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RESEARCH ARTICLE

PRELIMINARY PHYTOCHEMICAL SCREENING AND PROTECTIVE EFFECT OF *ERYTHRINA SENEGALENSIS* IN AN *IN VIVO* MOUSE MODEL OF ALZHEIMER'S DISEASE

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ABSTRACT

Erythrina senegalensis is a therapeutic plant of the African Pharmacopoeia, widely used by populations in the treatment many pathologies, including mental disorders and cognitive impairments. We qualitatively analyzed crude methanol, acidic extracts and selective extracts from bark of *E. senegalensis* by identification tests, and evaluated its anti-radical activity (DPPH test) and compared to vitamin C. Finally, we examined its neuroprotective potential in vivo in a pharmacological mouse model of

Alzheimer's disease at the behavioral and biochemical level. The neuroprotective activity was evaluated in vivo in mice intracerebroventricularly injected with oligomeric amyloid-β25-35 peptide. The different tests were analyzed and compared to donepezil, an acetylcholinesterase inhibitor. Sterols, terpenes, coumarins, alkaloids, flavonoids, polyphenols and tannins were identified in the plant. The best inhibitory profile of DPPH radical was observed for the chloroform extract. The crude methanol extract had high neuroprotective activities *in vivo*.

INTRODUCTION

Medicinal plants are an important source of bioactive ingredients. Their study presents a logical strategy that can lead to the discovery of new compounds offering treatments against several pathologies. *Erythrina senegalensis* DC. (*Fabaceae*), is a therapeutic plant of the African pharmacopoeia, widely used by populations in the treatment of many pathologies, such as dysentery, jaundice, hepatitis, cirrhosis, hemorrhoids, wounds, dermatological diseases, rheumatism, tooth decay, headache and asthma. [1, 2]

Alzheimer's disease is one of the most common neurodegenerative disorders in the elderly. The number of cases of sick people is increasing (26.6 million). This prevalence will quadruple around 2050. [3] Currently, the available therapies are only symptomatic and not very effective in view of the imprecise etiology of the disease. However, the current hypothesis incriminates the toxicity of an amyloid-β peptide that accumulates during the pathology and is responsible for the formation of insoluble amyloid/senile plaques. These extracellular plaques, together with the intracellular aggregation of hyperphosphorylated Tau protein causing neurofibrillary tangles, are responsible of an irreversible neurodegeneration in the brain and consequently trigger the disease. [4,5] Numerous pharmacological and genetic rodent models exist for this disease. Maurice *et al.* [6] have, for instance, described a model based on the intracerebroventricular (ICV) injection of oligomeric $A\beta_{25-35}$ peptide in mice or rats which results, one week after injection, in memory deficits and onset of numerous toxicity markers. Meunier *et al.* [7] have shown that the model is particularly suitable for the screening of putative neuroprotective compounds, using simple tests of behavioral analysis and biological assays performed *ex vivo*, for a

multifactorial reading of the efficacy of the compounds.

It is well known that for most diseases, effective natural remedies could be found that present fewer side effects. In the present study, we selected *E. senegalensis* a medicinal plant of the floristic heritage of Côte d'Ivoire whose bark is used by healers to treat people with mental disorders. We performed a methanol extraction of *E. senegalensis* bark (ESH), evaluating its antioxidant potential in vitro and neuroprotective activity was evaluated in vivo compared to a reference clinical drug, donepezil.

MATERIALS AND METHODLOGY

Reagents

The solvents and reagents used were purchased from Polychimie (Côte d'Ivoire) or Sigma-Aldrich (France) and were of analytical grade quality.

Plant material

The bark of *Erythrina senegalensis* was harvested in Toumodi (Center of Côte d'Ivoire, Region of Aries, 6° 55' North, 5° 03' West) in December 2014 and February 2015. They were identified according to the herbarium available at the National Center of Floristic at Felix Houphouët-Boigny University (Abidjan-Cocody) (N° 7769). After cleaning and drying under continuous air conditioning for 7 days, the bark was crushed until powders were obtained.

