Vol. 13(11), pp. 252-268, 10 June, 2019
DOI: 10.5897/JMPR2018.6728
Article Number: 3FF026261151
ISSN 1996-0875

http://www.academicjournals.org/JMPR

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Journal of Medicinal Plants Research

Full Length Research Paper

Chemical fingerprint of *Bacopa monnieri* L. and *Rosmarinus officinalis* L. and their neuroprotective activity against Alzheimer's disease in rat model's putative modulation *via* cholinergic and monoaminergic pathways

Nermien E. Waly[1], Nora M. Aborehab[2*] and Mahitab H. El Bishbishy[3]

[1]Department of Physiology, Faculty of Medicine, Helwan University, Cairo, 11795, Egypt.
[2]Department of Biochemistry, Faculty of Pharmacy, MSA University, Giza 11787, Egypt.
[3]Department of Pharmacognosy, Faculty of Pharmacy, MSA University, Giza 11787, Egypt.

Received 26 December, 2018; Accepted 30 January, 2019

Alzheimer's disease is characterized by progressive degeneration of cortical and hippocampal neurons. This study aims to characterize the metabolic profiles of the hydro-ethanolic extracts of *Bacopa monnieri* L. (BM) and *Rosmarinus officinalis* L. (RO) cultivated in Egypt *via* UPLC–ESI/MS analyses and reveal their possible mechanism of the prophylactic effect(s) on neuro-degeneration in rat model of Alzheimer's disease (AD). Here, UPLC–ESI/MS analyses were employed for the characterization of hydro-ethanolic extracts. Forty-two male albino rats were intra-peritoneally injected with Aluminum chloride at a dose of 4.2 mg/kg to induce AD. The extracts of BM and RO were separately orally administered at doses of 300 and 450 mg/kg, and Donazil® was orally administered at dose 2.5 mg/kg. Serum levels of malondialdehyde (MDA), and total antioxidant capacity (TAC) were measured using ELISA. Further, Amyloid β-protein, acetylcholinesterase (AChE), τ-protein and serotonin levels were measured in brain tissue using ELISA. The UPLC–ESI/MS analyses revealed the presence of fifteen and seventeen active metabolites in BM and RO extracts respectively which may account for their effects on neuro-degeneration. Serum level of MDA, amyloid β-protein, AChE and τ-protein were significantly decreased in herbal treated groups when compared to AD group (P value < 0.0001). On the other hand, TAC and serotonin levels were significantly elevated in groups treated with BM and RO compared to AD group (P value < 0.0001). Consequently, BM and RO extracts were found to have a potential neuroprotective effect in AD rat model due to their variety of active metabolites.

Key words: Alzheimer's, serotonin, anti-AChE, antioxidant, *Bacopa monnieri, Rosmarinus officinalis.*

INTRODUCTION

Aluminum (Al) is the most abundant metal on earth, it can enter the body through diet, drinking water, aluminum containing drugs and so enters the brain; deposited in the cortex, hippocampus and cerebellum which are crucial for

*Corresponding author. E-mail: naborehab@msa.eun.eg.

memory and cognition. Al was reported as a main risk factor for the cause and development of neuro-degenerative diseases as: Alzheimer's disease (AD), amyotrophic lateral sclerosis and Parkinson's disease (PD) (Thenmozhi et al., 2016).

Aluminum induced neurotoxicity was previously reported by many authors in which administration of aluminum chloride hexahydrate 25 mg/kg/day for one month orally (Zaky et al. 2013) and daily treatment with $AlCl_3$ at dose 100 mg/kg orally for 42 days was observed (Lin et al., 2015).

Alzheimer's disease is characterized by progressive degeneration of cortical and hippocampal neurons. This results in deterioration of the persons' memory and cognitive ability (Ghoneim et al., 2015). AD is the most common cause of dementia among elderly population (Zhang et al., 2016) and has a progressive and devastating nature that poses a huge financial and social burden on families and caregivers of the elderly (Sica, 2015; Goren et al., 2016). Precise worldwide prevalence of AD is difficult to estimate; however, prevalence of AD is expected to increase in the coming decades as a consequence of aging of the world population (Goren et al., 2016; Brookmeyer et al., 2007; Carter, 2008).

Although not well understood, it has been postulated that loss of cholinergic function at the central nervous system as well as accumulation of reactive oxygen species are the key components implicated in AD pathophysiology (Bartus, 2000; Murphy and Steenbergen, 2008). Accumulation of amyloid β (Aβ) protein and appearance of neurofibrillary tangles of tau (τ) protein are the most prominent pathological hallmarks of AD (Iqbal and Grundke-Iqbal, 2010). To date, there is no effective treatment for AD that is why finding preventive measure to reduce the disease incidence is of crucial significance (Sica, 2015).

Bacopa monnieri L. (BM), commonly known as "Brahmi" named after Brahma, the creator god of the Hindu pantheon of deities, is an herb that is used in traditional Indian medicine for its antioxidant and anti-inflammatory as well as memory-enhancing effect. This effect is believed to date back more than 3000 years in India (Rathee et al., 2008; Chaudhari et al., 2017).

Rosmarinus officinalis L. (RO) is used since antiquity to enhance the memory. The ancient Egyptians had a famous tradition of laying rosemary across the coffin or upon a tombstone during the embalming process and it was considered sacred to ancient Egyptians, Romans and Greeks (Burlando, 2010). It was traditionally burned for the Greek students prior to their exams to boost their mental performances and was considered a loyalty symbol between lovers due to this trait (Rathee et al., 2008). Rosemary is an herb reportedly known for its antioxidant and anti-inflammatory effects (Aruoma et al., 1996; Lipton et al., 2016; Posadas et al., 2009).

Herbal extracts pose a potential hope for prevention and treatment of many age-linked diseases such as atherosclerosis (Kabiri et al., 2012), hypertension (Reinhart et al., 2008), type 2 *Diabetes mellitus* (Li et al., 2004) and osteoarthritis (Aborehab et al., 2017). Focusing on nootropic herbal extracts, it is well established that *Ginkgo biloba* (Birks and Grimley, 2007), *Piper nigrum* (Subedee et al., 2015), *Hericium erinaceus* (Zhang et al., 2016), *Withania somnifera* (Bhattacharya et al., 2001) and *Panax ginseng* (Petkov et al., 1993) extracts improve learning and memory deficits and relieve the neuropsychological symptoms associated with animal models of AD. Recently, BM showed cholinergic effects in mice similar to current treatments of AD (Le et al., 2013). Also, attention has been drawn to use RO in treatment of AD as its metabolite, carnosic acid (CA), was found to enhance memory and learning of AD mice model (Lipton et al., 2016).

The objective of this study is to characterize the metabolic profiles of the hydro-ethanolic extracts of BM and RO cultivated in Egypt and to investigate their neuroprotective effects compared to standard medication Donazil® as a selective acetylcholinesterase inhibitor as well its potential mechanism of its neuroprotective actions in a rat model of AD.

METHODOLOGY

Plant material

The aerial parts of "Brahmi" *B. monnieri* L. (Plantaginaceae) and "Rosemary" *R. officinalis* L. (Lamiaceae) were provided from El Orman Botanical Garden, Giza, Egypt and kindly identified by Dr. Mohamed El-Gebaly (National Research Institute, Dokki, Giza, Egypt). Voucher specimens of both collected samples were kept at Pharmacognosy Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA) with codes (MSA-2017-8 and MSA-2017-9, respectively). The Fresh aerial parts (500 g) were air dried at room temperature, then ground into fine powder. The extracts were obtained using a Soxhlet extraction over a period of 8 h using 70% ethanol, filtered, and the solvent was evaporated under reduced pressure in a rotatory evaporator at a temperature as low as 45°C. The dried extracts were kept in a desiccator for further chemical and biological investigations.

