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RESEARCH ARTICLE

Neuroprotective effect of *Salvia splendens* extract and its constituents against $AlCl_3$-induced Alzheimer's disease in rats

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Abstract

Salvia splendens is a species of the genus *Salvia* that is known for its neuro-therapeutic properties. The present study aimed to investigate the effect of two fractions from the methanolic extract of the aerial parts of *S. splendens* cultivated in Egypt, the petroleum ether-soluble (PES) and *n*-butanol-soluble (BS) fractions, against $AlCl_3$-induced Alzheimer's disease (AD) in rats. Rats treated with $AlCl_3$ (100 mg/kg b.wt. *p.o.*) for 4 weeks developed behavioral, biochemical and histological changes similar to that of AD. Behavioral deficits were assessed by T-maze test and percentage changes in oxidative stress and AD markers in brain. Extent of DNA damage and histopathological changes were also evaluated. Results revealed that both fractions; PES and BS (at dose of 500 mg/kg b.wt), significantly attenuated $AlCl_3$-induced behavioral impairment in rats. This effect was accompanied by acetylcholinesterase (AChE) activity inhibition (53.18% and 68.66%, respectively), and Aβ deposition reduction (33.3% and 34.3%, respectively). Both fractions markedly decreased oxidative stress markers level (lipid peroxide, protein carbonyl, reduced glutathione and nitric oxide), and inhibited catalase and caspase-3 activities. Also, the content of noradrenaline, adrenaline, 5-HT and dopamine were significantly increased. The fractions preserved the histo-architecture pattern of the hippocampus and cortex from the $AlCl_3$-induced damage. Bioactivity-guided fractionation led to the isolation of two sterols; β-sitosterol and β-sitosterolpalmitate from PES fraction, and 6 phenolic compounds (acacetin, chrysoeriol, apigenin, luteolin, rosmarinic acid and caffeic acid) from BS fraction. Rosmarinic acid and caffeic acid significantly inhibited AChE in vitro (IC_{50} values of 0.398 mg/mL and 0.327 mg/mL, respectively) compared to physostigmine (IC_{50} 0.227 mg/mL). The BS fraction is standardized (HPLC–DAD) to contain not less than 0.0254% (w/w)of rosmarinic acid and 0.0129% (w/w) of caffeic acid. These findings suggest that *S. splendens* is beneficial in attenuating $AlCl_3$-induced neurotoxicity in rats.

Keywords Alzheimer's disease · Acetylcholinesterase · Oxidative stress · *Salvia splendens*

Abbreviations

ME	Methanolic extract
PES	Petroleum ether-soluble fraction
BS	*n*-Butanol-soluble fraction
VLC	Vacuum liquid chromatography
MAO	Monoamine oxidase
GSH	Glutathione
HPLC	High pressure liquid chromatography
ECD	Electrochemical detector
ESI	Electrospray ionization
ELISA	Enzyme linked immunosorbent assay
DAD	Diode array detector
AChE	Acetylcholinesterase
AD	Alzheimer's disease
CE	Catechin equivalent
GAE	Gallic acid equivalent

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that mainly affects the aged population and leads to behavioral changes Peters (2002). Two major competing hypotheses exist to explain the cause of the disease: the cholinergic hypothesis, which suggests that AD is due to reduced synthesis of the neurotransmitter acetylcholine (Ach) (Eikelenboom et al. 2010), and the amyloid hypothesis where oxidative stress induces β amyloid (Aβ) deposition that is manifested by lipid peroxidation, protein and DNA oxidation, free radical formation and neurotoxicity (Zhu et al. 2004).

Aluminum (Al), one of the most abundant metals in the earth crust, is an environmental neurotoxin that causes behavioral, chemical and pathological changes similar to AD in both humans and animals, where several studies showed a noticeable similarity between the neurofibrillary deterioration seen in experimental aluminum-induced AD and that found in patients with AD, so nowadays all the care was given to study the possible role of aluminum in Alzheimer disease (McDermott et al. 1979). Al has been concerned in the etiology of AD and other neurodegenerative diseases. Al has the facility to generate neurotoxicity by many mechanisms; it can promote accumulation of insoluble Aβ and hyperphosphorylated tau. Also, Al can increase oxidative stress and decreases the cortical cholinergic neurotransmission seen in AD (Yokel 2000).

Salvia splendens (family: Lamiaceae), commonly known as red sage or scarlet sage (Edward and Teresa 1999; Lowell 1997) is a member of genus *Salvia* that is known for its neurotherapeuticactivities. The role of *S. splendens*in AD and especially against $AlCl_{3n}$-induced neurotoxicity has not been so far studied. Therefore, this study was designed to investigate the effect of *S. splendens* extract and its successive fractions against $AlCl_3$-induced AD in rats (Kumar et al. 2011). Relevant behavioral and biochemical markers will be assessed, and extent of DNA damage and histopathological changes of the brain tissues will be evaluated. Chemical constituents from the bioactive fractions; PES and BS fractions will be isolated and evaluated in vitro for their AChE inhibitory activity. HPLC profile of the most active fraction (BS) and its content of two major active compounds; rosmarinic acid and caffeic acid will be determined.

Materials and methods

Plant material

Salvia splendens aerial parts were collected during January 2015 from the Central Botanical Garden, National Research Centre, Giza, Egypt. The plant material was kindly identified by Eng. Therese Labib (consultant of plant taxonomy, Ministry of Agriculture, Egypt). A voucher specimen (Reg. No. 5.1.2016) was deposited at the herbarium of the Faculty of Pharmacy, Cairo University.

Chemicals and apparatus

Silica gel H (10–40 µm) for vacuum liquid chromatography (VLC) and silica gel 60 for column chromatography (CC) were from Merck, Germany. Sephadex LH 20 (25–100 µm) for CC, vitamin C, physostigmine, $AlCl_3$, catechin and gallic acid standards, chemicals and reagents for Ellman's assay, and Specific ELISA kits for different biomarkers were from Sigma-Aldrich, Germany, and specific Biodiagnostic kits were purchased from Biodioagnostic Co. (Giza, Egypt). NMR: Bruker NMR spectrometer 400 MHz. HPLC analysis was conducted on an Agilent Technologies 1100 series liquid chromatograph equipped with a diode-array detector. An Eclipse XDB-C18 (150×4.6 µm; 5 µm) column with a C18 guard column (Phenomenex, Torrance, CA) was used with a gradient mobile phase composed of water-formic acid (20:1) (A) and methanol (B) as follows; at 0 min: 40% B, 15 min: 40% B, 20 min: 50% B, 30 min: 55% B, 50 min: 70% B, 51 min: 100% B, 53 min: 100% B. The flow rate was kept at 1 mL/min for a total run time of 54 min. The injection volume was 20 µL.

Evaluation of the anti-Alzheimer's activity

In vitro study

Acetylcholinesterase inhibitory activity The acetylcholinesterase inhibitory activity ofextract/fractions/compoundswasassessed using Ellman'smethod (Ellman et al. 1961). The AChE inhibitory activity was measured according to the following equation: $[1-(A_{(sample)}/A_{(control)}]\times 100$ (where $A_{(sample)}$ is the absorbance of the sample and $A_{(control)}$ is the absorbance of the control (having all reagents except for the tested sample that was replaced by acetylthiocholine iodide (the substrate). Physostigmine (five serial concentrations from 0.0625 to 1.0 mg/ml) was used as a positive control.