Preparation of crude and selective extracts

- ✓ *Preparation of crude extracts of aglycones*

Powder (100 g) was homogenized in 600 ml HCl (2N) and then heated under reflux for 4 h. After cooling and filtration with Büchner, the marc was washed with distilled water and dried at ambient temperature. Dry marc was taken up in 500 ml of ethyl acetate (AcOEt) and then left macerated under continuous stirring for 3 h. After filtration, concentration on a rotary evaporator at 40° C (Büchi R210). The residue obtained constituted the crude extract (ESG).

- ✓ *Preparation of crude glycoside extracts*

Powder (100 g) was homogenized in 600 ml of 80% methanol and then macerated under constant stirring for 3 h. After filtration, concentration on a rotary evaporator and freeze-drying, the lyophilisate obtained constituted the crude extract (ESH).

- ✓ *Preparation of glycoside selective extracts*

ESH (10 g) was subjected to two successive extractions with (2 × 20 ml) with hexane, chloroform ($CHCl_3$), AcOEt and

n-butanol (n-BuOH). The various organic fractions were concentrated on a rotary evaporator to lead to ESH1~ESH4 extracts, respectively.

The preliminary organic composition of ESH1~ESH4 extracts was established by phytochemical characterization tests used by Bekro *et al.* [8] and Mohammed *et al.* [9].

Evaluation of the antioxidant activity by the DPPH test

The antioxidant potential of ES1~ES4 extracts was evaluated according to the method of Blois[10] modified by Kabran *et al.*[11]. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was solubilized in absolute ethanol (EtOH) to obtain a solution of concentration 0.3 mg/ml. Different ranges of concentrations (0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 mg/ml) were prepared in the same solvent. After stirring, samples were protected from light for 30 min. The absorbance of the mixture was then read at 517 nm against a control consisting of DPPH (1 ml) in pure EtOH (2.5 ml). The positive reference was vitamin C. The percentages of inhibition of DPPH are calculated according to:

$$I\% = (A_b - A_e)/A_b \times 100$$

with I%: percentage of inhibition, A_b: absorbance of the control, and A_e: absorbance of sample. The 50% inhibitory concentrations (IC_{50}) of DPPH were determined using Prism (GraphPad Sofware Inc., USA). The maximum effect was determined graphically and the equivalent concentration (EC_{50}) was determined after normalization of the extract effect.

Animals

Male *Swiss* mice (90), from Janvier breeding center (France), were received at the animal facility of the University of Montpellier (France), at the age of 6 weeks and weighing 30-32 g. They were housed in a regulated environment (conventional cages; temperature: 22 ± 1°C; humidity: 50 ± 2%; light/dark cycle: 12 h/12 h) with free access to food and water. The animals were treated according to the requirements of bioethics and according to the procedures of the European directive 2010/63/EU.

Drugs and injections procedures

The non-toxic control peptide (Sc.Aβ) and amyloid $A\beta_{25-35}$ peptide were solubilized in sterile distilled water at 3 mg/ml and stored at -20° C. Prior to injection, the peptides were incubated at 37° C for 4 days that induces oligomerisation of $A\beta_{25-35}$, but not Sc.Aβ. The crude extracts were solubilized in distilled water (for ESH) or in DMSO 10% in distilled water (for ESG) to

provide a test solution at 40 mg/ml. From day 1 to day 7 day, ESH, its vehicle solution (V1), ESG, or its vehicle solution (V2) were administered to the mice orally (100 µl/20 g of body weight), b.i.d., at 9:00 AM and 5:00 PM, as a preventive treatment. Mice therefore received a daily dose of 400 mg/kg. On day 8, were anesthetized with isoflurane (2.5%) and peptides $A\beta_{25-35}$ and Sc.Aβ (9 nmol/mouse) were injected ICV in a final volume of 3 µl/mouse. [6;7] The administration of ESG, ESH or vehicle solutions continued for a week after the injection of the peptides in curative treatment. Body weight of the animals was therefore checked o.d. before the morning injection.

