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Research paper

Antioxidant-mediated neuro-protective and GABAergic calming effect of *Stephania japonica*

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Abstract

Stephania japonica, a tropically-habituated plant is widely distributed in Bangladesh. Traditionally, it has been used in the treatment of inflammation, asthma, fever, sleep disturbance, painful conditions, and rheumatism. However, scientific evidence of its biological activities are very limited. This study evaluated neuroprotctivity along with a possible anxiolytic-*like* effect of the methanol leaf extract of *S. japonica* (MSJ). The antioxidant test was performed by using the scavenging capacity of hydroxyl ($^{\bullet}$OH) and nitric oxide ($^{\bullet}$OH) radicals; and an inhibition of lipid peroxidation assay. *In vitro*, egg albumin denaturation (IELD) and anti-acetylcholinesterase (anti-AChE) tests were performed to evaluate the anti-inflammatory and anti-cholinesterase activity, respectively. Additionally, a possible anxiolytic action of the MSJ was investigated in Swiss mice by taking diazepam (DZP) and flumazenil (FLU) as gamma amino butyric acid (GABA) receptor agonist and antagonist, respectively. The MSJ concentration (50-200 µg/mL)/dose (50-200 mg/kg, oral)-dependently exhibited antioxidant, anti-inflammatory, anti-AChE and anxiolytic-*like* effects. The MSJ also increased the activities of the standards used in antioxidant and anti-AChE (trolox), anti-inflammatory (acetyl salicylic acid), and anxiolytic (DZP: agonist) test. The MSJ exhibited antioxidant and anti-inflammatory activities. It also potentiated the action of DZP, while antagonizing the effect of the FLU, suggesting a possible GABAergic, DZP-agonistic anxiolytic-like action in experimental animals. *S. japonica* may be one of the good sources of neuroprotective phytochemicals.

Keywords: *AChE; Anxiolytic-Like Effect; GABA-Agonistic Action.*

1. Introduction

Traditionally the plant, *Stephania japonica* L. (Fam.: Menisperaceae) has been used in inflammation, cancer, asthma, fever, sleep on disturbance, edema, bone fracture, painful conditions, and rheumatism (Kirtikar and Basu 1981; Rahman et al. 2007; Jahan et al. 2010; Seraj et al. 2013). The ethanol and methanol extracts of the plant are evident for antioxidant and analgesic activities (Rahman et al. 2011; Uddin et al. 2014).

Some important alkaloids such as oxostephamiersines and stebisimine were isolated from the methanol extract of *S. japonica* leaves (Matsui et al. 1982), and have been suggested to have an affinity to the human δ-and µ-opioid receptors (Carroll et al. 2010). In a recent study, Moniruzzaman et al (2016) suggests that the methanol extracts of *S. japonica* leaf did not produce any toxic effect to the Swiss mice up to 3000 mg/kg (orally administered). The preliminary phytochemical screening report in the same study revealed the presence of alkaloids, glycosides, tannins, flavonoids, saponins and carbohydrates. The authors concluded a promising anti-nociceptive activity of the extract at 50, 100 and 200 mg/kg (p.o.), possibly *via* opioid receptors and glutametergic system involvement. Del Valle-Laisequilla et al (2012) suggested that, the alkaloidal groups, atropine, hyoscyamine, hyoscine, or/and ephedrine may be responsible for such type of activity.

However, flavonoids, especially the polyhydric alcohols, including essential oils are also known for their potential antioxidant-mediated anti-inflammatory and organ-protective capacities (Islam et al. 2016a). Oxidative stress may trigger inflammation and etiology of a number of neurological diseases (more than 100), including anxiety and neurodegenerative disorders (NDs) such as Parkinson's diseases, Alzheimer's diseases, anxiety and so on (Islam 2016).

The enzyme, acetylcholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter acetylcholine to terminate signals across cholinergic synapses, including those at neuromuscular junctions. Thus, an inhibition of AChE may serve as a strategy for the treatment of NDs, especially the AD (Ota et al. 2015). Although, anxiety is a normal reaction to stress, but it causes a remarkable discomfort. To date, benzodiazepines (BDZs) are the widely used medications in anxiety. However, they can produce some dangerous problems, including sedation, muscle relaxation, anterograde amnesia and risk of accidents. Furthermore, chronic use of BDZs may lead BDZ tolerance and dependence, psychomotor effects, paradoxical reactions and teratologic risk (Costa et al. 2014).