In vivo study

Animals Male Wister albino rats (120–150 g) were obtained from the animal house, National Research Centre, Egypt and kept in a control environment of air and temperature with access to water and standard diet (El-Kahira Co. for Oil and Soap). All procedures and handling of animals were performed in accordance with the ethical guidelines of Medical Ethical Committee of National Research Centre in Egypt (Approval No. 15122).

Determination of acute toxicity (LD_{50}) The median lethal doses (LD_{50}) was determined according to the method described by Al-Jubory (2013) for evaluating the safety of the active fractions; PES and BS obtained from the methanolic extract of *S. splendens*. Briefly, each fraction (at a dose of 500, 1000, and 1500 mg/kg b.wt./day) was given orally to a group of six animals (for each dose). The mice were observed for 24 h and LD_{50} was calculated.

Experimental design Induction of AD was proceeded by $AlCl_3$ (100 mg/kg b.wt./day, oral) for four consecutive weeks to give AD rats (Kumar et al. 2009). For the anti-AD activity; the PES and BSfractions (500 mg/kg b.wt./day) and physostigmine (0.3 mg/kg b.wt./day) (Sonkusare et al. 2005) were administered orally by gastric tubes for another four consecutive weeks as a treatment period.

Animal groups Seventy male albino rats weighing approximately 250 ± 50 g were randomly allocated into 7 groups (n = 10). Group 1: received saline and served as the-normal control group. Group 2: normal animals received PES fraction (500 mg/kg b.wt./day). Group 3: normal animals received BS fraction (500 mg/kg b.wt./day). Group 4: received $AlCl_3$ (100 mg/kg b.wt./day, oral) and served as AD rats group. Group 5: AD rats received PES fraction (500 mg/kg b.wt./day). Group 6:AD rats received BS fraction (500 mg/kg b.wt./day). Group 7: AD rats received physostigmine (0.3 mg/kg b.wt./day) and served as a reference drug for AD.

Behavioral assessment of cognitive abilities using T-maze test The neurocognitive ability of all animal groups was assessed by T-maze test according to the method of Deacon and Rawlins (2006). Before doing this experiment, the animals were food deprived for 24 h. Cognitive ability of animals in the seven groups was assessed three times; at zero time before starting $AlCl_3$-induction of AD, 24 h. after AD-induction period (for one month), and 24 h. following the last drug administration.

Tissue preparation for biochemical estimations At the end of exposure, brains of animals in each group were, separately dissected, kept on ice and divided into two parts. The first part was homogenized using an electric homogenizer (Remi 8000 rpm) in 50 mM phosphate buffered saline (PBS) pH 7.0 containing 0.1 mmol/L ethylene-diamine-tetra-acetic acid (EDTA). The homogenate was centrifuged at 4000 rpm for 30 min at 4°C and the supernatant was used for estimation of relevant biochemical markers. The second part was fixed in 10% formaldehyde and kept for histopathological analysis. Whole brains of two rats in each group were kept in ice-chilled saline for Comet assay.

Determination of biochemical markers in brain tissues Glutathione (GSH), lipid peroxidation, nitric oxide levels and catalase enzyme activity were measured using specific Biodiagnostic kits. The level of Aβ and activity of caspase-3 and AChE were evaluated using Sandwich ELISA technique. The content of noradrenaline (NA), dopamine (DA) and serotonin (5-HT) were determined by HPLC (HPLC-ECD) according to the method of Zagrodzka et al. (2000). Adrenaline level was measured by a competitive ELISA technique, and protein carbonyl content was determined spectrophotometrically according to the method reported by Zusterzeel et al. (2000).

Detection of DNA damage by Comet assay Whole brains of two rats in each group were kept in ice-chilled saline to detect DNA damage by Comet assay under alkaline conditions (pH > 13) according to Singh et al. (1988).

Histopathological analysis Brain tissues fixed in 10% formaldehyde were embedded in paraffin and then sectioned using sledge microtome at 4–5 micron thickness. Dewaxed brain sections were stained with hematoxylin and eosin (H&E) and examined by light electric microscope (Drury and Wallington 1980).

Phytochemical study of the aerial parts of *S. splendens*

Preparation of total extract and fractions

The air-dried aerial parts were powdered and 3 kg were macerated with 80% aqueous methanol till exhaustion (7 L × 8) to yield 240 g of dry methanolic extract (ME). The extract was suspended in water (900 ml) by sonication and successfully fractionated with petroleum ether (60–80 °C) (600 mL × 3), chloroform (500 mL × 3) and then *n*-butanol (500 mL × 3). Each fraction was evaporated under reduced pressure to give PES (75 g), CS (110 g), and BS (40 g) fractions, respectively.

Phytochemical investigation of the active fractions

Further investigation of the active fractions; PES and BS was conducted as follows to identify major chemical constituents.

Isolation of major chemical constituents from PES fraction Vacuum liquid chromatography (VLC, silica gel H) of PES fraction (20 g) was conducted using hexane as mobile phase with 5% increase in percentage of chloroform (from 5 to 90%, 200 ml each), and similar fractions were pooled together. Fraction I [eluted with 10% chloroform in hexane] was purified by CC (silica gel 60) using 2.5% EtOAc in hexaneto obtain compound 1 (260 mg). Fraction II [eluted with 70% chloroform in hexane] was purified by CC (silica gel 60) using 10% EtOAc in hexane to yield compound 2 (400 mg).

Isolation of major chemical constituents from BS fraction VLC (silica gel H) of BS fraction (10 g) was conducted using chloroform as mobile phase with 5% increment of ethyl acetate in chloroform, and then 5% increment of meth-

anol in ethyl acetate (200 mL each). Fraction I [eluted with 30% EtOAc in chloroform] was further purified by Sephadex LH-20 CC (eluted with methanol) to yield 3 (15 mg) and 4 (38 mg). Similarly, fraction II [eluted with 35% EtOAc in chloroform] gave 5 (21 mg), fraction III [eluted with 50% EtOAc in chloroform] gave 6 (26 mg), fraction IV [eluted with 55% EtOAc in chloroform] gave 7 (49 mg), fraction V [eluted with 60% EtOAc in chloroform] gave 8 (42 mg).

Estimation of total phenolic content in BS fraction The total phenolic content of BS fraction was determined according to Folin–Ciocalteu procedure and was expressed as mg of gallic acid equivalent (GAE) [21].

Estimation of total flavonoid content in BS fraction The total flavonoid content of BS fraction was determined calorimetricallyand expressed as mg of catechin equivalent (CE) per g of the BS fraction (Marinova et al. 2005).

HPLC–UV DAD of BS fraction HPLC analysis was conducted on an Agilent Technologies 1100 series liquid chromatograph (as under 2.2) and peaks were monitored simultaneously at 280, 320 and 350 nm. All samples were filtered through a 0.45 µm syringe filter and the mobile phase was degassed before injection. Chromatographic identification and confirmation of phenolic compounds were based on comparing retention times with authentic standards and on-line ultraviolet absorption spectral data.

Statistical analysis

Statistical analysis was carried out using computer software, Statistical Package for the Social Sciences (SPSS) version 16 (SPSS Inc. Released 2007, SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). Simple one-way analysis of variance (ANOVA) and Duncan's multiple range tests were used. All data were expressed as mean ± S.D. (n = 10 for in vivo studies and n = 3 for in vitro studies). % Change = [(Treated mean − Normal control mean)/Normal control mean] × 100. % Improvement = [Treated mean − AD diseased mean)/Normal control mean] × 100.