Spontaneous alternation in the Y-maze test

Spatial working memory was evaluated 7 days after injection of the peptides, using spontaneous alternation performances in the Y-maze, as previously described by Maurice *et al.*[6], Meunier *et al.* [7]. The performance index of the working memory of the mice was calculated according to:

%Alt = Alt/(NAE-2) x 100

with %Alt: percentage of spontaneous alternation, Alt: number of actual alternations, and NAE: total number of arm entries.

Animals with extreme behaviour (%Alt < 20% or > 90%, or NAE < 10) were excluded from the calculation. In this study, 14 mice were discarded accordingly, corresponding to 12.9% attrition.

Step-through type passive avoidance test

The long-term contextual memory of the mice was evaluated using a passive avoidance procedure, with the acquisition session on day 8 and the retention session on day 9, as previously described by Maurice *et al.* [6] and Meunier *et al.* [7],

Animals showing training and retention latencies < 10 s and a sensitivity to shock = 0 were considered as no-responders and excluded from the calculations. In this study, 6 mice were discarded accordingly, corresponding to 5.6% attrition.

Measurement of reactive oxygen species (ROS) in hippocampus extracts

At the end of the behavioral period, mice were sacrificed, their brain extracted and hippocampus and cortex dissected out. The hippocampus were homogenized in 20 mM Tris buffer, 40 mM KCl, 2 mM ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 250 mM sucrose, and then centrifuged at 1,000 *g* at 4° C for 5 min. Supernatants (10 µl) were diluted 10-fold in phosphate buffer in the

presence of 0.5 µM 2',7'-dichlorofluo-resceindiacetate (DCF-DA). The samples were incubated for 30 min at 37° C. The fluorescence of the DCF formed (excitation at 485 nm; emission at 530 nm) was measured using an Ascent Fluoroskan spectrofluorimeter (ThermoScientific, France). The absorbances, minus blank control, were calculated according to the amount of protein per sample. Proteins were assayed using a BCA kit (Interchim, France), with bovine serum albumin as a standard.

Statistical analyses

The results were analyzed using a type I ANOVA, followed by a Dunnett's *post-hoc* test. Passive avoidance data are non-parametric since a maximum value is imposed. They were expressed as median and interquartiles 25%-75%. They were analyzed using a non-parametric Kruskal-Wallis ANOVA, followed by a multiple-comparison Dunn's test. The level of significance was $p < 0.05$. For reading clarity all statistical data are added in the figure legends.

RESULTS AND DISUSSION

Organic composition of ESH and ESG

The organic composition of *Erythrina senegalensis* was obtained from a preliminary phytochemical screening **(Table 1)** that showed the presence in ESH1~ESH4 extracts of secondary metabolites: terpenes, steroids, tannins, saponins, coumarins, reducing compounds, alkaloids, and flavonoids. ESG extract marked the absence of certain metabolites such as sterol, terpenes, saponins and a slight presence of alkaloids. As regards flavonoids specifically, Shinoda's test (Mg powder in HCl) showed the presence of flavanones and dihydroflavonols among the flavonoids characterized *stricto sensu* by Pew's test (Zn powder in HCl). These results don't contradict those reported by Bruneton (1999). [12]

The phytochemical screening of *E. senegalensis* was previously described by Saidu *et al.* [13] and Fofana *et al.* [14]. We did not confirm Saidu *et al.* [13] data, since they reported the presence of alkaloids, glycosides and the absence of flavonoids, saponins, tannins and volatile oils in the aqueous macerate of the species bark of Nigeria. Moreover, Fofana *et al.* [14] detected the existence of steroids, triterpenes and coumarins in the dichloromethane and ethyl acetate extracts of the roots and bark of the species from Burkina Faso, when flavonoids and tannins were perceived in the

same bark extracts. These results and ours do not contradict each other.