Taken into together, this study aims to evaluate the neuro protective effect of the methanol leaf extract of *S. japonica* (MSJ) through conducting antioxidant and anti-inflammatory followed by an anti-AChE test. Additionally, an anxiolytic-*like* effect of the MSJ and the possible mechanism of action involved in this study were also evaluated in Swiss mice.

2. Materials and methods

2.1. Plant material and extraction

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The leaves of the plant were collected from Jessore, Bangladesh and were identified by a taxonomist at Forest Research Institute, Chittagong with a voucher specimen number: FRIH 4018. After collection, the plant materials were subjected for shade drying (temperature not exceeding 50 ^{O}C), following to course grinding. Methanol hot extraction (16 h) was carried out by using the Soxhlet apparatus. The extract was then filtered through a cotton plug followed by Whatman filter paper (No. 1). For a rapid evaporation of solvent, rotary evaporator (temperature not exceeding 50 °C) was used. The yield was 6.13%.

2.2. Chemicals and reagents

All the necessary reagents and chemicals were purchased from (Sigma-Aldrich, St. Louis, MO; USA.).

2.3. Sample preparation

Required amount of extract (MSJ) was soaked in distilled water (DW) and shaken vigorously for 30 minutes, which was then kept overnight, filtered through Whatman filter paper and stored in an amber color glass container. The standard, trolox (TRO: 6-Hydroxy-2, 5, 7, 8-tetramethyl-2-carboxylic acid, for antioxidant and anti-AChE tests), diazepam and flumazenil (DZP/FLU, for anxiolytic test) were dissolved in 0.05% tween-80 with 0.9% NaCl solution.

2.4. Experimental animals

For hydroxyl radical scavenging or hemolysis test, a male adult wister albino rat (*Rattus norvegicus*) of 2 months old and 220 gm body weight were collected from the Bangladesh Council for Scientific Research and Institute, Chittagong (BCSIR), Bangladesh. The animal was allowed to free access to water and food (Purina's pellets) ad libitum and was kept under controlled lighting (12 h dark/light cycle) and temperature (24 ± 2 ^{O}C). Total 55 Swiss albino mice (*Mus musculus*, both sex, 6 weeks old, 22-28 gm body weight) were also purchased from the same organization and housed in the same controlled environment prior to the test commenced.

2.4.1. H_2O_2-induced hemolysis (HL) test

This test is an adjustment of the earlier described methods of Ruch et al. (1989) and Girish et al. (2012). Briefly, blood was collected from the retro-orbital plexus of an adult male wister albino rat and immediately reconstituted 10% RBC suspension (RRBC) with the phosphate buffer saline (PBS, pH 7.4). To 0.5 mL 10% RRBC suspension, 0.1 mL of 40 mM hydrogen peroxide (H_2O_2) was added. The hydrogen peroxide was considered as a stressor (STR), which was prepared in the same PBS solution. The tubes were then incubated at 37 ^{O}C for 30 minutes. After the incubation period, 0.2 mL of supernatant was withdrawn and absorbance (A_{STR}) was measured at 475 nm by using a UV spectrophotometer after the addition of 2.8 mL PBS. Similarly, the tubes for NC (vehicle) and test sample (MSJ/ TRO/ MSJ + TRO) were treated with 0.1 mL of each, just after the addition of STR, following to incubation in the same way and taking the absorbance (A_{TS}) at 475 nm. The percentage inhibition of hemolysis (%IHL) was calculated as follows:

$$\% \text{ IHL} = [(A_{STR} - A_{TS}) \div A_{STR}] \times 100$$

2.4.2. Nitric oxide ($NO^{\bullet}$) scavenging test

In this test, the reaction mixture contains 0.375 mL test sample, 1.5 mL of sodium nitroprusside (10 mM) and 0.375 mL phosphate buffer saline (pH 7.4). The absorbance (A_{BR}) was taken at 546 nm. After incubating the reaction mixture at 37 °C for 1 h, 1 mL of supernatant was mixed with 1 mL of Griess reagent [(1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 2 mL of naphthylethylenediamine dichloride (0.1% w/v)]. Then the reaction mixture was kept at room temperature for 30 min and the final absorbance (A_{AR}) was measured at the same wavelength. For NC, 0.375 mL vehicle was used (Marcocci et al. 1994). The percentage of $NO^{\bullet}$ inhibition was calculated by using the following equation:

$$\% \text{ inhibition of } NO^{\bullet} = [(A_{BR} - A_{AR})/A_{BR}] \times 100$$