Results and discussion

Evaluation of the anti-Alzheimer's activity

In vitro study

Acetylcholinesterase inhibitory activity The results compiled in Table 1 revealed that ME of *S. splendens* and its two fractions; PES and BS, demonstrated potent in vitro AChE inhibitory activity (IC_{50} values of 1.60 mg/mL, 1.73 mg/mL and 1.25 mg/mL, respectively) when compared to physostigmine (IC_{50} value of 0.253 mg/mL). Accordingly, the neuroprotective effect of both PES and BS was evaluated in vivo using $AlCl_3$-induced AD in rats.

Table 1 *In vitro* AChE inhibitory activity of the total extract, fractions and aqueous extract of *S. splendens* aerial parts using Ellman's assay

Extracts/fractions	IC_{50} (mg/mL)
Total 80% methanolic extract	$1.60^{b} \pm 0.06$
PES	$1.73^{b} \pm 0.26$
Chloroform fraction	–
BS	$1.25^{b} \pm 0.13$
Physostigmine	$0.25^{a} \pm 0.02$

PES petroleum ether-soluble fraction, *BS n*-butanol-soluble fraction

Unshared superscripts (within each column) differ significantly from normal control at $P < 0.05$

Data are presented as mean ± S.D. (n = 3)

In vivo study

Determination of acute toxicity (LD_{50}) Both fractions proved to be safe up to 1500 mg/kg b.wt and the dose 500 mg/kg b.wt. was chosen for evaluating their anti-AD activity. The dose (500 mg/kg b.wt./day) is chosen according to the LD_{50} results. LD_{50} was determined by giving the fractions to the mice in graded doses up to 1500 mg/kg. The mortality rate was recorded 24 h. later. No mortality was recorded after 24 h. and the two fractions proved to be safe up to a dose of 1500 mg/kg (this is not the LD_{50} but our achieved safe dose), so the dose 500 mg/kg (1/3 of the achieved safe dose) was selected for our experiment and this result comes in parallel with Moharram et al. (2012) who determined that 80% aqueous methanol extract of salvia splendens is safe up to 5000 mg/kg (this is not the LD_{50} but his achieved safe dose), so he also used a dose of 500 mg/kg in his experiments (Moharram et al. 2012).

Generally, we consider a higher dose for extracts and fractions than positive standards where an extract or fraction for a natural substance is considered not to be pure and contains a lot of impurities other than the active constituents, so it doesn't represent the actual weight like the positive control which is a pure standardized active compound.

Behavioral assessment of cognitive abilities using T-maze test The results shown in Table 2 demonstrated that there is significant difference ($P < 0.05$) among all groups in the time taken to reach food in the T-maze. Results showed that there is a significant increase in time taken by AD rats (20.00 s.), relative to those in the normal control group (12.52 s.) indi-

Table 2 Effect of PES and BS of *S. splendens*extract on the time spent by AD rats to reach the food in the T-maze

Groups	Time (s)
Normal control	$12.52^{a} \pm 2.12$
Normal rats + PES	$13.25^{a} \pm 1.22$
Normal rats + BS	$12.64^{a} \pm 1.98$
AD rats	$20.00^{b} \pm 1.57$
AD rats + PES (500 mg/kg b.wt./day)	$17.43^{c} \pm 1.65$
AD rats + BS (500 mg/kg b.wt./day)	$14.45^{c} \pm 1.98$
AD rats + physostigmine (0.3 mg/kg b.wt./day)	$16.57^{c} \pm 2.21$

PES petroleum ether-soluble fraction, *BS n*-butanol-soluble fraction

Unshared superscripts differ significantly from normal control at $P < 0.05$

Data are presented as mean ± S.D. of ten rats in each group

cating deterioration in the neurocognitive functions. These findings are in accordance with the results reported by Yassin et al. (2013), indicating that aluminum is a neurotoxic agent. Whereas AD rats treated with either PES or BS fractions (500 mg/kg b.wt./day) or physostigmine (0.3 mg/kg b.wt./day) showed significant decrease ($P < 0.05$) in time taken to reach food in T-maze from normal control (17.43 s., 14.45 s. and 16.57 s., respectively) indicating improvement in its cognitive abilities.

Determination of biochemical markers in brain tissues As a neurotoxin, aluminum (Al) is known to affect several enzymes and other biomolecules related to neurotoxicity and AD (Shati et al. 2011). In vivo antioxidant activity of both PES and BS fractions was evaluated by measuring the activity of catalase enzyme and the level of reduced glutathione (GSH), oxidative stress biomarkers; lipid peroxide and nitric oxide levels in brain tissue homogenate of rats in all groups. Table 3 revealed significant difference ($P < 0.05$) among all groups in GSH level, catalse activity, lipid peroxide and nitric oxide levels. Although, insignificant change ($P < 0.05$) was shown in catalase enzyme activity and GSH level in normal rats treated with PES and BS fractions as compared to untreated normal control rats, AD rats showed significant decrease ($P < 0.05$) in GSH level and catalase enzyme activity (53.8% and 45.68%, respectively from normal control). Aluminum has been reported to enhance peroxidative damage to lipids, proteins and possibly cause decrease in the level of GSH and the activity of catalase enzyme (Julka and Gill 1996). Treatment of AD rats with PES fraction led to significant increase ($P < 0.05$) in the level of GSH and the activity of catalase enzyme (29.44% and 25.83%, respectively from normal control). While treatment of AD rats with BS fraction revealed insignificant change ($P < 0.05$) when compared to normal control (with percentage of improvement of 50.4% and 37.3%, respectively from AD rats group). Physostigminedemonstrated insignificant

Table 3 Effect of PES and BS of *S. splendens* extract on oxidative stress markers in AD rats

Groups	GSH (mg/g tissue)	Catalase (µg/g tissue)	Lipid peroxide (mmol/g tissue)	Nitric oxide (µg mol/g tissue)
Normal control	$8.66^{a} \pm 0.80$	$120.87^{a} \pm 9.19$	$8.66^{a} \pm 0.17$	$36.88^{a} \pm 2.88$
Normal rats + PES	$13.25^{a} \pm 1.67$	$122.95^{a} \pm 12.19$	$8.22^{a} \pm 0.68$	$33.7^{a} \pm 2.44$
% Change	53.00	1.72	−5.08	−8.62
Normal rats + BS	$10.70^{a} \pm 1.22$	$126.88^{a} \pm 10.19$	$8.16^{a} \pm 0.70$	$32.40^{a} \pm 3.21$
% Change	23.55	4.97	−5.77	−12.14
AD rats	$4.00^{b} \pm 0.19$	$65.65^{b} \pm 8.14$	$20.11^{b} \pm 1.34$	$54.98^{b} \pm 3.18$
% Change	−53.8	−45.68	132.2	49.07
AD rats + PES	$6.55^{c} \pm 1.40$	$96.88^{c} \pm 7.77$	$13.56^{c} \pm 0.19$	$43.55^{c} \pm 4.23$
% Change	−24.36	−19.84	56.58	18.08
% Improvement	29.44	25.83	75.63	30.99
AD rats + BS	$8.37^{a} \pm 1.22$	$110.85^{a} \pm 8.54$	$9.40^{a} \pm 0.23$	$39.88^{a} \pm 3.69$
% Change	−3.34	−8.28	8.54	8.13
% Improvement	50.46	37.39	123.67	40.94
AD rats + physostigmine	$7.50^{a} \pm 0.90$	$99.10^{c} \pm 6.34$	$10.25^{a} \pm 0.90$	$38.74^{a} \pm 2.78$
% Change	−13.39	−18.01	18.36	5.04
% Improvement	40.41	27.67	113.85	44.03

PES petroleum ether-soluble fraction, *BS n*-butanol-soluble fraction

Unshared superscripts (within each column) differ significantly from normal control at $P < 0.05$. Data are presented as mean ± S.D. of ten rats in each group. % Change = [(Treated mean − Normal control mean)/Normal control mean] × 100. % Improvement = [Treated mean − AD diseased mean)/Normal control mean] × 100

change ($P < 0.05$) in GSH level from normal control group, butameliorated catalase activity with percentage of 27.67% from AD rats group.