UPLC-Electro spray ionization-mass spectroscopy (UPLC–ESI-MS) apparatus

40 µg of BM and RO hydro-ethanolic extracts were separately dissolved in 1 mL HPLC grade methanol, filtered using a 0.2 µm membrane disc filter and degassed by sonication prior to injection. UPLC–ESI-MS analyses were performed on an Agilent® 1100 Series using ACQUITY UPLC - BEH C18 column, (1.7 µm - 2.1 × 50 mm, i.d.), with an integrated pre-column. 10 µL of each extract was eluted using gradient mobile phase composed of two eluents: eluent A is nano-pure H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid with the flow rate of 0.2 mL/min for 35 min. A XEVO TQD triple quadruple instrument, Waters® Corporation, Milford, MA01757, U.S.A mass spectrometer connected to a PDA detector with standard flow cell (10 mm path length, 14 µL volume, 40 bar maximum pressure) was used for mass spectrometric analysis. ESI interface was employed in both negative and positive ion modes using N_2 as a drying and nebulizing gas. At 250°C capillary temperature, the spray and capillary voltages were 4.48 kV and 39.6 V respectively and the full scan mode was in mass range of *m/z* 100–2000. The peaks and

spectra were interpreted using the Masslynx 4.1® software and tentatively assigned by comparing their mass spectrums with the reported data.

Chemicals and drugs

Aluminum chloride ($AlCl_3$) was purchased from Sigma-Aldrich Chemicals Co, Egypt, dissolved in saline 0.9% and injected intraperitonealy (I.P) at dose 4.2 mg/kg/day for 28 days according to methodology of Bitra et al. (2014) and Nayak and Chatterjee (2001). Ethanol (analytical grade) was purchased from El Gomhoreya Co., Egypt. Donepezil hydrochloride, is the main active ingredient in Donazil®; it acts as a selective acetylcholinesterase inhibitor and hence, enhancing the cholinergic activity in the brain which is insufficient with AD (Seltzer, 2005). Donazil was purchased from Eva Pharma, Egypt, and was dissolved in Carboxy Methyl Cellulose (CMC) (0.25%) and administered orally.

Animals

Forty-two male albino rats, weighting 200 ± 20 g at the start of the experiments were used. Prior to the initiation of the studies, the animals were randomized and assigned to treatment groups. Four rats were housed per cage (size 26 × 41 cm) and placed in the experimental room for acclimatization 24 h before the test. The animals were fed with standard laboratory diet and with tap water ad libitum, and kept in an air-conditioned animal room at 23 ±1°C with a 12 h light/dark cycle. Animal care and handling was performed in conformity with approved protocols of MSA University, Faculty of Pharmacy, Research Ethics Committee and Egyptian Community guidelines for animal care.

Experimental groups

The dose of BM extract was determined according to methodology of Sathiyanarayanan et al. (2010). The LD_{50} of RO extract was determined previously (Anadon et al., 2008) which is 2000 mg/kg of body weight. Rats were randomly allocated into seven groups of six animals each. Rats were orally given the hydro-ethanolic extracts of BM and *RO* for a period of 2 weeks prior to injection of $AlCl_3$.

Group 1: Control group injected (I.P) by 0.9% saline.
Group 2 ($AlCl_3$): Rats injected $AlCl_3$ at dose 4.2 mg/kg/day (I.P) for 28 days.
Group 3 (BM 300): Rats received BM extract 300 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 4 (BM 450): Rats received BM extract 450 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 5 (RO 300): Rats received RO extract 300 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 6 (RO 450): Rats received RO extract 450 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.
Group 7 (Donazil® 500): Rats received Donazil 2.5 mg/kg/day, orally for 28 days and injected by the same dose of $AlCl_3$ for 28 days.

Blood samples and biochemical analysis

Preparation of blood samples

At the end of the study, rats were fasted overnight, anesthetized with thiopental sodium (50 mg/kg) (Vogler, 2006) and blood samples were collected in the morning (5 ml per rat). Blood samples were centrifuged at 3000 rpm for 15 min after 30 min of collection and stored at –80°C until analyzed for the analysis of Malondialdehyde (MDA), and Total anti-oxidant capacity (TAC).

Preparation of brain samples

Animals were euthanized by cervical dislocation, and then the brain was rapidly removed from each rat. Part of each brain was fixed in formalin-saline for 48 h for histopathological study. Another part of the brain was homogenized, using glass homogenizer (Universal Lab. Aid MPW-309, mechanika precyzyjna, Poland), with 5 ml phosphate buffer saline (PBS) then centrifuged using cooling ultra-centrifuge. The homogenate was divided into four aliquots for measuring Amyloid β (Aβ) - peptide, acetylcholinesterase (AChE), tau (τ) protein and serotonin.

Biochemical analysis

Analysis of serum was carried out for measuring MDA, and TAC levels using corresponding colorimetric Cell Biolabs, Inc, USA and Zen-Bio, ABTS antioxidant assay kit, Inc., USA respectively. Aβ-peptide, tissue AChE, τ-protein and serotonin were measured using corresponding rat enzyme immunoassay kits Wuhan EIAAB Science Co, Ltd, China, Kamiya Biomedical Company, USA, Elabscience, USA, and Lifespan Biosciences Inc. USA, respectively.

Histopathological examination

As mentioned earlier, brain tissue was fixed in 10% formalin and then routinely processed and embedded in paraffin. Five microns' sections were cut and stained with hematoxylin and Eosin (H&E) and Congo Red.

Statistical analyses

All data were expressed as mean ± SD and analyzed using Prism program version 6. For all parameters, comparisons among groups were carried out using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. All P values reported are two-tailed and $P < 0.05$ was considered significance.

Ethics approval

Animal care and handling was performed in conformity with approved protocols of MSA University and Egyptian Community guidelines for animal care.

RESULTS AND DISCUSSION

Characterization of major metabolites in BM and RO extracts *via* UPLC-ESI-MS

Chromatographic fingerprints of the hydro-ethanolic extracts of BM and RO were obtained using UPLC-ESI-MS as depicted in Figures 1 and 2 respectively. The identities, elemental compositions, relative percentages and observed molecular and product ions for individual components in both positive and negative modes are presented in Tables 1 and 2 respectively. With the optimized LC and MS conditions, a total of 15 and 17

