trypsin-EDTA, DNAse I grade II, L-glutamine, brain-derived neurotrophic factor (BDNF) and inactivated calf serum were purchased from PAN Biotech (Germany). Neurobasal medium was purchased from Invitrogen (USA). $A\beta_{1-42}$ peptide was purchased

from Bachem (Germany).

Preparation of plant extract

The plants were purchased from "Acegem Biological Technology Wuhu - China" (Batch number 20160929, Certificate of Analysis NO.: ASG-BG-16-1008) and deposited in the Laboratory of Pharmacognosy of Aix Marseille University (Marseille, France). AT00X extract was prepared as described previously with some modification (Iskandar et al., 2018).

A total of 4.5 g of the mixture of *Liquidambar orientalis* (500 mg), *Saussurea lappa* (500 mg), *Aquilaria agallocha* (500 mg), *Santalum album* (500 mg), *Boswellia carteri* (500 mg), *Eugenia caryophyllata* (500 mg), *Cyperus rotundus* (500 mg), *Styrax benzoin* (500 mg), and *Cinnamomum camphora* (500 mg) was pulverized and extracted once with 45 ml of a water/ethanol mixture (70:30, v/v) at 80 to 85°C with reflux condenser for 3 h. The extract was then vacuum filtered using a Büchner flask and evaporated using a rotary evaporator at 60°C to achieve a sticky brown oil. The quantity of AT00X resin extract produced in the laboratory was limited, for which we used the AT00X powder extract from the supplier to carry out the evaluation study of biological activities.

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 (Brand-Williams et al., 1995). Briefly, 0.05 mM solution of $DPPH^{\bullet}$ was prepared in methanol, and 193.3 µl of this solution was added to 6.66 µl 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 min, and the absorbance was recorded at 517 nm using a Multiskan^{TM} Multiplate Photometer UV-Vis spectrophotometer. Control was prepared by adding 193.3 µl of the DPPH solution (0.05 mM) to 6.63 µl 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.

Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was assessed in microplates as described by Ou et al. (2001) with some modifications. The plant extract, AAPH, and fluorescein were diluted in 100 mM potassium phosphate buffer (pH 7.4). 25 µl of each extract (0.94 to 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 to 12.5 µM) as standard. The ORAC values were calculated using the neat AUCs and expressed as µmol of Trolox equivalent per 100 g of extract (µmol TE/100 g). Trolox was used as a control standard.

KRL test

The “Kit Radicaux Libres” (KRL) test allows evaluation of the overall resistance of the blood subjected to a radical attack. The intra- and extracellular antioxidant defenses contribute to the maintenance of membrane integrity and cellular functions until the lysis of blood cells. This biological test, patented by the laboratory Spiral Couternon - France, thus allows a dynamic measurement of the global potential of antiradical defense of an individual (Prost, 1992). Concretely, an aqueous or alcoholic extract of the product to be tested is added to a standardized suspension of red blood cells (or EDTA blood) which is then incubated for several hours at 37°C in the presence of a free radical generator. The intermediate results are expressed in half-hemolysis time. Using this test, we can also determine in vitro, under biological conditions, the "anti-oxidant" or "pro-oxidant" action of different compounds. Thus, by adding a compound with an anti-radical action to the medium, we increase the overall defence potential against the free radical aggression of control blood. On the contrary, by adding a pro-radical compound, we reduce the overall antiradical capacity of the control blood. The overall resistance of the control blood to the radical attack in the presence or absence of the product is expressed by the time after which 50% of the blood cells are lysed ($T_{1/2}$ in minutes). The test was carried out on the extract in a concentration range of 0 to 100 mg per litre of reaction medium for the extract.

The antiradical efficacy is expressed as a percentage of the total antiradical defence potential of the control blood (% $T_{1/2}$ of the control blood) and the results are standardized in Trolox equivalents (water-soluble vitamin E analog) and gallic acid equivalents (phenolic acid).

ABTS test

This test is based on the ability of an antioxidant to stabilize the $ABTS^{\bullet+}$ cationic radical. 2,2-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) reacts with potassium persulfate ($K_2S_2O_8$) to form the $ABTS^{\bullet+}$ blue-green color radical. The radical cation $ABTS^{\bullet+}$ reacts with antioxidants and reconverts to its colorless neutral form by trapping a proton. The antioxidant activity of AT00X extract was measured according to the ABTS test using the method described by Re et al. (1999) with some modifications. The $ABTS^{\bullet+}$ working solution was prepared by reacting ABTS (9.5 ml, 7 mM) with potassium persulfate (245 µl, 100 mM) and raising the volume to 10 mL with distilled water. The solution was kept in the dark at room temperature for 18 h, then diluted with potassium phosphate buffer (0.1 M, pH 7.4) until an absorbance of 1 (± 0.02) at 734 nm. AT00X samples at different concentrations (0.025 to 10 mg/ml) were prepared in methanol. The control was prepared by adding 2.9 ml of the $ABTS^{\bullet+}$ solution to 0.1 ml of methanol. The absorbance of the resulting clear mixture was recorded at 734 nm. The IC_{50} values, which represent the concentration of the extract that causes the stabilization of 50% of the $ABTS^{\bullet+}$ radicals, were calculated from the percentage of stabilization as a function of the concentration curve. The stabilization of $ABTS^{\bullet+}$ by AT00X extract was calculated using the following equation:

$$P.S\ (\%) = [1 - (A_1 / A_0)] \times 100$$

Where A_0 is the absorbance of the control reaction and A_1 is the

absorbance in the presence of the sample. Trolox was used as a positive control.