With respect to oxidative stress parameters, insignificant change ($P < 0.05$)from normal control was detected in the levels of lipid peroxide and nitric oxide in normal rats treated with both PES and BS fractions. However, significant increase ($P < 0.05$) was recorded in the level of lipid peroxide and nitric oxide in AD rats (132.2% and 49.07%, respectively) from normal control. Oxidative stress caused by $AlCl_3$ in AD rats can be explained by its ability to increase nitric oxide synthase in neuronal brain tissues which led to increase in the nitric oxide level (Bondy et al. 1998). In addition, high aluminum exposure induced the production of reactive oxygen species and caused lipid peroxidation in brain tissues (Katyal et al. 1997). Treatment of AD rats with PES fraction led to significant decrease ($P < 0.05$)in both lipid peroxide and nitric oxide levels with improvement percentages of 75.63% and 30.99%, respectively from AD rats group. While, insignificant change ($P < 0.05$) from normal control in lipid peroxide and nitric oxide levels was evident in AD rats treated with BS fraction with improvement percentage of 123.6% and 40.9%, respectively from AD rats group. Treatment of AD rats with physostigmine also revealed insignificant change ($P < 0.05$) from normal control in lipid peroxide and nitric oxide levels with improvement percentages of 113.85% and 44.03%, respectively from AD rats group.

Different neuro biomarkers were estimated in brain tissue homogenates of all groups to evaluate the potential of PES and BS fractions of *S. splendens* in controlling AD. Table 4 revealed that there is significant difference ($P < 0.05$) among all groups in noradrenaline, adrenaline, serotonin, dopamine, protein carbonyl and β amyloid levels, also there is significant difference among all groups at $P < 0.05$ in AChE and caspase-3 enzymes activity. Results showed that the activities of acetylcholinesterase and caspase-3 enzymes were enhanced, and the levels of protein carbonyl and β amyloid were increased in brain homogenates of $AlCl_3$-induced AD rats, while the levels of noradrenaline, adrenaline, serotonin and dopamine were decreased. On the other hand, insignificant change ($P < 0.05$) from normal control in the level/or activity of all biomarkers was observed in normal rats treated with PES and BS fractions. However, AD rats showed significant reduction ($P < 0.05$) in the levels of noradrenaline, adrenaline, serotonin and dopamine with percentages of 52.96%, 46.41%, 48.1% and 38.23%, respectively from normal control. This can be explained by oxidative stress caused by aluminum which resulted in down regulation of the neurotransmitters; adrenaline and noradrenaline in the AD rats (Adolfsson et al. 1979). Also, aluminum can decrease dopamine level by altering expression of tyrosine hydroxylase (TH); a principle enzyme for

Table 4 Effect of PES and BS of *S. splendens* extract on AD-relevant markers in AD rats

Groups	Noradrenaline (ng/gm)	Adrenaline (ng/gm)	AChE (ng/mL)	Serotonin (ng/gm)	Dopamine (ng/gm)	Caspase-3 (pg/mL)	Ptn carbonyl (nmol/mg ptn)	β Amyloid (pg/mL)
Normal control	$195.72^{a} \pm 10.97$	$305.02^{a} \pm 13.90$	$96.62^{a} \pm 3.05$	$87.97^{a} \pm 5.60$	$64.10^{a} \pm 6.00$	$7.46^{a} \pm 0.69$	$3.90^{a} \pm 0.46$	$3.02^{a} \pm 0.65$
Normal rats + PES	$194.90^{a} \pm 8.85$	$295.10^{a} \pm 11.54$	$88.20^{a} \pm 2.89$	$87.65^{a} \pm 3.0$	$62.60^{a} \pm 1.76$	$6.07^{a} \pm 0.22$	$3.94^{a} \pm 0.44$	$2.85^{a} \pm 0.45$
% Change	− 0.41	− 3.25	− 8.71	− 0.36	− 2.34	− 18.63	1.02	− 5.62
Normal rats + BS	$198.47^{a} \pm 7.80$	$295.55^{a} \pm 13.32$	$82.92^{a} \pm 3.00$	$89.27^{a} \pm 1.86$	$61.77^{a} \pm 3.41$	$6.08^{a} \pm 0.28$	$3.94^{a} \pm 0.23$	$2.63^{a} \pm 0.34$
% Change	+ 1.4	− 3.1	− 14.17	1.47	− 3.63	− 18.49	1.02	− 12.91
AD rats	$92.06^{b} \pm 6.12$	$163.45^{b} \pm 10.22$	$161.82^{b} \pm 2.45$	$45.65^{b} \pm 1.60$	$39.59^{b} \pm 1.55$	$17.89^{b} \pm 1.00$	$10.22^{b} \pm 0.96$	$30.16^{b} \pm 2.10$
% Change	− 52.96	− 46.41	67.48	− 48.1	-38.23	139.81	162.05	898.67
AD rats + PES	$155.10^{c} \pm 7.35$	$252.89^{c} \pm 11.31$	$110.43^{a} \pm 3.12$	$61.57^{c} \pm 3.22$	$46.60^{c} \pm 1.65$	$9.13^{c} \pm 0.54$	$7.07^{c} \pm 0.96$	$10.05^{c} \pm 0.98$
% Change	− 20.75	− 17.09	14.29	− 30.01	− 27.3	22.38	81.28	232.78
% Improvement	32.2	29.32	53.18	18.09	10.9	117.42	80.76	665.89
AD rats + BS	$156.22^{c} \pm 5.10$	$260.48^{c} \pm 13.91$	$95.48^{a} \pm 2.66$	$77.59^{a} \pm 4.87$	$54.75^{a} \pm 4.49$	$8.70^{c} \pm 0.87$	$5.72^{c} \pm 0.39$	$10.38^{c} \pm 0.77$
% Change	− 20.18	− 14.6	− 1.17	− 11.79	− 14.5	16.62	46.66	243.70
% Improvement	32.77	31.8	68.66	36.3	23.65	123.19	115.38	654.96
AD rats + physostigmine	$163.67^{c} \pm 10.76$	$273.16^{a} \pm 6.89$	$103.36^{a} \pm 3.25$	$74.70^{a} \pm 1.96$	$54.66^{a} \pm 1.55$	$8.57^{c} \pm 0.32$	$6.45^{c} \pm 0.70$	$10.43^{c} \pm 0.65$
% Change	− 16.37	− 10.44	6.97	− 15.08	− 14.72	14.87	65.38	245.36
% Improvement	36.58	35.9	60.5	33.02	23.51	124.9	96.66	653.31

PES petroleum ether-soluble fraction, *BS n*-butanol-soluble fraction, *Ptn carbonyl* protein carbonyl

Unshared superscripts (within each column) differ significantly from normal control at $P < 0.05$. Data are presented as mean ± S.D. of ten rats in each group. % Change = [(Treated mean − Normal control mean)/Normal control mean] × 100. % Improvement = [Treated mean − AD diseased mean)/Normal control mean] × 100

dopamine synthesis (Lee et al. 2011). Aluminum accumulation in the brain may also decrease the level of serotonin in diseased brain regions through neuro-degeneration of serotonin synapses (Ravi et al. 2000).