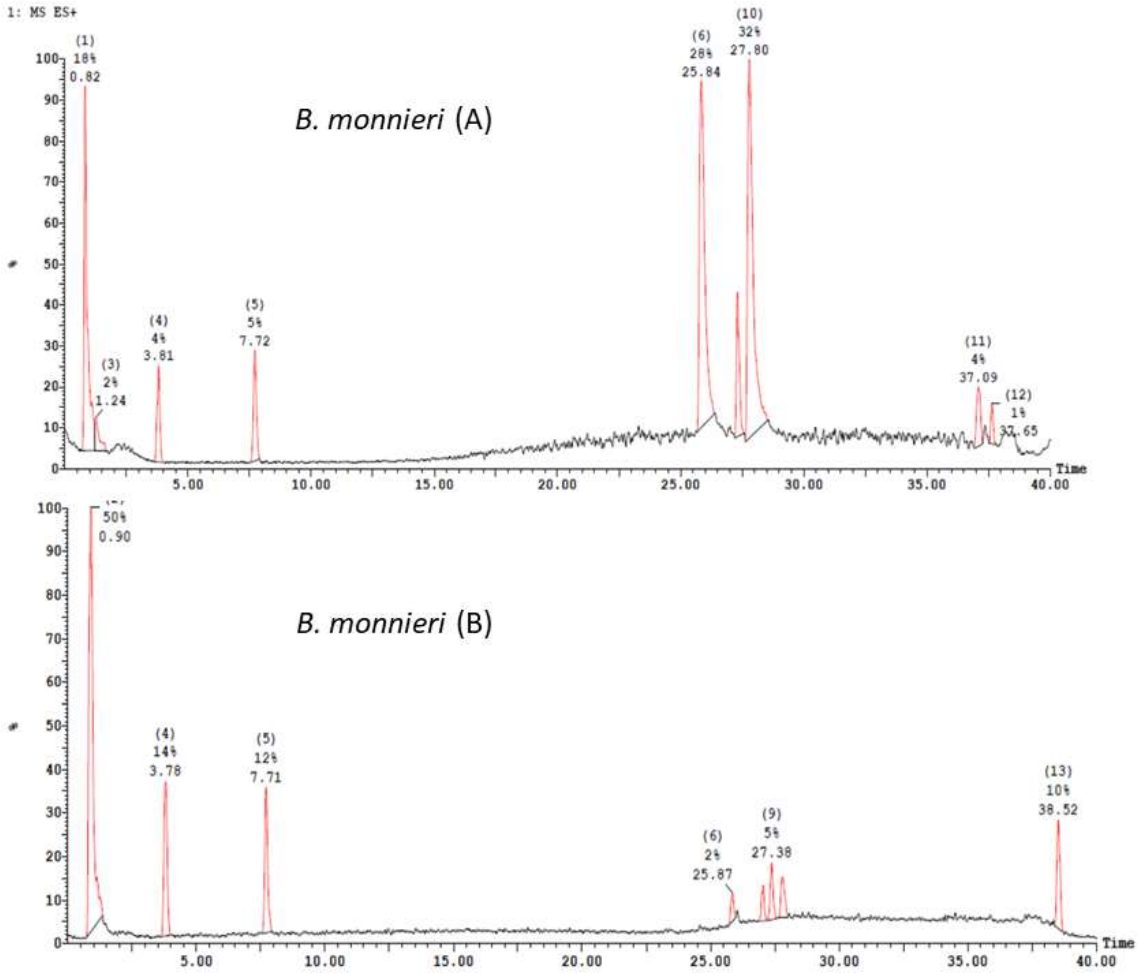

Figure 1. The LC–ESI/MS Chromatograms of the hydro-ethanolic extracts of *B. monnieri* (A): positive mode and (B): negative mode.

metabolites were tentatively characterized in BM and RO extracts respectively on the basis of their elemental compositions and MS fragmentation patterns compared to the data previously reported in literature.

The major metabolites of BM are dammarane-type of steroidal saponins that are categorized into jujubogenin saponin glycosides and pseudojujubogenin saponin glycosides. Pseudojujubogenin is the isomer of jujubogenin having different position of the prenyl side chain (Nuengchamnong et al., 2016), in addition to, cucurbitacins and sterol glycosides. The UPLC-ESI-MS analyses revealed the presence of 5 jujubogenin saponin glycosides, namely; bacoside A1, bacopasaponin F, bacopaside N1, deoxy-jujubogenin-Ara-Glc and deoxy-jujubogenin-2-Glc; and 7 pseudojujubogenin saponin glycosides, namely; bacopaside (I, II and III), oxy-bacopaside I, bacopasaponin (C and D) and pseudojujubogenin-Glc-Glu-Ara. Cucurbitacins (bacobitacin B and C) and bacosterol-Glc were also identified in the hydro-ethanolic extract of BM.

The UPLC-ESI-MS analyses of RO hydro-ethanolic extracts led to the characterization of rosemary landmarks represented in 6 phenolic diterpenes, namely; carnosol, carnosic acid, rosmanol, epirosmanol, rosmadial and methyl carnosate, 3 phenolic acids namely; Gallic acid, caffeic acid and rosmarinic acid. Flavonoids of flavone and flavanone types were also detected such as apigenin, luteolin, cirsimaritin, hesperidin and their glycosides, in addition to the dihydrochalcone (phloridzin) and the lignan (medioresinol).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on serum MDA and TAC levels

Mean serum level of MDA was significantly increased in $AlCl_3$ induced Alzheimer's group compared to the control group (*P* value was < 0.001). Mean serum level of MDA was significantly reduced in BM 300, BM 450, RO 300,

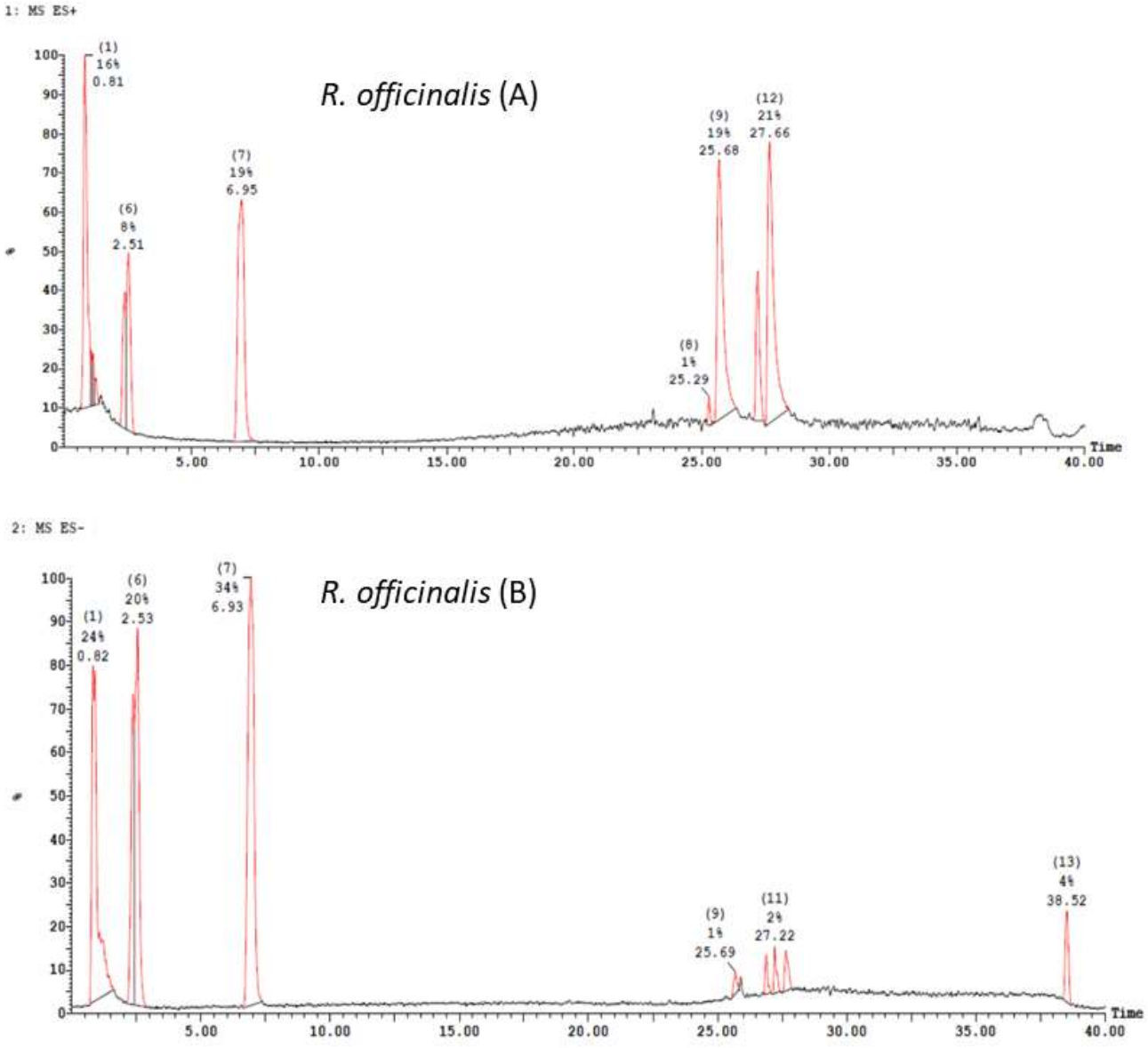

Figure 2. The LC–ESI/MS Chromatograms of the hydro-ethanolic extracts of *R. officinalis* (A): positive mode and (B): negative mode.

RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (*P* value < 0.001). RO 450 treatment reduced MDA level compared to BM 450 and Donazil groups (*P* value was < 0.001).

Similarly, mean serum level of TAC was significantly decreased in $AlCl_3$ induced Alzheimer's group compared to the control group (*P* value was < 0.0001). The mean serum level of TAC was significantly raised in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.001). TAC was increased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figures 3 and 4; Table 3).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue amyloid beta protein and τ-protein levels

Mean tissue level of amyloid β peptide was significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value was < 0.0001). On the other hand, mean tissue level of amyloid β peptide was significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Amyloid β peptide was decreased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figure 5 and Table 4).

Table 1. Peak assignment of metabolites in the hydro-ethanolic extract of *Bacopa monnieri* using LC–ESI/MS in the positive and negative modes.

Peak No.	Positive Ionization		Negative Ionization		Elemental composition	Tentative compound assignment	Relative (%)	References
	$[M+H]^+$ *(m/z)*	Product ion fragments *(m/z)*	$[M-H]^-$ *(m/z)*	Product ion fragments *(m/z)*				
Jujubogenin saponin glycosides								
1	736.69	455	n.d.	n.d.	$C_{40}H_{64}O_{12}$	Bacoside A1	2.65	Nuengchamnong et al. (2016)
2	n.d.	n.d.	1059.38	765, 633	$C_{52}H_{84}O_{22}$	Bacopasaponin F	2.13	
3	797.47	779 , 635, 455	795.39	633	$C_{42}H_{68}O_{14}$	Bacopaside N1	1.47	
4	749.43	437	747.32	435	$C_{41}H_{64}O_{12}$	Deoxy-Jujubogenin-Ara-Glc	4.39	
5	779.62	437	n.d.	n.d.	$C_{46}H_{66}O_{10}$	Deoxy- Jujubogenin -2 Glc	2.81	
Pseudojujubogenin saponin glycosides								
6	979.1	767, 605, 473	977.46	845, 241	$C_{46}H_{74}O_{20}S$	Bacopaside I	32	Sookying et al. (2017)
7	n.d.	n.d.	927.48	795, 633	$C_{47}H_{76}O_{18}$	Bacopaside II	5.11	
8	847.41	765, 515, 393	n.d.	n.d.	$C_{41}H_{66}O_{16}S$	Bacopaside III	14.18	
9	n.d.	n.d.	993.51	861, 505, 389	$C_{46}H_{74}O_{21}S$	Oxy-Bacopaside I	4	Nuengchamnong et al. (2016)
10	n.d.	n.d.	897.03	765, 603	$C_{46}H_{74}O_{17}$	Bacopasaponin C	3.5	
11	767.5	605, 473	n.d.	n.d.	$C_{41}H_{66}O_{13}$	Bacopasaponin D	7.71	
12	n.d.	n.d.	941.53	809, 647	$C_{47}H_{74}O_{19}$	Pseudojujubogenin-Glc-Glu-Ara	28.06	
Cucurbitacins								
13	599.37	479, 443	n.d.	n.d.	$C_{34}H_{46}O_9$	Bacobitacin B	6.23	Bhandari et al. (2006)
14	1113.47	981, 835	n.d.	n.d.	$C_{54}H_{80}O_{24}$	Bacobitacin C	17.5	
Sterol glycosides								
15	575.89	414	n.d.	n.d.	$C_{35}H_{60}O_6$	Bacosterol-Glc prevent	9.72	Bhandari et al. (2006)

*n.d.: Not detected, Glc: glucose, Glu: glucouronide, Ara: arabinose.

Similarly, mean tissue level of tau-protein was significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value < 0.001). Also, the mean tissue levels of τ -protein was significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.001). τ -protein was decreased in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.01 (Figure 8 and Table 5).

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue acetylcholinesterase (AChE) levels

Mean tissue levels of AChE were significantly increased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value < 0.0001). AChE were significantly reduced in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Also, AChE was reduced in RO 450 group compared to Donazil and BM 450 groups at *P* value < 0.0001 (Figure 6).

Table 2. Peak assignment of metabolites in the hydro-ethanolic extract of *Rosmarinus officinalis* using LC–ESI/MS in the positive and negative modes.

Peak No.	Positive Ionization		Negative Ionization		Elemental composition	Tentative compound assignment	Relative (%)	References
	$[M+H]^+$ *(m/z)*	Product ion fragments *(m/z)*	$[M-H]^-$ *(m/z)*	Product ion fragments *(m/z)*				
Phenolic diterpenes								
1	331.34	287	n.d.	n.d.	$C_{20}H_{25}O_4$	Carnosol	8.32	Hossain et al. (2010)
2	n.d.	n.d.	331.24	287, 244	$C_{20}H_{27}O_4$	Carnosic acid	19.47	
3	347.19	285	345.25	283	$C_{20}H_{26}O_5$	Rosmanol	1.87	Achour et al. (2018)
4	347.18	285	345.16	283	$C_{20}H_{26}O_5$	Epirosmanol	0.85	Borras-Linares et al. (2014)
5	n.d.	n.d.	343.37	315, 300	$C_{20}H_{23}O_5$	Rosmadial	16.28	Hossain et al. (2010)
6	347.2	303, 288	n.d.	n.d.	$C_{21}H_{29}O_4$	Methyl carnosate	0.72	
Phenolic acids								
7	171.01	127	169.03	125	$C_7H_5O_5$	Gallic acid	0.93	Hossain et al. (2010)
8	n.d.	n.d.	179.12	161, 135	$C_9H_7O_4$	Caffeic acid	0.95	
9	n.d.	n.d.	359.07	197, 161	$C_{18}H_{16}O_8$	Rosmarinic acid	19.39	Borras-Linares et al. (2014)
Flavonoids: Flavones								
10	n.d.	n.d.	269.1	269	$C_{15}H_{10}O_5$	Apigenin	3.75	Achour et al. (2018)
11	n.d.	n.d.	576.95	269	$C_{27}H_{29}O_{14}$	Apigenin-7-O-rutinoside	5.36	Hossain et al. (2010)
12	n.d.	n.d.	285.05	263, 191	$C_{15}H_{10}O_6$	Luteolin	24.36	Achour et al. (2018)
13	n.d.	n.d.	478.91	315	$C_{22}H_{22}O_{12}$	Nepetrin	1.5	Borras-Linares et al. (2014)
14	n.d.	n.d.	313.09	298, 283	$C_{17}H_{13}O_6$	Cirsimaritin	12.19	
Flavonoids: Flavanone								
15	611.07	463, 303	n.d.	n.d.	$C_{28}H_{34}O_{15}$	Hesperidin	20.24	Achour et al (2018)
Dihydrochalcone								
16	437.15	275, 169	435.13	273, 167	$C_{21}H_{23}O_{10}$	Phloridzin	0.73	Hossain et al. (2010)
Lignan								
17	389.23	209, 165	n.d.	n.d.	$C_{21}H_{24}O_7$	Medioresinol	1.28	Mena et al. (2016)

*n.d.: Not detected.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on tissue serotonin level

Mean tissue level of serotonin was significantly decreased in $AlCl_3$ induced Alzheimer's group compared to control group (*P* value < 0.0001). Also, mean tissue levels of serotonin was significantly elevated in BM 300, BM 450, RO 300, RO 450, and Donazil groups compared to $AlCl_3$ induced Alzheimer's group (P value < 0.0001). Serotonin was increased in RO 450 group compared to Donazil and BM 450 groups at *P*

Table 3. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extracts on serum MDA and TAC levels.