Where, A_{BR} and A_{AR} are the absorbance of $NO^{\bullet}$ free radicals before and after reaction with Griess reagent, respectively.

2.4.3. Lipid peroxidation test

The thiobarbituric acid substances (TBARS) assay was adopted to measure the quantity of inhibition of lipid peroxidation (ILP) capacity of the test sample and controls. Briefly, 0.1 mL of sample was added to the test tube containing 1 mL of 1% w/v homogenized egg yolk (in 20 mM phosphate buffer at pH 7.4). Lipid peroxidation was induced by the addition of 0.1 mL of 2,2'-azobis(2-methylpropionamidine) dihydrochloride solution (AAPH; 0.12 M). The reaction mixture was then incubated at 37 °C for 15 min. After cooling, 0.5 mL of supernatant mixed with 0.5 mL of trichloroacetic acid (15%) was centrifuged at 1,200 × g for 10 min. An aliquot of 0.5 mL of supernatant was mixed with 0.5 mL TBA (0.67%) and heated at 95 °C for 30 min. After cooling, absorbance was measured by using a spectrophotometer at 532 nm. The results were expressed as percentage of inhibition of TBARS formed by AAPH alone (induced blank) (Esterbauer and Cheeseman 1990). The antioxidant activity by TBA method was calculated as follows:

$$\% \text{ ILP} = [1- \{(A_{TS} - A_{BL})/A_{TS}\}] \times 100$$

Where, A_{TS} and A_{BL} are the absorbance of test sample and blank, respectively.

2.4.4. Anti-AChE activity test

Ellman's method with the adaptation of Pohanka et al (2011) was used in this study. A disposable cuvette was consequently filled with 0.4 mL of 0.4 mg/mL DTNB, 0.025 mL of AChE solution (0.5 μkat in 1 mM acetylthiocholine), 0.425 mL of PBS, and 0.050 mL of sample (NC/ MDA/ TRO/ MDA + TRO). The reaction was started by adding 0.1 mL of acetylthiocholine chloride at a given concentration for the assessment of K_m and V_{max} or 1 mM for toxicological and pharmacological investigations. Absorbance at 412 nm was measured immediately and after one minute. Enzyme activity was calculated estimating extinction coefficient ε = 14,150 M/cm.

2.4.5. Anxiolytic activity test

The description (design, work principle and mode of operation) of the swing apparatus had been already described in a previous study (Islam et al. 2014). In this study, total, 55 Swiss mice were used (five in each group). A short-term physical stress (STPS) was given to the experimental animals just after the last treatment to produce angina. For this, animals were placed on an electronic shaker for 30 seconds (90 shakes/min).

2.4.5.1. Animal groups and treatments

In the first zone, 35 mice were randomly divided into 7 groups like- NC (STPS) (normal saline: 10 mL/kg; p.o.); NC (NSTPS) (vehicle + non-STPS); DZP (2 mg/kg, i.p.), FLU (2.5 mg/kg, i.p.) and three groups for MSJ (50, 100 and 200 mg/kg, p.o.). After 10 min, each animal was placed into the swing box and number of swings was counted manually for 3 min. The swing box was cleaned before placing a new animal with 70% ethanol solution.
In the second zone, 15 mice were treated with FZL (2/2.5 mg/kg, i.p.). After 15 minutes, two groups were isolated as FZL + DZP

(2.5 mg/kg + 2 mg/kg, i.p.) and FZL + MSJ (2.5 mg/kg (i.p.) + 200 mg/kg (p.o.)). The animals were then placed individually in the swing box for 3 min after a 10 min interval.
Finally, 5 mice were treated with DZP (2 mg/kg, i.p.) prior to an administration of MSJ (200 mg/kg, p.o.) for further confirmation of the possible anxiolytic effect of MSJ. In this occasion, DZP (2 mg/kg, i.p.) was administered 10 min prior to the MSJ treatment. After 10 min, the number of swings of each animal was counted and then followed by an administration of FZL (2.5 mg/kg, i.p.) and counting again the number of swings after 15 min as above.