Treatment of AD rats with PES and BS fractions resulted in significant increase ($P < 0.05$) from normal control in noradrenaline level with improvement percentages of 32.2% and 32.77%, respectively, and increase in adrenaline levels with improvement percentages of 29.32% and 31.8%, respectively, from AD rats group. On the other hand, treatment of AD rats with physostigmine showed significant increase ($P < 0.05$)from normal control in noreadrenaline level with improvement percentage of 36.58% from AD rats group, while insignificant change ($P < 0.05$)from normal control in adrenaline level was recorded.

Treatment of AD rats with PES fraction led to significant increase ($P < 0.05$) in serotonin and dopamine levels from normal control with improvement percentages of 18.09% and 10.9%, respectively, from AD rats group. While insignificant change ($P < 0.05$) from normal control was recorded upon treatment of AD rats with BS fraction as well as physostigmine.

Aluminum exposure can increase activity of AChE through its reaction with the enzyme molecule peripheral anionic site, resulting in structural alteration of the enzyme and accordingly its activity (Alleva et al. 1998). Oxidative stress resulted when the generation of free radicals exceeds the capacity of anti-oxidizing system; subsequently leads to cellular dysfunction, cell membrane degradation and higher activity of caspase-3 enzyme that is responsible for neuronal death in the brain (Lucca et al. 2009). Also, elevated protein carbonyl and lipid peroxidation products may promote Aβ peptide formation and deposition (Praticò et al. 2002). Insignificant change ($P < 0.05$) from normal control in AChE activity was observed upon treatment of AD rats with PES and BS fractions.

Treatment of AD rats with PES, BS fractions or standard physostigmine demonstrated significant decrease ($P < 0.05$) from normal control in caspase –3 activity and protein carbonyl level, with improvement percentages of 117.42% and 80.76%, respectively, for PES, 123.19% and 115.38%, respectively for BS, and 124.9% and 96.66%, respectively for physostigmine from AD rats group. Treatment of AD rats with both PES and BS fractions showed marked reduction in the deposition of Aβ in brain tissues by 665.89% and 654.96%, compared to AD rats, and similar to that demonstrated by standard physostigmine (653.31%).

Detection of DNA damage by Comet assay The Comet assay is a method for measuring DNA strand breaks in brain cells.

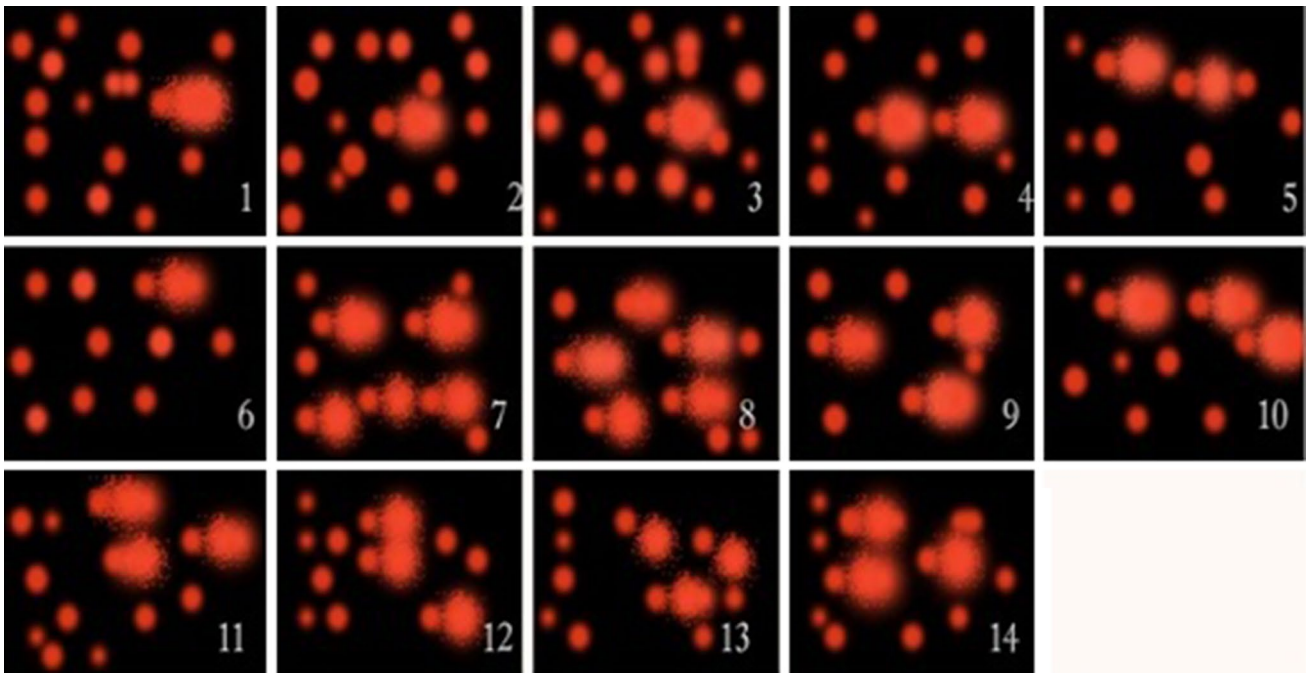

Fig. 1 Ethidium bromide-stained AD rat brain cells after treatment with PES and BS fractions of *S. splendens* extract. (1 and 2): photos of brain cells of normal control. (3 and 4): photos of brain cells of normal rats administered PES fraction. (5 and 6): photos of brain cells of normal rats administered BS fraction. (7 and 8): photos of brain cells of AD rats. (9 and 10): photos of brain cells of AD rats treated with PES fraction. (11 and 12): photos of brain cells of AD rats treated with BS fraction. (13 and 14): photos of brain cells of AD rats treated with physostigmine