Groups	Serum MDA (umol/ml)	Serum TAC (umol/ml)
Control	12.4 ± 1.40	100 ± 7.43
ALZ	38 ± 4.56 [a]	41.9 ± 3.80 [a]
BM 300	26.5 ± 0.87 [ab]	53 ± 3.80 [ab]
BM 450	20.9 ± 1.24 [abc]	68 ± 3.82 [abc]
RO 300	32.5 ± 3.66 [ab]	54.4 ± 4.39 [ab]
RO 450	17.7 ± 1.32 [ab]	84.2 ± 5.52 [ab]
Donazil	21.4 ± 1.28 [abc]	76.7 ± 4.94 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.001$, b = Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.001$.

Table 4. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue amyloid beta protein and acetylcholinestrase levels.

Groups	Tissue amyloid beta peptide (Pg/gm tissue)	Tissue acetylcholinestrase (ng/gm tissue)
Control	9.3 ± 0.92	0.81 ± 0.08
ALZ	30.9 ± 4.01[a]	3.14 ± 0.26[a]
BM 300	21.8 ± 1.37[ab]	2.2 ± 0.08 [ab]
BM 450	17.3 ± 1.09[abc]	1.79 ± 0.08 [abc]
RO 300	21.8 ± 1.74[ab]	2.34 ± 0.15 [ab]
RO 450	13.2 ± 0.95[ab]	1.21 ± 0.11 [ab]
Donazil	18.9 ± 0.9[abc]	1.67 ± 0.11 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.01$.

value < 0.0001 (Figure 7).

Histopathological changes associated with herbal treatment

Brain sections from the control group (C) showed normal histological appearance in both the neurons and the blood vessels. The ALZ group showed marked neuronal degenerative changes and marked amyloid deposits on the blood vessels. All other groups showed different histological changes in both neurons and blood vessels illustrated in Figures 9 and 10.

The present study showed that *Bacopa monnieri* (BM) and *Rosmarinus officinalis* (RO) have a neuroprotective effect in AD rat model. This effect is possibly mediated *via* anti-AChE and antioxidant, and monoaminergic pathways modulation. Both extracts are potential candidates in the management of AD.

BM significantly increased serum TAC levels on the other hand significantly reduced MDA, a well-known oxidative stress biomarker, in $AlCl_3$ induced AD rat model. This clearly indicates the enhancement of the antioxidant activity, which is a key component in AD pathogenesis (Bartus, 2000). This result comes in agreement with several studies that have shown that BM exerts antioxidant activity both *in vivo* and *in vitro* (Russo et al., 2003; Bhattacharya et al., 2000; Chaudhari et al., 2017). Jyoti et al. (2007) reported that BM inhibited the reduction of superoxide dismutase (SOD) activity in $AlCl_3$ induced neurotoxicity of rat brain. It also showed that BM inhibited thiobarbituric acid reactive substance (TBARS), an index of lipid peroxidation (LPO) and its accumulation associated with $AlCl_3$ neurotoxicity of rat brain (Jyoti et al., 2007; Jyoti and Sharma, 2006). Our results further elaborate and confirm these reports of the antioxidant effect of BM as a neuroprotective agent in AD that is comparable to current medications used (Figures 3 and 4). In addition, it was shown that BM reduced Aβ and τ - protein levels in AD rat model. It was also observed that

Table 5. Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on tissue serotonin in Alzheimer's rats.

Groups	Tissue serotonin (ng/gm tissue)	Tissue Tau-protein (Pg/gm tissue)
Control	47.7 ± 6.27	8.2 ± 1.02
ALZ	6.07 ± 0.77 [a]	30.7 ± 2.91 [a]
BM 300	23.6 ± 2.31 [ab]	23.5 ± 0.97 [ab]
BM 450	21.9 ± 3.24 [abc]	17.3 ± 1.17 [abc]
RO 300	12.8 ± 1.22 [ab]	22.8 ± 1.78 [ab]
RO 450	37.5 ± 2.93 [ab]	14.7 ± 1.14 [ab]
Donazil	14.9 ± 0.73 [abc]	17.3 ± 1.42 [abc]

C = control; ALZ = Alzheimer's; BM 300 = *Bacopa monnieri* extract 300 mg/kg; BM 450 = *Bacopa monnieri* 450 mg/kg; RO 300 = *Rosmarinus officinalis* extract 300 mg/kg; RO 450 = *Rosmarinus officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.0001$.

Effect of *Bacopa monnieri* and *Rosmarinus officinalis* extract on serum MDA of ALZ model in rats

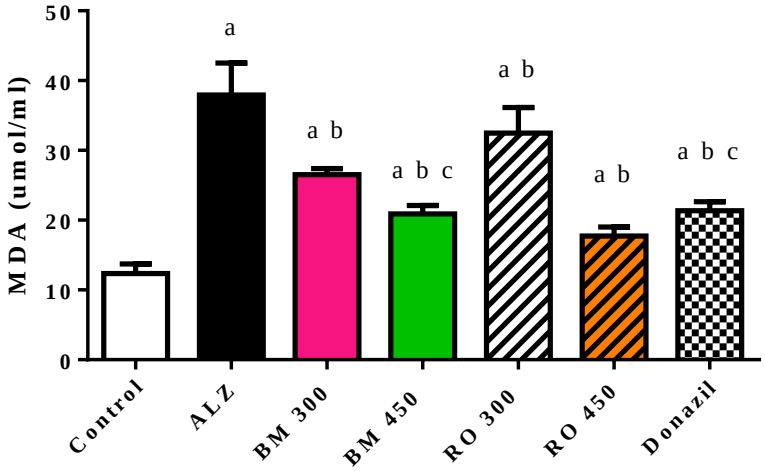

Figure 3. Serum level of MDA (µmol/ml) in the experimental groups. BM and RO extract reduced serum level of MDA in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.001$, b = Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.001$.

BM at tested doses reduced Aβ and τ -protein levels in AD rat model brain when examined histologically using H&E and Congo Red staining compared to control. Neurons exhibited unremarkable degenerative changes while blood vessels showed moderate and unremarkable amyloid thickening (Figures 9 and 10).

We also demonstrated that BM extract inhibited significantly acetylcholinesterase (AChE) activity in AD rat model, a result that further support its use as a neuroprotective agent in AD. It is well documented that cholinergic dysfunction is implicated in AD pathogenesis although the mechanism is not well understood (Bartus, 2000). Our results support the hypothesis that the observed neuroprotective effect of BM extract can be attributed to inhibition of AChE consequently preserving ACh longer at the synapses and compensating for the

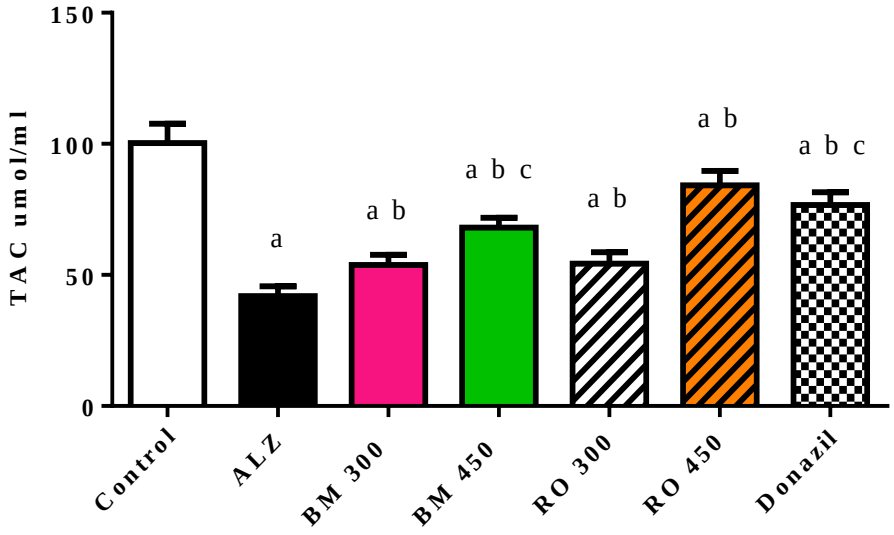

Figure 4. Serum level of TAC (µmol/ml) in the experimental groups. BM and RO extract raised serum level of TAC in Alzheimer's rats at the end of 2 month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.01$.