2.4.5.2. GABA quantification in mice brain

After the swing test, this process was followed by a rapid removal of the brain of mice, weighing and transferring to ice-cold trichloroacetic acid solution to obtain a 10% w/v constitution, which was then homogenized and centrifuged at 10,000 × g for 10 min at 0 ^{O}C. Then, 0.1 mL of brain tissue extract was added to 0.2 mL of 0.15 M ninhydrin solution in a 0.5 M carbonate-bicarbonate buffer (pH 9.95) and incubated in a water bath at 60 ^{O}C for 30 min. After cooling, the mixture was treated with 5 mL of copper tartrate reagent (0.16% di-sodium carbonate, 0.03% copper sulfate and 0.03% tartaric acid) (Lowe et al. 1958). Absorbance was measured at 360 nm in a UV spectrophotometer (Lu et al. 2010). The results were expressed as brain GABA levels (µg/g of wet brain tissue).

2.5. Statistical analysis

The results are expressed as mean ± standard deviation (SD); analysis of variance (ANOVA) followed by Newman-Keul's post hoc *t*-student test using GraphPad Prism software (version 6.0, San Diego, California, U.S.A., Copyright©) considering $p < 0.05$.

3. Results

Table 1 suggests that MSJ concentration-dependently scavenged hydroxyl ($^{\bullet}$OH) and nitric oxide (NO$^{\bullet}$) radicals along with an inhibition of lipid peroxidation (ILP). The highest %IHL, %SNO$^{\bullet}$ and %IHL were observed at 200 µg/mL of MSJ by 67.54 ± 2.23, 56.62 ± 1.49 and 62.63 ± 2.94, respectively. In comparison to the TRO 100 µg/mL, the MSJ exhibited a better antioxidant capacity at 200 µg/mL. Furthermore, MSJ 200 µg/mL when co-treated with TRO 100 µg/mL, significantly ($p < 0.05$) increased the %IHL, %SNO$^{\bullet}$ and %IHL than the other groups. The negative control (NC: vehicle) produced negligible antioxidant capacity.

Table 1: Antioxidant Capacity of Methanol Extract of *Stephania Japonica*

Treatments (conc.)	$^{\bullet}$OH scavenging test (%IHL)	NO$^{\bullet}$ scavenging test (%SNO$^{\bullet}$)	TBARS test (%ILP)
NC	2.01 ± 0.58	2.21 ± 1.08	1.29 ± 1.85
TRO (100 µg/mL)	60.59 ± 2.78^{acd}	45.67 ± 1.33^{ac}	61.61 ± 1.37^{acd}
MSJ (50 µg/mL)	19.23 ± 3.01^{a}	31.93 ± 2.13^{a}	29.94 ± 2.34^{a}
MSJ (100 µg/mL)	41.50 ± 2.11^{ac}	48.21 ± 2.57^{ac}	41.28 ± 2.75^{ac}
MSJ (200 µg/mL)	67.54 ± 2.23^{abcd}	56.62 ± 1.49^{abcd}	62.63 ± 2.94^{acd}
TRO 100 + MSJ 200	78.02 ± 2.53^{abcde}	62.63 ± 1.69^{abcde}	67.35 ± 1.07^{abcde}

Values are mean ± SD (n = 5); NC: negative control (vehicle: distilled water); TRO: trolox; MSJ: methanol extract of *S. japonica*; $^{\bullet}$OH: hydroxyl radical; IHL: inhibition of hemolysis; NO$^{\bullet}$: nitric oxide radical; SNO$^{\bullet}$: scavenge of NO$^{\bullet}$; TBARS: thiobarbituric acid substances; ILP: inhibition of lipid peroxidation; $p < 0.05$ when compared to ^{a}NC, ^{b}TRO 100, ^{c}MSJ 50, ^{d}MSJ 100 and ^{e}MSJ 200 (ANOVA followed by *t*-Student-Neuman-Keul's as a post hoc test).