Anti-inflammatory and cytotoxicity assays

In vitro anti-inflammatory assay was determined by measuring the capacity of macrophages to generate a strong inflammatory response when stimulated with antigens, inducing NO release (Garayev et al., 2017). Cells were seeded into 48-well tissue culture plates at a concentration of 1.10^5 cells/ml (200 µl/well) for 24 h at 37°C (5% CO_2). Then the culture medium was replaced by 200 µl of medium containing the appropriate concentrations of AT00X extract (Table 1), 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 h. 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 supernatant were transferred into the wells of a 96-well tissue culture plate, and 100 µl of the Griess modified reagent was 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 AT00X 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 AT00X 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:

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

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

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

Evaluation of neuroprotective activity *in vitro*

Primary culture of cortical neurons

Rat cortical neurons were cultured as described by Callizot et al. (2013). Briefly, fetuses were collected and immediately placed in ice-cold L15 Leibovitz medium with a 2% penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solution (PS) and 1% bovine serum albumin (BSA). Cortex was treated for 20 min at 37°C with a trypsin-EDTA solution at a final concentration of 0.05% trypsin and 0.02% EDTA. The dissociation was stopped by the addition of Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L of glucose, containing DNAse I grade II (final concentration 0.5 mg/ml) and 10% fetal calf serum (FCS). Cells were mechanically dissociated by three forced passages through the tip of a 10 ml pipette. Cells were then centrifuged at 515 g for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in a defined culture medium consisting of neurobasal medium with a 2% solution of B27 supplement, 2 mmol/L of L-glutamine, 2% of PS solution, and 10 ng/ml of brain-derived neurotrophic factor (BDNF). Viable cells were counted in a Neubauer cytometer, using the trypan blue exclusion test. The cells were seeded at a density of 20,000 per well in 96-well plates previously coated with poly-L-lysine and cultured at 37°C in air (95%)/CO_2 (5%) incubator. The medium was changed every 2 days. Cortical neurons were intoxicated with Aβ solutions after 11 days of culture.

Table 1. Different concentrations of AT00X extract tested *in vitro*.

Control (vehicle)
+ Aβ (20µM 24H) / vehicle
+ Aβ (20µM 24H) / AT00X 500 ng/ml
+ Aβ (20µM 24H) / AT00X 1 µg/ml
+ Aβ (20µM 24H) / AT00X 5 µg/ml
+ Aβ (20µM 24H) / AT00X 10 µg/ml
+ Aβ (20µM 24H) / AT00X 30 µg/ml
+ Aβ (20µM 24H) / AT00X 60 µg/ml
+ Aβ (20µM 24H) / AT00X 90 µg/ml
+ Aβ (20µM 24H) / BDNF (50 ng/ml)

Test compound and human $A\beta_{1\text{-}42}$ exposure

The $A\beta_{1\text{-}42}$ preparation was done following the procedure described by Callizot et al. (2013). Briefly, $A\beta_{1\text{-}42}$ peptide was dissolved in the defined culture medium mentioned above, devoid of serum, at an initial concentration of 40 µmol/L. This solution was gently agitated for 3 days at 37°C in the dark and immediately used after being properly diluted in culture medium to the concentrations used (20 µM, corresponding to 2 µM of AβO quantified with Western Blot).

AT00X extract (500 ng/ml to 90 µg/ml) was dissolved in a culture medium and then pre-incubated with primary cortical neurons for 1 hour before the $A\beta_{1\text{-}42}$ application. $A\beta_{1\text{-}42}$ preparation was added to a final concentration of 20 µM diluted in the control medium in presence of AT00X extract or BDNF (used as gold standard).

Organization of culture plates

AT00X plant extract was tested on a culture in a 96-well plate (6 wells per condition). AT00X extract was pre-incubated for 1 hour before Aβ application. The following conditions will be assessed:

Staining on live cells for ROS evaluation

24 hours after intoxication, the live cells were incubated with cellROX green reagent for 30 min at 37°C, then cells were fixed by a cold solution of ethanol (95%) and acetic acid (5%) for 5 min at -20°C.

Neuron survival and neurite network

After permeabilization with 0.1% of saponin, cells were incubated for 2 hours with mouse monoclonal antibody anti-microtubule-associated-protein 2 (MAP-2) at dilution of 1/400 in PBS containing 1% fetal calf serum and 0.1% of saponin (this antibody stains specifically cell bodies and neurites, allowing the study of neuronal cell death and neurite network).

This antibody was revealed with Alexa Fluor 568 goat anti-mouse IgG at the dilution of 1/400 in PBS containing 1% FCS, 0.1% saponin, for 1 hour at room temperature.