Anti-oxidant profile of ES2-ES4

The ESH2~ESH4 and ESG extracts, but not ESH1, exhibited a good antioxidant profile with respect to the DPPH radical **(Fig.1a)**, although less than that of vitamin C **(Fig.1b)**. IC_{50}, maximum effect and EC_{50} values are presented in **Table 2**. The most important antioxidant activity (80%) was observed with ESH2 with a max effect of 80% and IC_{50} of 46 µg/ml **(Table 2)**. The activity was close to that of vitamin C (max effect 90% and IC_{50} of 16 µg/ml). ESH3 and ESH4 presented a lower max effect of 50-60%, but ESH4 appeared more active with an IC_{50} value of 91 µg/ml **(Table 2)**. Fofana *et al.*[14] showed that the EtOAc extract of bark of the Burkinabe species had an obvious antioxidant potential (IC_{50} = 11.4 µg/ml). These findings in substance did not contradict each other. Moreover, they confirmed that the bark of *E. senegalensis* possesses a non-negligible antioxidant power which appears to originate from the position of the phenolic OH contained in the flavonoids whose presence was detected by Pew's and Shinoda's tests **(Table 1)**.

The preliminary organic composition of ESH1~ESH4 that we have established seems to explain *a priori*, the medicinal virtues of *E. senegalensis*. While the ESG extract has shown limitations in biological tests, this may lead us to believe that the compounds present in the ESH extract act synergistically and the compounds that have responded in ESG are not responsible for the activity shown by ESH.

Behavioral and biochemical analyzes

The animal body weight was checked daily during the extract injection period. As shown in **Fig. 2a**, ESH administration did not affect body weight gain as compared to V1-treated groups and particularly the V1+Sc.Aβ-treated control group. On the contrary, ESG administration resulted in a significant decrease in body weight gain, contrarily to V2-treated groups **(Fig. 2a)**. Animals lost 7% of the initial body weight after the 2-weeks treatment with ESG while control animals gained 1 %. The decrease remained limited but whether the extract presents an aversive or a satiety effect remains to be determined.

ICV injection of $A\beta_{25-35}$ in mice caused after one week significant alterations of spontaneous alternation **(Fig. 2a)** and passive avoidance **(Fig. 2b)**. Repeated treatment of mice with ESH reduced significantly the $A\beta_{25-35}$-induced spontaneous alternation deficit **(Fig. 2a)** and the passive avoidance deficit **(Fig. 2b)**.

Repeated treatment with ESG did not significantly affect the $A\beta_{25-35}$-induced spontaneous alternation deficit **(Fig. 2a)**. The compound however significantly attenuated the passive avoidance deficit **(Fig. 2c)**. A reference drug was used in a separate set of experiment, the acetylcholinesterase inhibitor donepezil. The drug was administered at 1 mg/kg IP o.d. during one week after the peptide injection. The drug blocked both $A\beta_{25-35}$-induced spontaneous alternation deficit **(Fig. 2d)** and the passive avoidance deficit **(Fig. 2e)** and mice performances were similar as observed for control V+Sc.Aβ-treated animals.

$A\beta_{25-35}$ injection resulted in a marked oxidative stress in the mouse hippocampus, measured by increased ROS levels **(Fig. 3)**. The ESH treatment blocked the $A\beta_{25-35}$-induced increase in ROS **(Fig. 3a)**. The ESG treatment was without effect **(Fig. 3a)**. The reference drug donepezil also fully prevented the $A\beta_{25-35}$-induced increase in ROS.

The extracts were then tested in a pharmacological mouse model of Alzheimer's disease. Amyloid toxicity is induced in the model by an acute ICV injection of $A\beta_{25-35}$ peptides that have been oligomerized by incubation. The peptide is therefore in the form of short amyloid fibrils, the form toxic form of amyloid aggregates, and diffuses through the choroid plexus and vasculature throughout the parenchyme in brain structures such as the cortex, hippocampus, hypothalamus, caudate putamen and limbic structures[15]. The toxicity develops in 1-2 weeks and mice present learning and memory deficits, neuroinflammation, oxidative stress, cellular stress and apoptosis, and progressive accumulation of amyloid $A\beta_{1-42}$ protein and hyperphosphorylated tau protein [16,17,6]. The model is therefore highly pertinent to provide a rapid *in vivo* screening approach to test potential neuroprotectants in AD. Its predictive value towards transgenic mouse model of AD is excellent. Indeed, two recent studies allowed a direct comparison of a candidate drug performance in the acute $A\beta_{25-35}$ mouse model and in a transgenic line overexpressing human APP protein carrying selected mutations. First, Neuro-EPO, a low sialic form of erythropoietin that can be administered intranasally, was shown superior to recombinant human EPO in the $A\beta_{25-35}$ model and was then tested successfully, under a two-month chronic regimen, in APP_{Swe} mice [18]. Second, a novel drug combination, acamprosate + baclofen, was tested in the $A\beta_{25-35}$ model and an $APP_{Swe,Ldn}$ line. Behavioral readouts