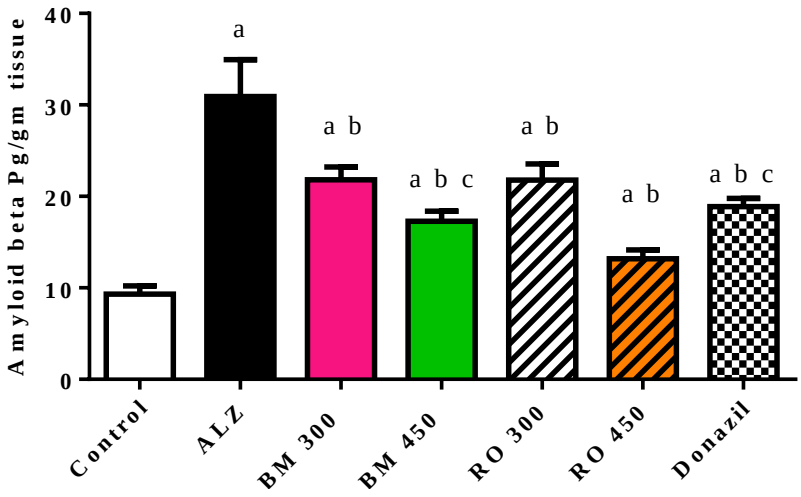

Figure 5. Tissue level of amyloid beta peptide (Pg/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of amyloid beta in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.01$.

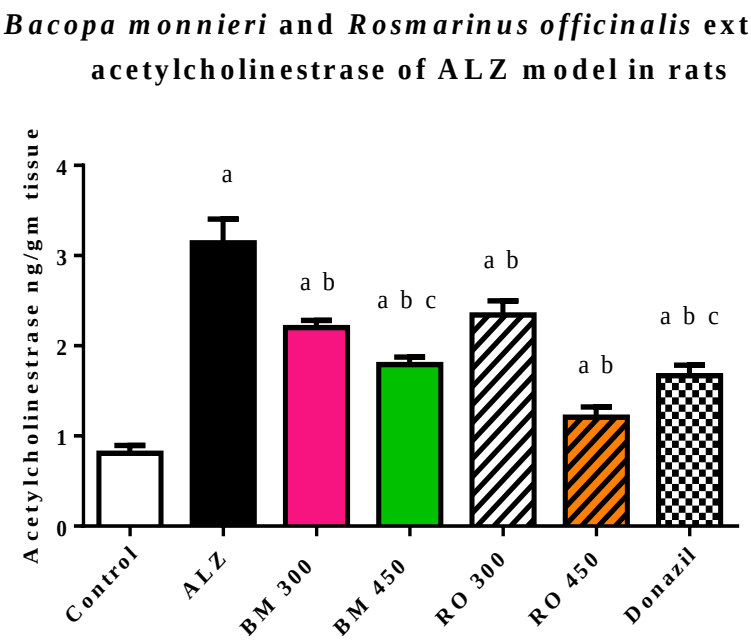

Figure 6. Tissue level of acetyl cholinestrase (ng/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of amyloid beta in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at $P < 0.0001$, b = Significant from ALZ at $P < 0.0001$, c = Significant from RO 450 at $P < 0.0001$.

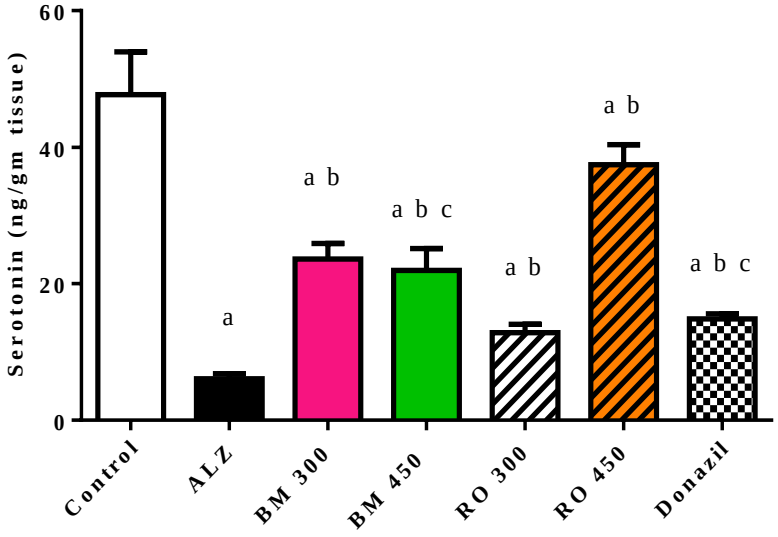

Figure 7. Tissue level of serotonin (ng/gm tissue) in the experimental groups. BM and RO extract raised tissue level of serotonin in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 = B. monnieri extract 300 mg/kg; BM 450 = B. monnieri 450 mg/kg; RO 300 = R. officinalis extract 300 mg/kg; RO 450 = R. officinalis extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a = Significant from control at P < 0.0001, b = Significant from ALZ at P < 0.0001, c = Significant from RO 450 at P < 0.0001.

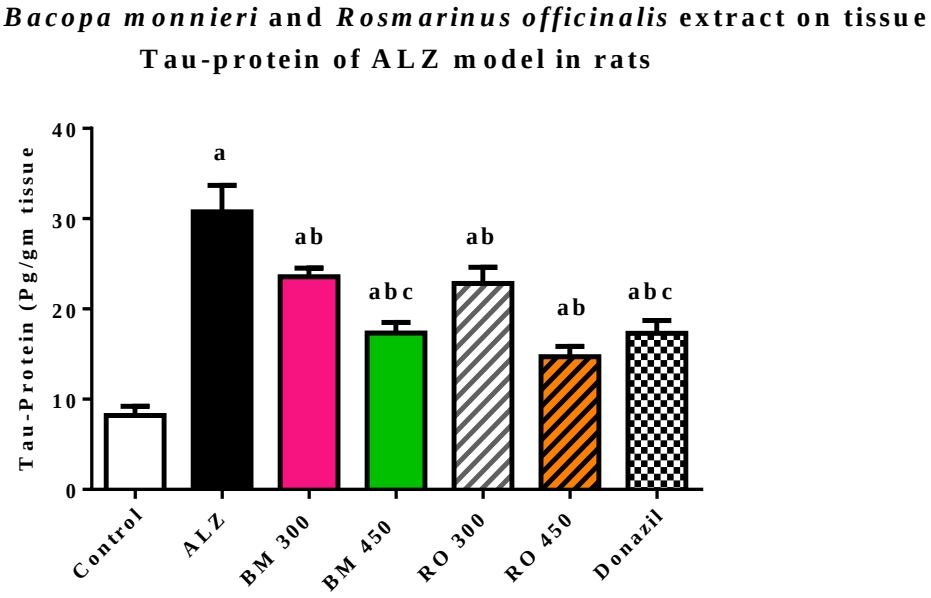

Figure 8. Tissue level of Tau-Protein (Pg/gm tissue) in the experimental groups. BM and RO extract reduced tissue level of tau-protein in Alzheimer's rats at the end of 2-month prophylaxis; C = control; ALZ = Alzheimer's; BM 300 **=** *B. monnieri* extract 300 mg/kg; BM 450 = *B. monnieri* 450 mg/kg; RO 300 = *R. officinalis* extract 300 mg/kg; RO 450 = *R. officinalis* extract 450 mg/kg; D = Donazil. 2.5 mg/kg. Results were expressed as mean ± SD and analyzed using one-way ANOVA followed by Bonferroni's post hoc test a **=** Significant from control at $P < 0.001$, b **=** Significant from ALZ at $P < 0.001$, c = Significant from RO 450 at $P < 0.01$.

lost cholinergic function. To our knowledge, this is the first demonstration of BM effect on AChE.