For each condition, 30 pictures (representative of all well area) per well were automatically taken using ImageXpress (Molecular Devices) with 20x magnification. All images were taken under the same conditions.

Statistical analysis

Data are expressed in the percentage of control conditions (no injury, no compound = 100 %). All values are expressed as mean +/- SEM (standard error of the mean) from 6 wells per condition per culture. Graphs and statistical analyses were performed by one-way ANOVA, followed by Dunnett's test when allowed, using GraphPad Prism software version 5.0. $P < 0.05$ was considered significant.

Identification by GC-MS

For qualitative and quantitative analysis of the components, gas chromatography-mass spectrometry (GC-MS) was performed. 10 mg of AT00X extract were weighed and added to 1 ml of MeOH. After solubilization, the solution was filtered and 100 µl of the filtered solution was dried with nitrogen, then 100 µl of BSTFA were added and the whole was incubated at 70°C for 20 min. 2 µl were injected for GC-MS analysis. The compounds were previously derivatized to make them volatile. The derivation was carried out in a 2mL CHROMACOL type tube sealed with a cap and an airtight septum. 100 µl of BSTFA (N,O-bis (trimethylsilyl trifluoroacetamide, SIGMA-ALDRICH M7891) were added directly to 0.1mg of dry sample. The solution was incubated for 20 min at a temperature of 70°C, and 2 µl were directly injected into GC-MS. The analyses were carried out with a GC-MS (Shimadzu GC-MS 2010) composed of a QP 2010S mass spectrophotometer detector equipped with an electronic impact ion source and a quadrupole type analyser, a GC-MS Solution acquisition software as well as banks (NIST, WILEY). The compounds were separated using a DB-1 capillary column (30 m × 0.32 mm × 0.25 µm, from JW Scientific) and whose temperature limits are between -60 and 350°C. The pressure in the column is 3 psi and the flow rate was 1.59 ml/min. The injector temperature is 280°C. The detection mode is the full scan. The programming of the temperature gradient is as follows: 1 min at 60°C, then increasing from 100 to 260°C at a rate of 4°C/min, then 10 min at 260°C. The total elution time is 62 min.

RESULTS

Antioxidant activity

The results of the DPPH, ORAC and ABTS tests are presented in Table 2. The antiradical power of AT00X extract evaluated by the DPPH test is represented by an IC_{50} value of 3498 µg/ml. According to the results, AT00X hydroalcoholic extract has a moderate antioxidant capacity, its IC_{50} is relatively low than those of standards Trolox, Ascorbic acid and Propyl gallate whose value is 298, 196.6 and 65.18 µg/ml respectively. The ORAC values are expressed in µmol Trolox equivalent per 100g of extract (TE µmol/100 g).

Results show the antioxidant potency of AT00X extract relative to foods known to have significant antioxidant activity (*USDA Database for Oxygen Radical Absorbance Capacity of Selected Foods*). AT00X extract has a potent antioxidant activity of 224010 equivalents of Trolox, compared to reference compounds.

Table 2 shows the result of the ABTS test applied to AT00X extract. The measured value 2819.2 µg/ml is moderate compared to Trolox (793.5 µg/ml).

KRL test

The results indicate that AT00X extract has a dose-dependent antiradical capacity and this antioxidant power is strongly increasing in a concentration range of 0 to 20 mg/L of the reaction medium. Thus, at a concentration of 20 mg/L, AT00X extract increases the resistance of the control blood to a radical attack up to 54.21% (Table 3). In the same conditions in vitro, one gram of AT00X extract has an antioxidant capacity respectively equivalent to 1058.67mg of Trolox® or 492.10 mg of gallic acid (Table 4).

Evaluation test of anti-inflammatory activity

The anti-inflammatory activity and cytotoxicity of AT00X extract were tested *in vitro* and compared to dexamethasone as a positive control. The results are summarized in Table 5. AT00X extract shows a NO release with an IC_{50} value < 20 µM on macrophages RAW 264.7 and cytotoxicity with an IC_{50} value > 50 µM.

Evaluation of the neuroprotective activity of AT00X extract

Effect of AT00X extract on the survival of cortical neurons in the rat and on the neurite network in the presence of the $A\beta_{1-42}$ peptide after 24 hours of application

The neurotoxic peptide $A\beta_{1-42}$ induces a significant neuronal death (by 30%) and a loss of the neurite network (~ 40%) as compared to the control group (Figure 1). The AT00X extract added 1h before the application of the peptide, showed a significant effect on the survival of neurons at 5 µg/ml (93% of the neurons were protected against amyloid damage). In addition, a

Table 2. Results of DPPH, ORAC and ABTS tests of AT00X extract, and references.

Sample	DPPH test IC_{50} (µg/ml)	ORAC test Equivalent Trolox (µmol/100 g)	ABTS test IC_{50} (µg/ml)
AT00X	3498	224010	2819.2
Trolox	298		793.5
Ascorbic Acid	196.6		
Propyl gallate	65.18		
Oregano spice		175295	
Rosemary spice		165280	
Dried thyme		157380	
Cinnamon spice		131420	
Turmeric spice		127068	
Pepper spice		118400	

Table 3. Percent change in $T_{1/2}$ hemolysis of control blood.