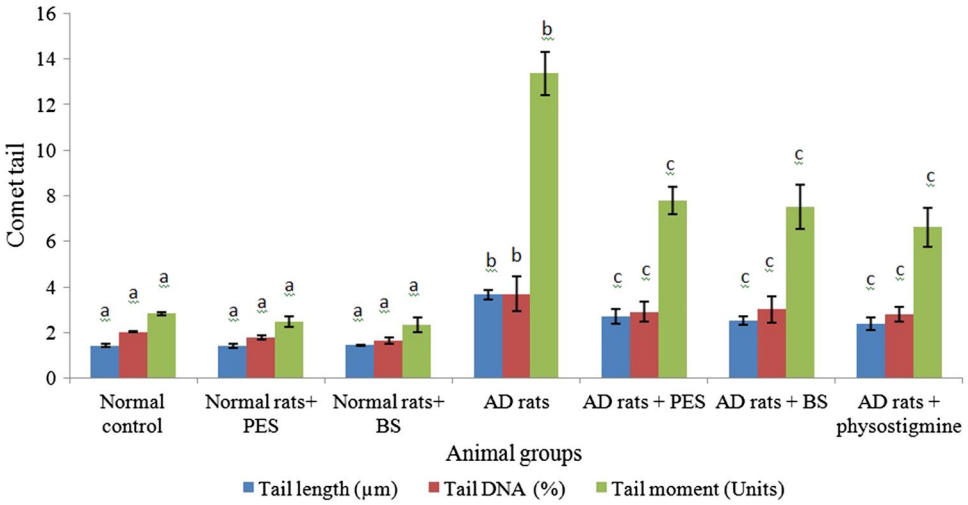

Fig. 2 Tail DNA intensity (%) = DNA % in tail/DNA % in comet. Tail moment (unit) = Tail length × Tail DNA intensity. Data are presented as mean ± S.D. of ten rats in each group. Unshared superscripts (within each column) differ significantly from normal control at $P < 0.05$. % Change = [(Treated mean − Normal control mean)/Normal control mean] × 100. % Improvement = [Treated mean − AD diseased mean)/Normal control mean] × 100. *PES* petroleum ether-soluble fraction, *BS* *n*-butanol-soluble fraction

The intensity of the comet tail relative to the head reflects the number of DNA breaks. The data obtained were evaluated based on tail length and tail moment of the brain cells investigated under fluorescent microscope after ethidium bromide staining (Figs. 1, 2). On the molecular level, DNA damage is one of the biomarkers of programmed cell death. The Comet assay showed that aluminum can lead to more DNA damage and apoptosis of neuron cells as indicated by the increased fragmentation of DNA and the number of comets (Sumathi et al. 2013). Results showed that there is significant difference among all groups at $P < 0.05$ in tail length, tail DNA and tail moment. When DNA damage was

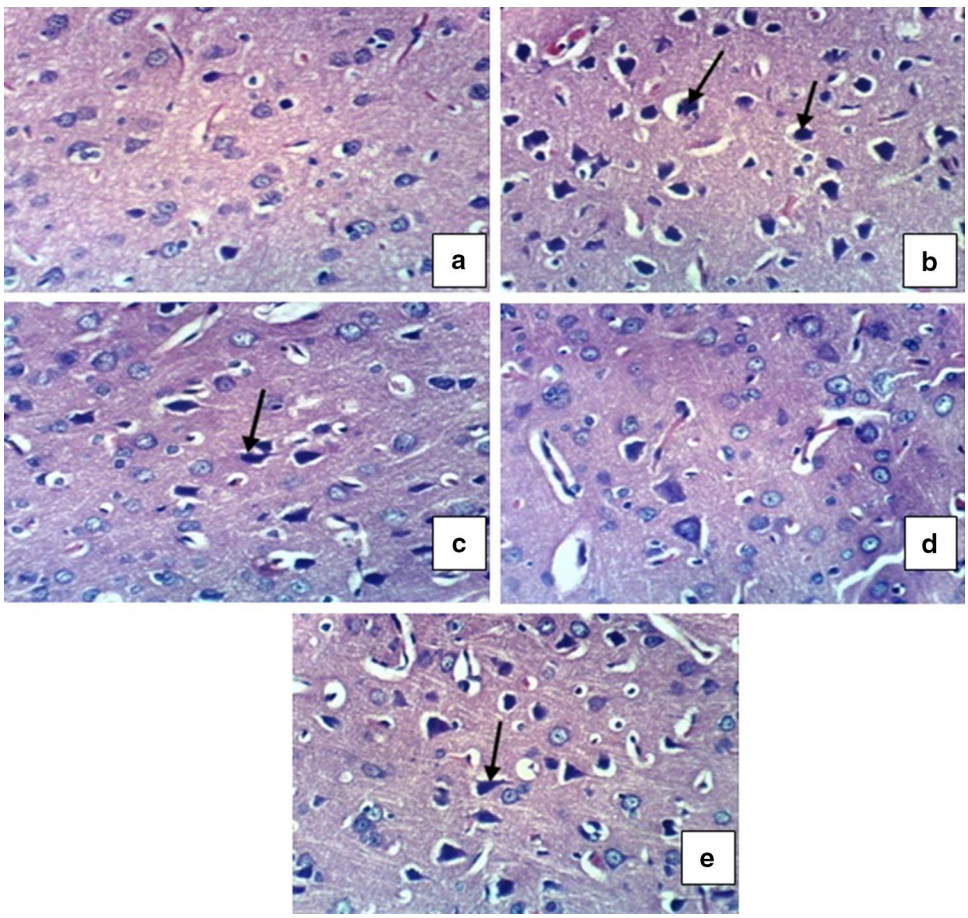

Fig. 3 Histopathology of cerebral cortex of control, diseased and treated rats. **a** photo of cerebral cortex cells of normal control rats. **b** photo of cerebral cortex cells of AD rats. **c** photo of cerebral cortex cells of AD rats administered PES fraction. **d** photo of cerebral cortex cells of AD rats administered BS fraction. **e** photo of cerebral cortex cells of AD rats administered physostigmine; Black arrows show necrosis of neurons