were highly similar, notably in the water-maze paradigm. The $A\beta_{25-35}$ mouse model is now widely used to screen both new drug candidate or natural extracts [19].

The extract composition in coumarins and flavonoids is likely to sustain its anti-oxidant action [20], but synergies with other compounds, including sterols and terpenes, must be examined. Other markers of the amyloid toxicity, adressing neuroinflammation, cellular stress and apoptosis, must also be examined, since the efficacy of ESH extracts in AD may rely on additive effects on several aspects of the toxicity. ESG extract showed some moderate effect at the behavioral level, particularly in the passive avoidance task, but the extract is devoid of efficacy on ROS increase and may therefore present, at best, a symptomatic effect.

Conclusion

The preliminary phytochemical screening of various organic extracts of bark of *Erythrina senegalensis* allowed to identify several groups of potentially active ingredients existing in the plant. The extract antioxidant potential was evaluated by the DPPH test, which showed a marked anti-radical activity of the chloroform extract.

A first observation of a protective effect of the crude methanol extract was provided using an *in vivo* mouse model of AD. The extract demonstrated a protective activity on behavioral and biochemical alterations (oxidative stress) induced by $A\beta_{25-35}$. The antioxidant efficacy of ESH, linked to its phytoconstituants, seems sufficiently intense, *in vivo*, to explain its neuroprotective potential highlighted on the behavioral tests.

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Conflict of interest

The authors declare that there is no conflict of interest in the present work.

Abbreviations

%Alt, percentage of spontaneous alternation; Alt, number of actual alternations; Aβ25-35, amyloid-β25-35 peptide; *b.i.d.*, twice-a-day; DCF-DA, 2',7'-dichlorofluoresceindiacetate; EGTA, ethylene glycolbis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; ESG, ethyl acetate/acidic crude extract of *E. senegalensis*; ESH, methanol extract of

E. senegalensis; ICV, intracerebroventricular; NAE, total number of arm entries; o.d., once-a-day; PO, *per os*; Sc.Aβ, scrambled Aβ25-35 peptide; YMT, Y-maze test; STPA, step-through passive avoidance.

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Table 1. Preliminary phytochemical screening of ESH1~ESH4 and ESG.

Identified compound	*Test*	*ESH1*	*ESH2*	*ESH3*	*ESH4*	*ESG*
Tannins	$FeCl_3$ (2%)	-	+ (green-black)	+ (dark blue)	+ (dark blue)	+ (dark blue)
Sterols	LB	+ (green)	+ (green)	-	-	-
Terpenes	LB	+ (red)	+ (red)	-	-	-
Saponins	foam	-	+	+	+	-
Reducing compounds	Fehling	-	+ (brick red)	+ (brick red)	+ (brick red)	-
Coumarins	KOH	-	+ (troubling)	+ (troubling)	+ (troubling)	+ (troubling)
	Zn (Pew)	-	+ (orange)	+ (orange)	+ (red)	+ (orange)
Flavonoids	Mg (Shinoda)	-	+ (red)	+ (red)	+ (red)	+ (orange)
	Wagner	-	+	+	-	+/-
Alcaloids	Mayer	-	+	+	-	+/-

(Abbreviations: LB,Libermann-Bürchard)

Table 2. Anti-oxidant activity of the different fractions ESH1~ESH4.