Concentrations (mg/L) *	% Variation of $T_{1/2}$ hemolysis of blood
	AT00X Extract
0	0
1	4.83
2	7.55
5	16.28
10	28.91
20	54.21
50	116.72
100	201.52

Table 4. Equivalents mg of Trolox and mg of gallic acid/g of extract.

Concentrations (mg/L) *	Antiradical activity of AT00X extract	
	Equivalents mg of Trolox and mg of Gallic acid/g of extract	
	Trolox	Gallic acid
0	0	0
1	1887.35	877.29
2	1474.13	685.22
5	1272.08	591.30
10	1129.12	524.84
20	1058.67	492.10
50	911.87	423.86
100	787.15	365.89

* : Concentration in mg of extract per litre of reaction medium

Table 5. Anti-inflammatory activity of AT00X extract.

	NO release IC_{50} (µM)	Toxicity IC_{50} (µM)	Anti-inflammatory ratio
AT00X	0.81	80.30	99.13
Dexamethasone	4.31 ± 1.45 µM	163.22 ± 74.96 µM	37.87

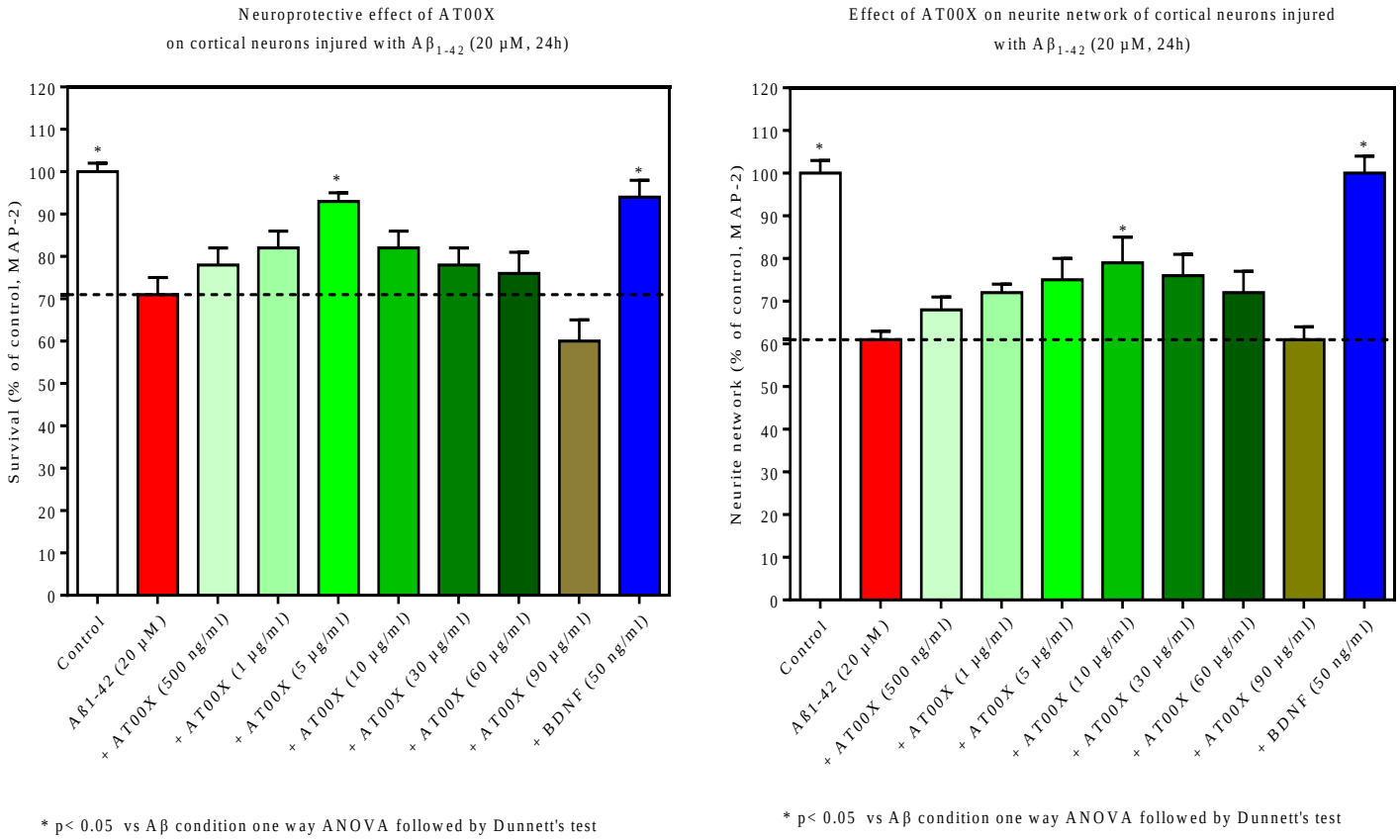


Figure 1. Effect of AT00X on the survival of neurons and the neurite network of rat primary cortical neurons intoxicated by peptide $A\beta_{1-42}$ (24 h, 20 µM corresponding to 2 µM of AβO). The data expressed as a percentage of control show the mean ± SEM (100% = no Aβ, no compound). One-way ANOVA followed by Dunnett's test, n = 4-6. * p < 0.05 was considered significant.

protective effect was observed on the neurite network. At 10 µg/ml, network neuroprotection was significant (Figure 1). However, the other doses of AT00X did not significantly impact neuronal survival. BDNF, used here as a positive control, completely reversed the glutamate-induced neuronal cell death and showed a significant protective effect (neuron survival and neurite network).