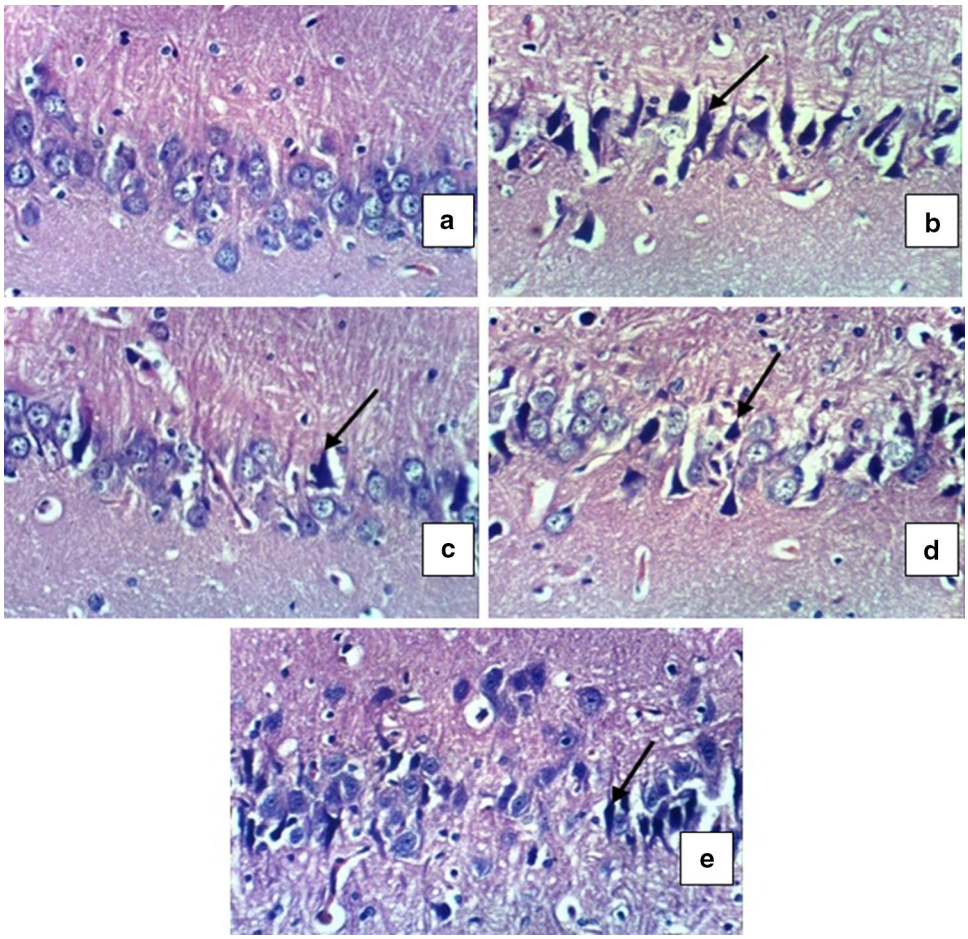

Fig. 4 Histopathology of hippocampus of control, diseased and treated rats. **a** photo of pyramidal cells of normal control rats. **b** photo of pyramidal cells of AD rats. **c** photo of pyramidal cells of AD rats administered PES fraction. **d** photo of pyramidal cells of AD rats administered BS fraction. **e** photo of pyramidal cells of AD rats administered physostigmine; Black arrows show necrosis of neurons

compared with respect to tail length and tail moment, DNA strand breaks in brain cells caused by $AlCl_3$ in AD rats was increased by 160% and 377.14%, respectively. Insignificant changes ($P < 0.05$) from normal controlwere recorded in all comet parameters in normal rats treated with either PES or BS fractions as compared to normal control rats. However, obvious changes were detected in DNA comet parameters upon treatment of AD rats with PES and BS, similar to that observed by standard physostigmine. Treatment of AD rats with PES and BS fractions decreased the level of tail length to 91.42% and 78.57%, respectively, when compared to normal control group with improvement percentages of 68.57% and 81.42%, respectively, when compared to AD rats group. With respect to tail moment; groups treated with PES and BS fractions showed improvement percentages of 200.35% and 209.285%, respectively, when compared to AD rats group. Also, standard physostigmine showed high percentage of improvement with respect to both tail length and tail moment (90.71% and 241.42%, respectively) when compared to AD rats group.

Histopathological analysis Histopathological examination of cerebral cortex (Fig. 3) under the microscope revealed that there were insignificant pathological alterations in the cerebral cortex of normal control rats (Fig. 3a). Meanwhile, cerebral cortex of rats treated with $AlCl_3$ showed atrophy and necrosis of neurons (Fig. 3b). On the other hand, cerebral cortex of rats treated with PES fraction showed less atrophy of the neurons (Fig. 3c), while some examined sections from rats treated with BS fraction showed less necrosis and some other sections revealed no pathological changes (Fig. 3d). Examined sections from rats treated with physostigmine revealed also less necrosis of some neurons (Fig. 3e).

Similarly, histopathological examination of hippocampus was conducted (Fig. 4) and the results revealed that hippocampus of normal control rats showed no pathological changes (Fig. 4a). However, hippocampus of rats treated with $AlCl_3$ showed necrosis of the pyramidal cells (Fig. 4b). Moreover, sections from rats treated with either PES, BS fractions orphysostigmine showed less atrophy and necrosis in some pyramidal cells (Fig. 4c–e, respectively).

The above findings suggested that administration of bothPES and BS fractions are beneficial in controlling

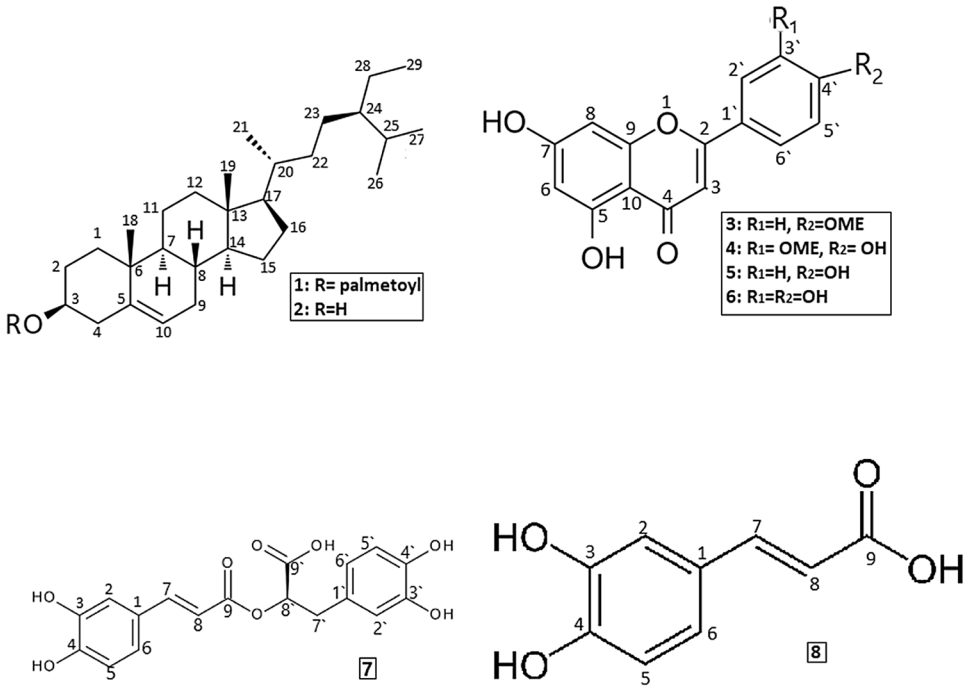

Fig. 5 Chemical structures of the isolated compounds

$AlCl_3$-induced AD disease in rats when compared to physostigmine.

Accordingly, bio-guided purification of these two active fractions was undertaken to isolate and identify their major active constituents.

Phytochemical investigation of the active fractions

Chromatographic purification of PES fraction resulted in the isolation of two compounds (1 and 2). Compound 1 was isolated as an oily residue and identified as β sitosterolpalmitate (Sun et al. 2003) (ESI–MS data, (positive mode): *m/z* 653.6 $[M+H]^{+)}$, while compound 2 was isolated as white powder and identified as β sitosterol (Chaturvedula and Prakash 2012). On the other hand, purification ofBS fractionresulted in isolation of 6compounds (3–8). The isolated compounds were identified as acacetin (3) (Gomes et al. 2011), chrysoeriol (4) (Park et al. 2007), apigenin (5) (Van Loo et al. 1986), luteolin (6) (Lin et al. 2015), rosmarinic acid (7) (Lu and Foo 1999), and caffeic acid (8) (Jeong et al. 2011). Identification of all compounds was assured by comparing their spectral data [additional data are given in Online Resource 1 (Tables 1, 2 and 3)] with those reported in literature. Chemical structures of the isolated compounds are depicted in Fig. 5. To the best of our knowledge, this is the first report on the presence of β sitosterol palmitate (1), acacetin (3) and chrysoeriol (4) in *S. splendens*.