Sample	*LogIC_{50}*	*IC_{50} (µg/ml)*	*Max Effect (%)*	*EC_{50} (µg/ml)*
ESH1	ND	ND	ND	ND
ESH2	1.66	46.3 ± 7.0	80%	27.9 ± 4.4
ESH3	2.72	542 ± 155	55%	47.7 ± 0.5
ESH4	1.96	91.3 ± 6.7	55%	24.1 ± 1.6
ESG	2.53	339 ± 22	65%	87.7 ± 0.9
Vitamin C	1.19	15.5 ± 0.6	90%	14.0 ± 0.1

Abbreviations: ND, not determined; IC_{50}, concentration inhibiting 50% of the oxidant response; EC_{50}, concentration equivalent to 50% of the extract response.

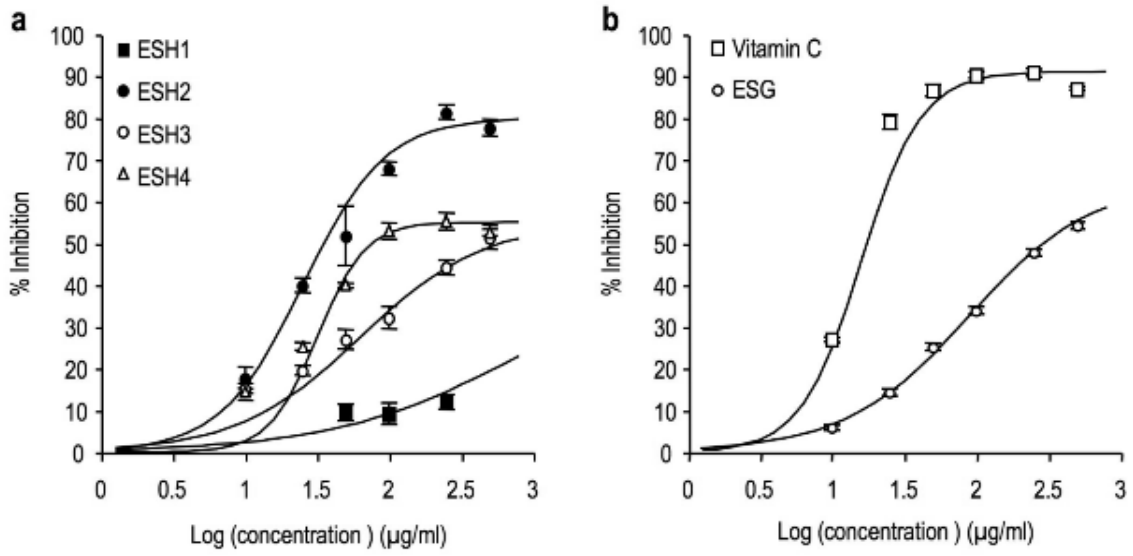

Fig. 1. Antioxidant activity of (a) ESH1-ESH4 and (b) ESG and vitamin C, expressed as percentage of DPPH inhibition.