Effect of AT00X extract on the production of ROS in rat cortical neurons in the presence of $A\beta_{1-42}$ peptide after 24 hours of application.

The neurotoxic peptide $A\beta_{1-42}$ induced a significant increase in ROS production in the cytoplasm of neurons, as previously described (Callizot et al., 2013). AT00X added 1h before peptide application, is able to reduce the generation of ROS in neurons (~ 25% reduction), the effect is significant for 5, 30 and 60 µg/ml (Figure 2). On the other hand, at 90 µg/ml, AT00X has slightly increased the production of ROS. Other doses of AT00X did not impact the production of ROS. The BDNF used as a standard, partially counter-acted the production of ROS after intoxication.

Coloration of neurons and neurites

Coloration of neurons shows the death of cells after intoxication with Aβ peptide (20 µM). AT00X extract treatment protects the neurons and improves their survival as well as neurite network reconstruction (Figure 3).

Identification by GC-MS

Mass-coupled gas chromatography (GC-MS) allows the identification of most compounds whose mass does not exceed 500 Daltons. The results of GC-MS analysis of AT00X extract are shown in Table . The relative amount of each component was determined by calculating the peak area of the TIC chromatogram. The percentage of similarity was given by NIST mass spectral library.

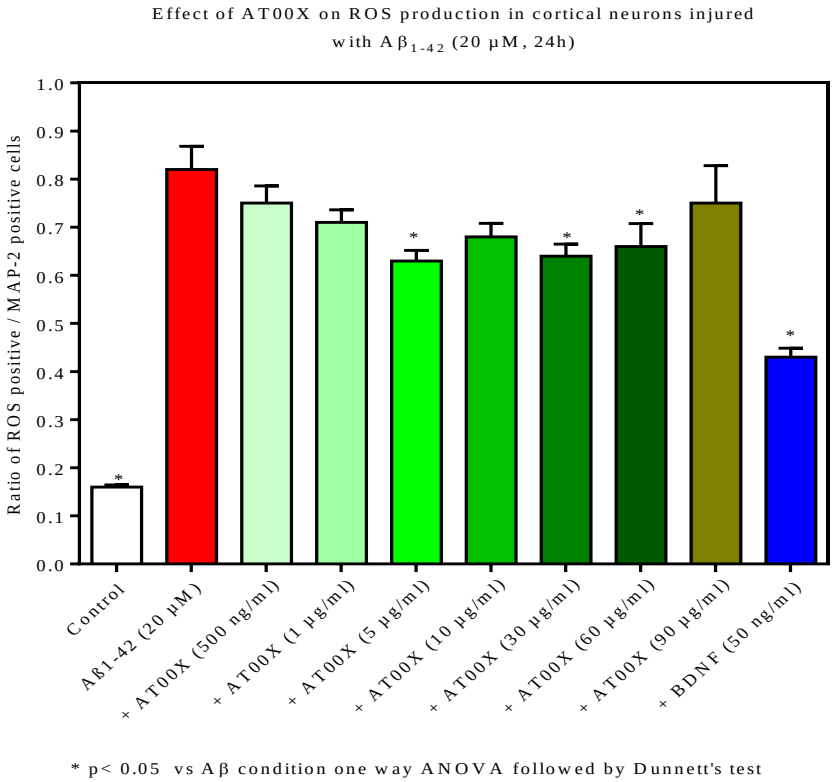
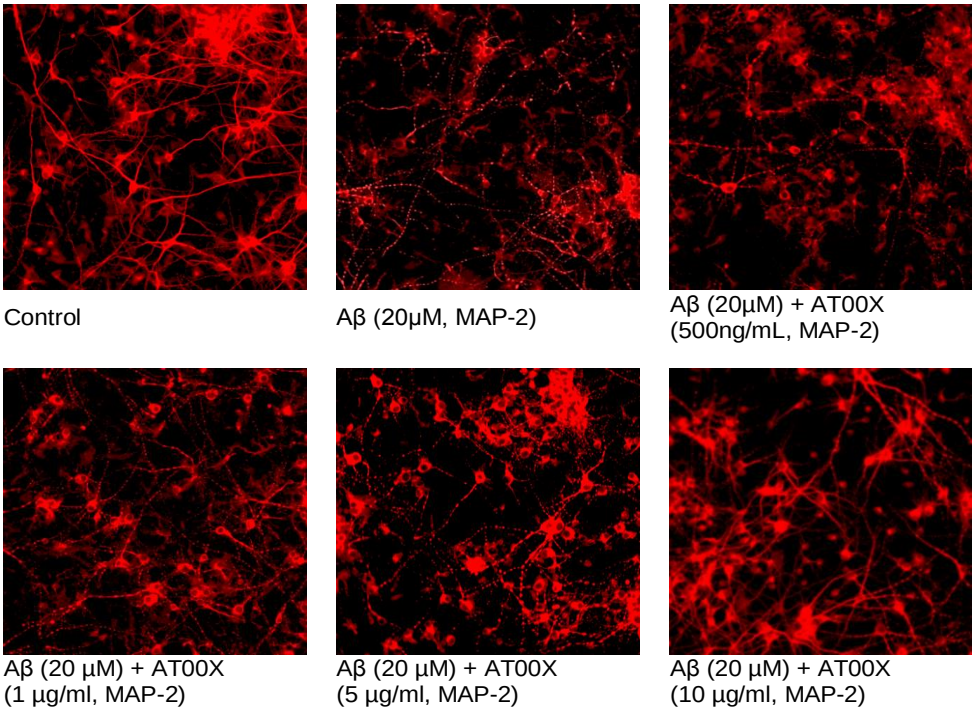