In vitro acetylcholinesterase inhibitory activity of the isolated compounds

The ability of the isolated compounds to inhibit AChE was evaluated in vitro using Ellman's assay and the results are expressed as IC_{50} or IC_{25} values (Table 5). IC_{50} could only be calculated for rosmarinic acid (7) and caffeic acid (8) (IC_{50}, 0.398 mg/mL and 0.327 mg/mL, respectively); the compounds showed insignificant difference ($P<0.05$) when compared to physostigmine (IC_{50}, 0.227 mg/mL). Both rosmarinicacid and caffeic acid were previously reported to inhibit AChE (Vladimir-Knežević et al. 2014) and β amyloid peptide-induced neurotoxicity (Iuvone et al. 2006; Sul et al. 2009). Under the experimental conditions, compounds 1, 2, 4, 5 and8 induced 25% inhibition of the enzyme (at concentration of 0.108, 0.142, 0.077, 0.193 and 0.073 mg/mL, respectively) that showed insignificant difference ($P<0.05$) from physostigmine (0.076 mg/mL), while compound 3 (acacetin) was almost inactive. However, acacetin (3) was previously reported for its anti-AD activity by being inhibitor of MAO enzyme (Chaurasiya et al. 2016), amyloid peptide precursor synthesis (Wang et al. 2015) and neuroinflammation (Ha et al. 2012). In addition, apigenin (5) andluteolin (6) were previously reported as anti β amyloidogenic (Zhao et al. 2013), antioxidant and anti-inflammatory agents (Rezai-Zadeh et al. 2008), and β-sitosterol (2) was reported to prevent β amyloid deposition in the brain (Novak 1999).

Table 5 *In vitro* AChE inhibitory activity of the isolated compounds

Compounds	IC_{50} (mg/mL)	IC_{25} (mg/mL)
βSitosterolpalmitate (1)	–	$0.108^{ab} \pm 0.016$
βSitosterol (2)	–	$0.142^{ab} \pm 0.027$
Acacetin (3)	–	–
Chrysoeriol (4)	–	$0.077^{ab} \pm 0.010$
Apigenin (5)	–	$0.193^{ab} \pm 0.023$
Luteolin (6)	–	$0.482^{b} \pm 0.096$
Rosmarinic acid (7)	$0.398^{a} \pm 0.056$	$0.064^{a} \pm 0.007$
Caffeic acid (8)	$0.327^{a} \pm 0.031$	$0.073^{ab} \pm 0.007$
Physostigmine	$0.227^{a} \pm 0.020$	$0.076^{ab} \pm 0.007$

Unshared superscripts (within each column) differ significantly from normal control at $P < 0.05$. Data are presented as mean ± S.D. (n = 3)

Although, both fractions (PES and BS) showed high in vivo anti AD effects through normalizing the levels or enzyme activities of neurobiomarkers, isolated compounds from both fractions did not show that high results as AChE inhibitors except for caffeic acid and rosmarinic acid, this can be explained by either there is a synergistic effect among all compounds in the crude fractions or they can control AD through any other mechanism.

Estimation of total phenolic and flavonoid content in BS fraction

Standardization of BS fraction (the most active fraction as AChE inhibitor) was made chemically and spectrophotometrically. Based on the chemical nature of the compounds isolated from BS fraction, its content of total phenols and flavonoids was chemically quantified. The total phenolic content was found to be 92.4 ± 3.6 mg GAE/g (expressed as mg gallic acid equivalent (GAE) per one gram of BS fraction), while the total flavanoidal content was found to be 36.2 ± 2.7 mg CEE/g [expressed as mg catechin equivalent (CE) per one gram].

HPLC–UV DAD of BS fraction

On the other hand, an HPLC profile of BS fraction was developed with major peaks identified (Fig. 6). The major peaks in BS fraction were identified as caffeic acid (*R*t 5.19 min), rosmarinic acid (Rt 20.7 min) and chrysoeriol (*R*t 42.9 min) by comparing their retention times with those of standards. Accordingly, standard calibration curve for each was constructed by HPLC and the BS fraction was found to contain not less than 0.0254 ± 0.0003% (w/w) of rosmarinic acid, 0.0129 ± 0.0004% (w/w) of caffeic acid and 0.0081 ± 0.0004% (w/w) of chrysoeriol.

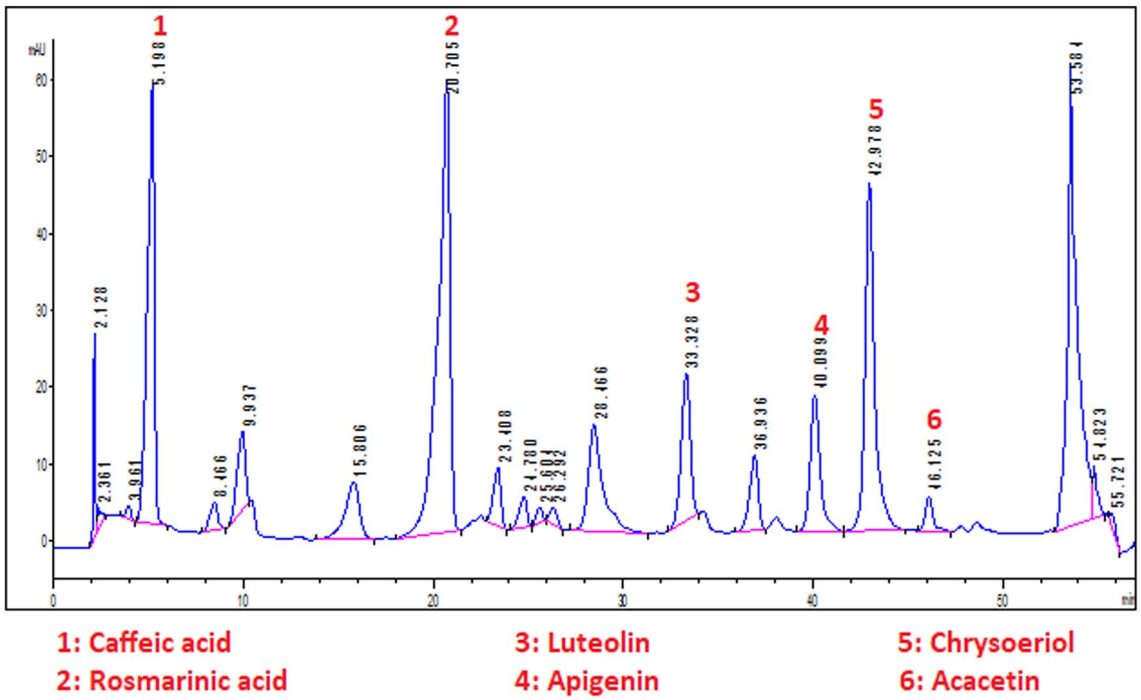

Fig. 6 HPLC chromatogram of BS fraction of the methanolic extract of *S. splendens*

Conclusion

Salvia splendens may have potential effect in controlling Alzheimer's disorders where both sterols isolated fromits PES fraction and phenolic compounds isolated from its BS fraction contribute, at least in part to its neuroprotective effect. Good results obtained from both fractions in controlling AD in vivo, can be explained by the synergistic effect found among compounds. Some isolated compounds did not show any in vitro AChE inhibition, but it seems that they can control AD through any other mechanism.

Recommendation

Further investigation of other possible neuroprotective mechanisms of *S. splendens* extract is warranted.

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Compliance with ethical standards

Ethical statement All procedures and handling of animals were performed in accordance with the ethical guidelines of Medical Ethical Committee of National Research Centre in Egypt (Approval No. 15122).

Conflict of interest Salma Ahmed El Sawi has no conflict of interest. Shahira Mohamed Ezzat has no conflict of interest. Hanan Farouk Aly has no conflict of interest. Rana Merghany Merghany has no conflict of interest. Meselhy Ragab Meselhy has no conflict of interest.

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