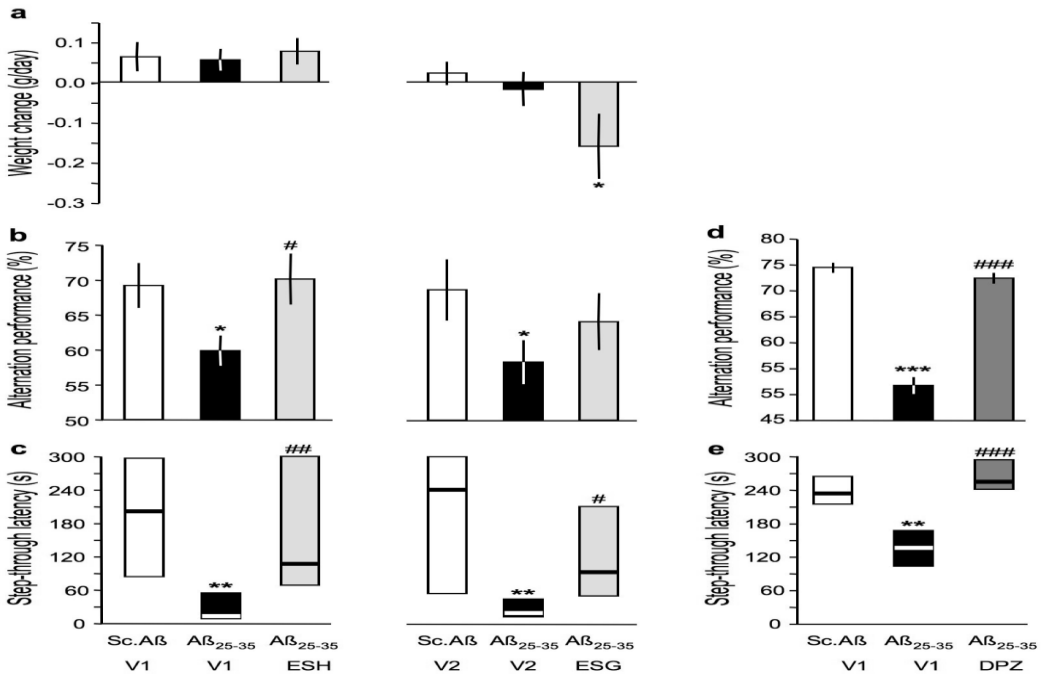

Fig. 2. Protective effects of the crude extract on the $A\beta_{25-35}$-induced deficits in mice. (a) Evolution of mouse body weights during the PO treatment with the extracts; (b) spontaneous alternation and (c) passive avoidance with the extracts; (d) spontaneous alternation and (e) passive avoidance with donepezil. Abbreviations: Sc.Aβ: scrambled peptide; V1: vehicle solution 1 (distilled water).; V2 vehicle solution 2 (DMSO 20% in water). The number of mice per group is 8-10 for ESH and 9-11 for ESG in (b, c) and 12 in (d, e). ANOVA: $F_{(2,35)} = 104$, $P < 0.0001$ in (b) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ with respect to group V+Sc.Aβ-treated

group; # P < 0.05, ## P < 0.01, ### P < 0.001 compared to V+Aβ$_{25-35}$-treated group; Dunnett's test in (b, d); Dunn's test in (c, e).

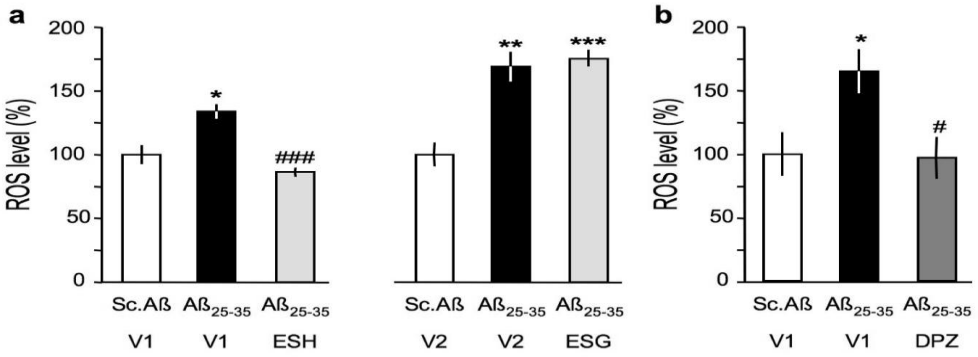

Fig. 3. Protective effects of the crude extract against the Aβ25-35-induced increases in ROS level in mouse hippocampus: (a) extracts and (b) donepezil. The number of mice per group is 4-8 in (a) and 6 in (b). ANOVA: F(2,15) = 21.4, P < 0.0001 for ESH, F(2,14) = 21.0, P < 0.0001 for ESG in (a); F(2,17) = 5.10, P < 0.05 in (b). * P < 0.05, ** P < 0.01 with respect to V+Sc.Aβ-treated group; # P < 0.05, ### P < 0.001 compared to V+Aβ25-35-treated group; Dunnett's test.