Figure 2. Effect of AT00X on the production of ROS in rat primary cortical neurons (MAP-2 positive) intoxicated by $A\beta_{1-42}$ peptide (24h, 20 µM corresponding to 2µM of AβO). The data expressed as a percentage of control show the mean ± SEM (100% = no Aβ, no compound). One-way ANOVA followed by Dunnett's test, n = 4-6. * p <0.05 was considered significant.



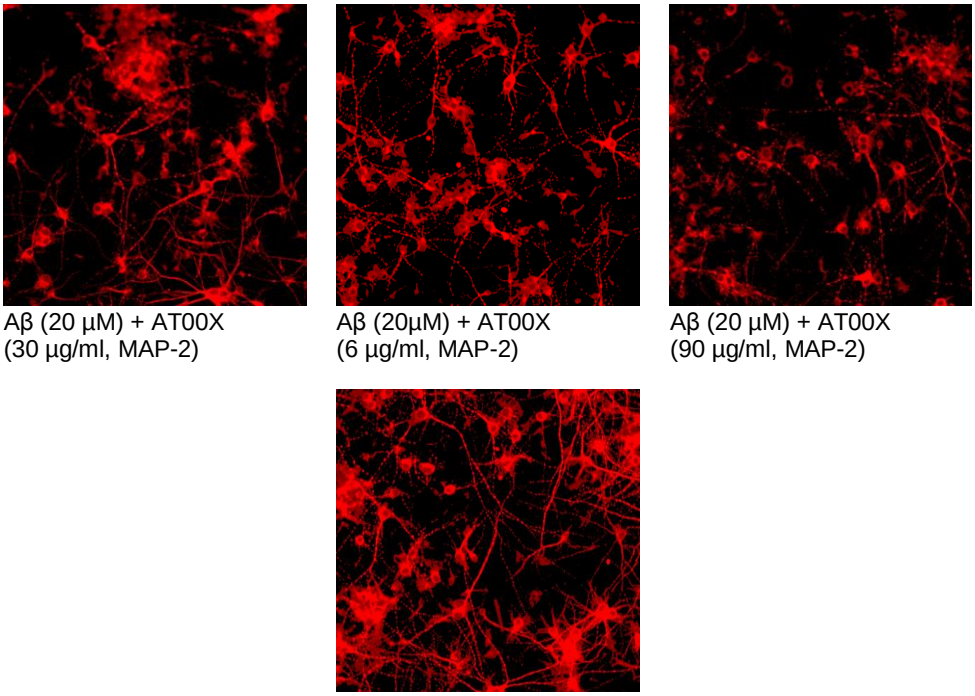


Figure 3. Coloration of neurons and neurite network after treatment with AT00X extract.

Table 6. Molecules identified in AT00X extract.

Peak	Retention time (min)	Area (%)	Compound	% Similarity NIST
1	13.26	14.85	Benzoic acid	86
2	15.38	2.85	Glycerol	91
3	15.74	0.39	Succinic acid	97
4	20.48	0.42	Isoeugenol	77
5	21.31	0.22	Eugenol	78
6	21.87	1.76	Vanillin	95
7	22.10	4.39	Malic acid	92
8	34.67	1.27	Gallic acid	91
9	37.77	0.44	Nerolidol isobutyrate	69
10	41.36	0.41	Pimaric acid	91
11	41.67	0.42	Pimaric acid	85
12	41.89	1.05	Isopimaric acid	89
13	42.78	1.08	Dehydroabietic acid	88
14	43.45	1.07	Abietic acid	90

DISCUSSION

In the present study, we formulated a new alimentary herbal extract by substituting *Dryobalanops aromatica* with *Cinnamomum camphora*. The biological activities of AT00X extract were studied. The results obtained show that this new extract has strong antioxidant, anti-inflammatory and neuroprotective capacities due to the richness of this extract in phenolic and terpenic compounds. Phenolic acids such as benzoic acid and gallic acid inhibit the neurodegeneration of the hippocampus by virtue of their powerful antioxidant, anti-inflammatory and anti-apoptotic properties(Latha and Daisy, 2011; Mansouri et al., 2013). They fight against

$A\beta_{25-35}$ peptide-induced neurotoxicity in PC12 cells through antioxidant and anti-apoptotic activities (Hong et al., 2012). They also contribute to a reduction in levels of lipid peroxidation (LPO), NO and ROS, markers of oxidative stress, the main actor of many neurodegenerative diseases (Dugan et al., 1995; Oyagbemi et al., 2016).