The mechanism underlying the protection of Brahmi against Aβ25–35-mediated neurotoxicity demonstrated that Aβ 25–35 induced neurotoxicity causes the elevation of intracellular AChE activity and so the elevation of AChE activity was diminished by co-treatment of cortical cells with Brahmi extract. Also, AChE was proved to be neurotoxic both *in vitro* and *in vivo* models. This observation suggests the neuroprotection of Brahmi through its inhibitory effect on amyloid peptide-activated intracellular AChE activity (Limpeanchoba et al., 2008).

The presence of the nootropic metabolite; Bacopaside I (Pseudojujubogenin- 3-O-[α-l-arabinofuranosyl-(1→2)]-6-O- sulfonyl-α-D-glucopyranosyl-(1→3)-α-l-arabinopyranoside) is believed to account for the neuroprotective effect of BM extract as it reverses the depressive-like symptoms caused by reserpine, which is mediated through antioxidant and noradrenergic activations. It also stimulates PI3/Akt signaling in organotypic hippocampal slice cultures (Yin et al., 2016). In accordance, a study conducted by Le et al. (2015) concluded that bacopaside I played a role in neuroprotective effects in both *in vitro* and *in vivo*, where in the *in vitro* experiment, the hippocampal slice cultures (OHSCs) were incubated with triterpenoid saponins from BM, where bacopaside I exhibited potent neuroprotective effects against OGD-induced neuronal cell damage (Le et al., 2015). However, the role of each dammarane steroidal saponin of BM in the neuroprotection is still uncovered.

The presence of Bacoside metabolite is thought to have anti-oxidant and free radical scavenging as it inhibits lipid peroxidation and elevates the anti-oxidant enzymes in prefrontal cortex, hippocampus, and striatum which also possess a significant iron chelating property; also, iron and other divalent metals interact with Aβ protein and modulate several effects that are thought to be the pathogenic effects of that protein (Chaudhari et al., 2017).

Similar to BM, *R. officinalis* (RO) extract also exhibited antioxidant effects in our $AlCl_3$ induced AD rat model. This effect was demonstrated by its significant reduction of the oxidative stress biomarker MDA and enhancement of TAC (Figures 3 and 4). This result complement previous reports that Carnosic acid (CA), a metabolite found in rosemary and sage, had antioxidant effects in both *in vitro* and transgenic mice (Lipton et al., 2016). Also, Rasoolijazi et al. (2013) reported on its role in memory and learning scores improvement. CA decreased

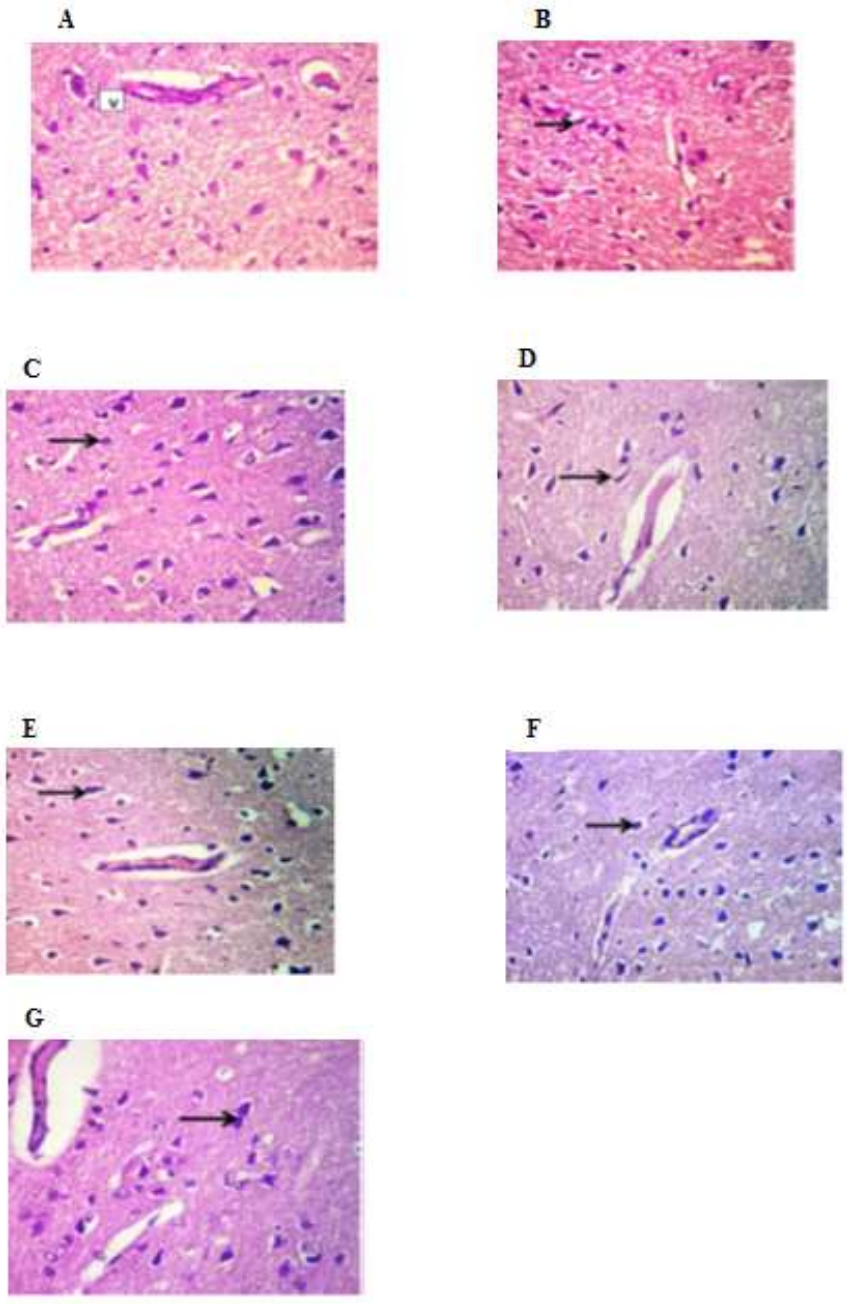

Figure 9. Histopathological sections of brain tissue illustrating herbal treatment effect in all experimental groups. **A:** negative control, illustrates unremarkable changes in both neurons and vessels (V). **B:** ALZ group exhibiting neuronal marked degenerative changes (thin arrow). **C:** RO 300 group showed minimal neuronal degenerative changes (thin arrow). **D:** RO 450 group showed unremarkable neuronal degenerative changes (thin arrow). **E:** BM 300 group demonstrating un remarkable neuronal degenerative changes (thin arrow). **F:** BM 450 group demonstrating un remarkable neuronal degenerative changes (thin arrow). **G:** Donazil group showing unremarkable degenerative changes of neurons (thin arrows). H&E staining. Magnification 200, arrow pointing at neurons, V blood vessels.

52% of the infarct volume from brains under ischemia/reperfusion *in vivo*, and also protected the PC12 cells from hypoxic injury *via* reducing oxidative stress biomarkers and enhancing cell viability *in vitro*

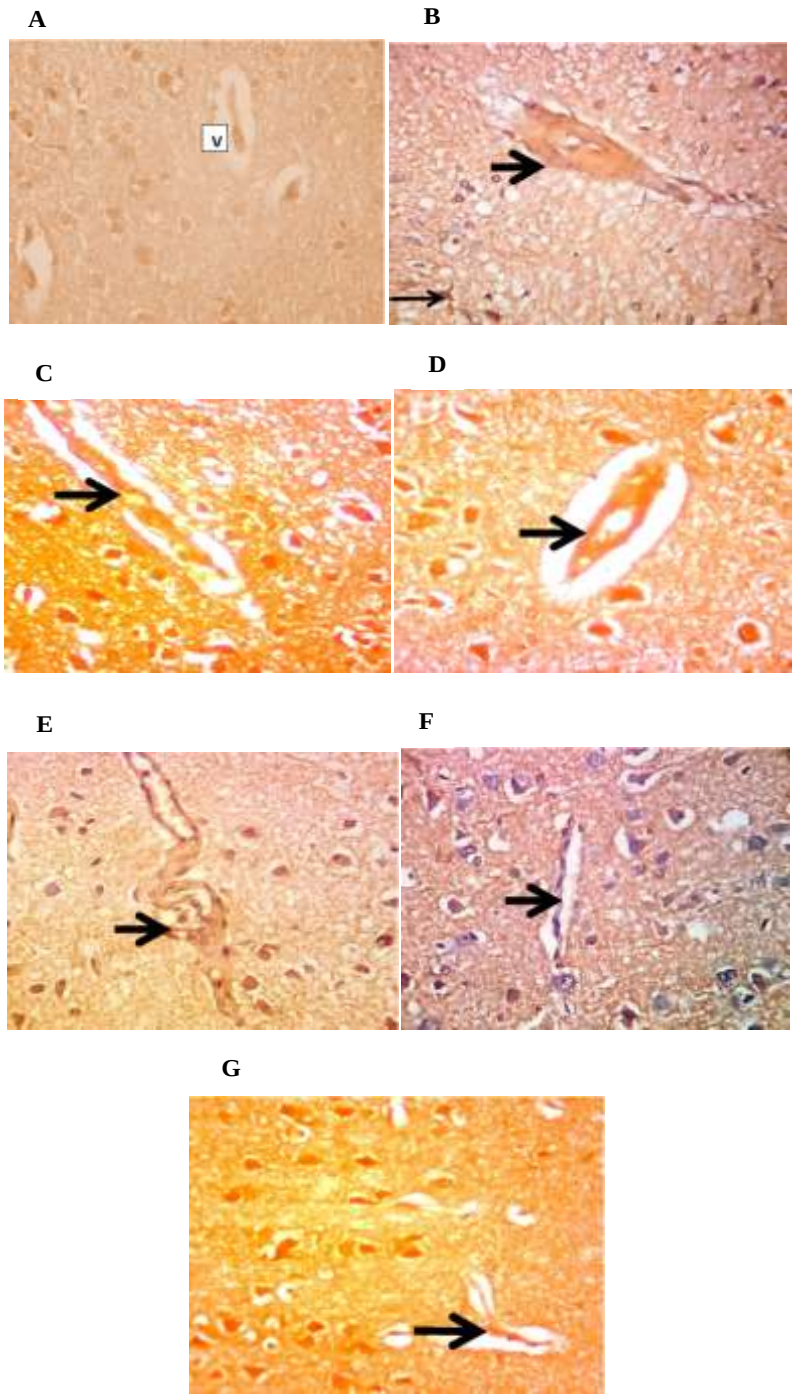

Figure 10. Histopathological sections of brain tissue illustrating herbal treatment effect in all experimental groups. **A:** negative control illustrates unremarkable changes in both neurons and vessels (V). **B:** ALZ group exhibited marked thickening of the vessel wall by the amyloid deposits on blood vessels (thick arrow). **C:** RO 300 group showed mild thickening of the vessel wall by the amyloid deposits (thick arrow). **D:** RO 450 group showed moderate thickening of the vessel wall by the amyloid deposits (thick arrow). **E:** BM 300 group exhibiting moderate thickening of the vessel wall by the amyloid deposits (thick arrow). **F:** BM 450 group exhibiting unremarkable thickening of the vessel wall by the amyloid deposits (thick arrow). G: Donazil group showing unremarkable thickening of the vessel wall by the amyloid deposits (thick arrow). Congo Red staining. Magnification 200, arrow pointing at neurons, V blood vessels.

(Hou et al., 2012).

Not only that CA has a nootropic effect, but also, the administration of Rosmarinic acid (RA) averted cognitive impairment induced by chronic ethanol (Hasanein et al., 2017). Actually, the mechanism of action of RO pertaining to neuroprotection could also be attributed to the synergistic effects of phenolic and polyphenolic metabolites that possess well-known antioxidant and anticholinesterase activities.

Our experiments also showed that RO at selected doses reduced Aβ and τ -protein levels in AD rat model brain, a result that comes in agreement with a previous report of the effect of CA on U373MG human astrocytoma cells probably *via* activation of α-secretase (Yoshida et al., 2014). This may also explain our histopathological examination of RO treated rat brains that revealed mild to unremarkable degenerative changes as well as mild to moderate amyloid thickening of vascular walls (Figures 9 and 10).

Since there seems to be an agreement that AD pathogenesis results primarily from defective brain cholinergic function (Bartus, 2000; Iqbal and Grundke-Iqbal, 2010; Murphy and Steenbergen, 2008), we investigated the possibility that RO neuroprotective effects in our AD rat model may be due to enhancement of cholinergic function. It was found that, similar to BM, RO significantly inhibited AChE. This also agrees with previous report that RO improved long-term memory and inhibited the AChE activity of rat brain (Ozarowski et al., 2013). These results may also explain the observed effectiveness of rosemary aromatherapy in human (Jimbo et al., 2009).

It is well-known that nitrocatechol derivatives exhibit anti-aggregation properties against Aβ protein. It is also documented that Rosmarinic acid had two cathecol moieties, which consequently induces morphological and signature changes in the secondary structure of tau-protein once it is interacted with, thus, preventing aggregation and β-sheets assembly and also reducing fibril progression (Cornejo et al., 2015). Both RO and BM effects on all afore-mentioned biochemical markers and histopathological results were comparable to Donazil®™, a standard selective inhibitor of brain cholinesterase commonly used in AD management (Bitra et al., 2014; Nayak and Chatterjee, 2001).

Serotonin (5-hydroxytryptamien, 5-HT) has been linked to emotional and motivational aspects of human behavior and memory (Meneses and Liy-Salmeron, 2012). Recently, it has been documented that the serotonin as a neurotransmitter is involved in the pathophysiology of AD (Ramirez, 2013; Maccioni et al., 2018). Hence, the 5-HT_6 receptor is a promising target for cognitive disorders AD (Amat-Foraster et al., 2017) where we aimed to evaluate tissue serotonin in both our AD model and with herbal therapy (Figure 7).

Indeed, our results have shown the diminution of tissue serotonin as well as its partial restoration with our herbal treatment.

Both extracts exhibited significant elevation of tissue serotonin levels with a prominent effect for RO 450 mg/kg compared to the controls. This result is the first demonstration that RO and BM extracts may exert their neuroprotective effects in AD rat model *via* serotoninergic system. It was previously reported that RO extract exhibited antidepressant-like activity in mice *via* the monoaminergic system (Machado et al., 2009). BM, on the other hand, elevated serotonin levels rats subjected to stress (Sheikh et al., 2007). Our results is in line with other reports of BM and RO influencing the monoaminergic system (Rajan et al., 2015; Machado et al., 2009), however, we are the first to report this effect in AD rat model.

Since both BM and RO have similar antioxidant and monoaminergic effects, combining both RO and BM can have synergistic effects in the treatment of AD. Ramachandra et al. (2014) reported its neuroprotective effectiveness in contrast to its individual use in embryonic cell line (Ramachandran et al., 2014). The effect in animal model still needs to be investigated.

This study showed that *B. monnieri* (BM) and *R. officinalis* (RO) have a neuroprotective effect in AD rat model at both histological and biochemical levels which is due to their interesting variety of bioactive metabolites. This effect is possibly mediated *via* anti-AChE, monoaminergic and antioxidant pathways modulation. Further pharmacological and clinical studies are needed to confirm these results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors appreciate Dr. Hebatallah Amin, Lecturer of Pathology, Helwan University for the invaluable technical assistance of preparing the histopathological sections micrographs, as well as Dr. Nashwa Waly, Professor of Small Animal Medicine, Assiut University, Egypt, for the invaluable editorial assistance.

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