The antioxidant properties of phenolic acids can be attributed to direct free radical scavenging activity, inactivation of enzymes responsible for ROS production and/or upregulation of antioxidant enzymes (Saibabu et al., 2015; Thyagaraju and Muralidhara, 2008).

Vanillin, a phenolic compound, offers neuroprotection because of its antioxidant, anti-inflammatory and anti-apoptotic properties. Vanillin neutralizes apoptosis by preserving mitochondrial functions and antioxidant action (Dhanalakshmi et al., 2015). A study conducted by Kim and Park (2017), showed that vanillin treatment significantly alleviated Scopolamine's learning and memory impairments.

According to recent reports, eugenol attenuates the neurotoxicity induced by beta-amyloid peptides in vitro. It inhibits the excessive influx of calcium ions induced by Aβ in neurons, responsible for neuronal death.

Eugenol has neuroprotective effects against various types of inflammatory events. Inducible cyclooxygenase (COX-2) is an important factor in driving inflammatory processes in many organs, including the brain (Marnett and Kalgutkar, 1999). Blocking this enzyme can reduce inflammation.

Eugenol exerts a dose-dependent inhibitory activity on PGE2 production by suppressing COX-2 gene expression in lipopolysaccharide-stimulated macrophages (LPS) (Kim et al., 2003). It also inhibits the enzymatic activity of COX-2 (Huss et al., 2002).

Eugenol has an antioxidant activity which can directly eliminate free radicals and reduce oxidative stress as well as the production of ROS (Ogata et al., 2000; Taira et al., 1992). Isoeugenol, the isomer of eugenol, inhibits the lipid peroxidation of ferric ions and cumene hydroperoxide in rat brain homogenates (Rajakumar and Rao, 1993).

Gallic acid reduces neuronal damage, cerebral amyloid neuropathology and improves cognitive function via free radical scavenging and inhibition of Aβ peptide oligomerization. It plays a neuroprotective role by involving the antioxidant and inflammatory pathways in animal models of neurodegenerative diseases (Farbood et al., 2013; Korani et al., 2014; Mansouri et al., 2013; Naghizadeh and Mansouri, 2015). This potential protective effect against neurotoxicity is due to the sensitivity of the NMDA receptors and to the excitotoxicity induced by glutamate after cerebral ischemia, followed by an influx of Ca^{+2} and therefore of an intracellular accumulation of Ca^{+2} induced by neuronal apoptosis. On the other hand, gallic acid, thanks to its antioxidant effect can oppose the activation of NMDA receptors and thus have a protective effect on neurotoxicity and/or excitotoxicity after brain injury (Korani et al., 2014). Gallic acid binds to key proteins and minerals such as iron, zinc and calcium and affects their bioavailability by forming insoluble complexes (Niho et al., 2001). It improves cerebral electrophysiological deficits through changes in pro and anti-inflammatory cytokine levels, antioxidant effects in brain tissue and enhancement of the permeability of the blood-brain barrier (Farbood et al., 2013; Mansouri et al., 2013).

Gallic acid has also been described as an excellent free radicals scavenger that allows the level of ROS to decrease in the brain (Yang et al., 2006). This indicates that gallic acid exerts antioxidant activity by modulating brain dysfunctions (Sun et al., 2014).

Aβ peptide disrupts synaptic plasticity in the hippocampus due to the formation of senile plaques and neuronal apoptosis. Gallic acid treatment improves synaptic failure and histological damage to the brain induced by the Aβ peptide and can be introduced as a promising multipotent pharmacological agent in the prevention or treatment of AD in the future (Hajipour et al., 2016).

These results, consistent with other research, have shown the potential therapeutic effect of antioxidants to protect neurons against Aβ-induced cell death and lipid peroxidation. Antioxidants have been shown to improve cognitive function in older rats and to prevent learning and memory deficits after brain injury (Farbood et al., 2013; Mansouri et al., 2014). Clinical studies have also described the positive effects of antioxidant treatments to slow the progression of AD (McDaid et al., 2005; Scuderi et al., 2014).

BK (Big Potassium) channels are voltage-gated potassium channels that drive large amounts of potassium (K^+) ions across the cell membrane. They help regulate physiological processes, such as neuronal excitability, neuron triggering and neurotransmitter release. They also play a role in modulating the activity of dendrites as well as astrocytes and microglia (Contet et al., 2016). Inhibition of BK channels by $A\beta_{1-42}$ inhibits potassium efflux and thus reduces the use of ATP, thereby allowing neuronal survival in oxygen-poor environments (Hermann et al., 2015). BK channels can also serve as a neuronal protector by limiting the entry of calcium into cells by the oxidation of methionine. Isopimaric acid blocks the effect of $A\beta_{1-42}$ in neurons of wild mice, acting as an activator of BK channels. It is a potential candidate to treat Alzheimer's disease (Yamamoto et al., 2011). The rich composition of the extract AT00X gives it its biological properties evaluated in this study.

The present study showed that AT00X extract added 1 h before the application of the $A\beta_{1-42}$ peptide acts as a preventive treatment and was able to protect cortical

neurons after intoxication at 5 µg/ml. Moreover, at this dose of AT00X, the production of ROS was significantly reduced. At this stage, and without mechanistic study, it is difficult to know the mode of action of this prevention and at what level the extract acts. Hypotheses are emitted to understand the mode of action:
The extract acts by interfering with the neurotoxic pathways of the peptide (on glutamate release, GSK3b activation, and mitochondrial stress).

The extract acts by activating "pro-survival" signaling pathways. So, it does not prevent $A\beta_{1-42}$ peptide from acting but rather, prevents neuronal death by activating neuron survival pathways.

The extract acts by releasing pro-survival factors by the glial cells (as a reminder in the cell model we have 20% of astrocytes in the culture).

Another hypothesis of neuroprotective activity is based on the generation of ROS and its triggering oxidative stress that leads to amyloid beta aggregation and neurodegeneration. Mitochondria are damaged because of hyperphosphorylation of proteins, such as heme oxygenase-1 and biliverdin reductase A (Butterfield et al., 2013, 2014; Petersen et al., 2018). This damage coincides with the decrease in the activity of mitochondrial energy-related proteins, including pyruvate dehydrogenase complex and alpha-ketoglutarate dehydrogenase. Defective mitochondria in turn trigger the overproduction of ROS (García-Escudero et al., 2013; Hardas et al., 2013; Yan et al., 2013).

The highest dose of AT00X (90 µg/ml) has no protective effect against $A\beta_{1-42}$, in terms of neuronal cell death and ROS production. At this dose, AT00X could have an effect on neurite elongation. We can assume the presence of possible interactions between some plants of the AT00X extract at doses higher than 5 and 10 µg/ml which are manifested by a decrease in neuronal survival and reconstitution of the neurite network. To form a definitive conclusion on that aspect, additional work should be performed.

Indeed, several studies on traditional herbal treatments have reported problems of toxicity or interaction that may cause therapeutic failures or accidents (Hmamouchi, 1998). On the other hand, the toxic principles of plants are not well known, mainly due to their natural complexity. These medicinal plants must, therefore, like "conventional medicines", obey strict rules for cultivation, control and dispensing.

Medicinal plants are complex mixtures of various molecules. Their composition, often poorly defined, is made up of molecules with known biological activity, including heterosides, alkaloids, anthocyanins, tannins, and steroids. Like all bioactive molecules, these constituents can, at a certain concentration, present intrinsic toxicity (Zekkour, 2008).

It has been demonstrated in a study on the same model (Combes et al., 2015) that low concentrations of Aβ (at a non-toxic level) induced significant glutamate production. These results suggest that glutamate is involved in the process of Aβ-induced degeneration and that Aβ could be an important element triggering an excitotoxicity event.

These results are consistent with the notion that brain accumulation of Aβ is the primary influence in Alzheimer's disease. This abnormal accumulation triggers the following pathophysiological processes: formation of neurofibrillary tangles, oxidation and lipid peroxidation, glutamatergic excitotoxicity, inflammation and activation of the apoptotic cell death cascade which are considered secondary consequences of generation and deposition of Aβ (Querfurth and LaFerla, 2010).

Our results suggest, in conjunction with previous findings, that Aβ targeting may be a promising avenue in the design of new treatments for Alzheimer's disease.

Conclusion

The results of the biological evaluation tests of AT00X herbal extract, show a great ability to delay or even stop a chain of oxidative reactions according to the Oxygen Radical Absorbance Capacity Assay (ORAC), and a moderate anti-radical power according to the Diphenyl-1-PicrylHydrazyl Radicals test (DPPH) and Azino-Bis (3-ethylbenz-Thiazoline-6-Sulfonic acid) test (ABTS) in order to prevent the formation of oxidative damage and stabilize free radicals. Several tests must be performed to reflect the total antiradical and antioxidant capacity of a sample. For this purpose, we used the “Kit Radicaux Libres” test (KRL), a biological technique that allows us to evaluate the ability of a product to improve cellular defenses preventively to a radical attack. The AT00X extract has a strong antiradical activity according to the KRL test. AT00X has a high anti-inflammatory effect superior to that of dexamethasone (positive control), inhibiting NO release from Lipopolysaccharides-stimulated murine macrophages and reducing induced cytotoxicity.

Also, AT00X extract attenuates the deficits induced by the neurotoxic peptide $A\beta_{1-42}$ in vitro thus improving the neuronal survival, the neurite network and reducing the production of reactive oxygen species (ROS) in the neurons. These results indicate that AT00X extract has the potential as a potent therapeutic agent for neuroprotection and prevention of Alzheimer’s disease.

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