The MSJ also concentration-dependently inhibited the denaturation of EAL, where highest %IEALD (57.59 ± 2.34) was observed with MSJ 200 µg/mL. The standard, ASA at 100 µg/mL produced %IEALD by 55.31 ± 2.53. Moreover, ASA 100 µg/mL when co-treated with MSJ 200 µg/mL, significantly increased %IEALD than the MSJ and ASA alone treated groups. The NC produced negligible %IEALD (Table 2).

Table 2: Anti-Inflammatory Activity of Methanol Extract of *Stephania Japonica*

Treatments (conc.)	EALD test (%IEALD)
NC	2.19 ± 1.03
ASA (100 µg/mL)	55.31 ± 2.53^{acd}
MSJ (50 µg/mL)	26.43 ± 2.17^{a}
MSJ (100 µg/mL)	39.14 ± 1.93^{ab}
MSJ (200 µg/mL)	57.59 ± 2.34^{acd}
ASA 100 + MSJ 200	63.25 ± 1.78^{abcde}

Values are mean ± SD (n = 5); NC: negative control (vehicle: distilled water); ASA: acetyl salicylic acid; MSJ: methanol extract of *S. japonica*; EALD: egg albumin denaturation; IEALD: inhibition of EAL denaturation; $p < 0.05$ when compared to ^{a}NC, ^{b}TRO 100, ^{c}MSJ 50, ^{d}MSJ 100 and ^{e}MSJ 200 (ANOVA followed by *t*-Student-Neuman-Keul's as a *post hoc* test).

In the AChE inhibitory test, TRO at 50 µg/mL exhibited better anti-AChE capacity than the 50, 100 and 200 µg/mL of MSJ, although, the MSJ concentration-dependently inhibited AChE and exhibited significant anti-AChE capacity as compared to the NC group. The co-treatment group of TRO 50 + MSJ 200 was found to increase in %IAChE significantly (Table 3).

Table 3: Anti-Ache Activity of Methanol Extract of *Stephania Japonica*

Treatments (conc.)	AChE inhibition test (%IAChE)
NC	1.11 ± 0.34
TRO (50 µg/mL)	66.71 ± 2.08^{acde}
MSJ (50 µg/mL)	26.54 ± 2.13^{a}
MSJ (100 µg/mL)	35.12 ± 2.85^{ac}
MSJ (200 µg/mL)	59.89 ± 2.57^{acd}
TRO 50 + MSJ 200	72.17 ± 2.93^{abcde}

Values are mean ± SD (n = 5); NC: negative control (vehicle: distilled water); TRO: trolox; MSJ: methanol extract of *S. japonica*; AChE: acetylcholinesterase; IAChE: inhibition of AChE; $p < 0.05$ when compared to ^{a}NC, ^{b}TRO 100, ^{c}MSJ 50, ^{d}MSJ 100 and ^{e}MSJ 200 (ANOVA followed by *t*-Student-Neuman-Keul's as a post hoc test).

Table 4 says a clear, calming effect in the STPS-induced anxiety of the animals with DZP and MSJ treatments. MSJ at 100 and 200 mg/kg significantly ($p < 0.05$) reduced the number of swings in the animals than the DZP (2 mg/kg). Most reduction in swings was observed in DZP 2 + MSJ 200 group. The FLU alone or co-treated with DZP and/or MSJ augmented the number of swings in comparison to the other groups. The NC (NSTPS) group produced almost 50% reduction in number of swings as compared to the NC (STPS) group.
The brain GABA levels in the MSJ-treated groups were found to augment dose-dependently. More augmentation in brain GABA level was observed in DZP 2 + MSJ 200, which was then, followed by DZP 2 + FLU 2.5 + MSJ 200 and MSJ 200 mg/kg groups. There was a relation between the decreased swing numbers with an increase in the brain GABA level.

Table 4: Anxiolytic-Like Activity of Methanol Extract of *Stephania Japonica*

Treatments (dose(p.o.))	STPS-induced anxiolytic test	
	Number of swings	Brain GABA levels (µg/g of wet brain tissue extract)
NC (10 mL/kg) (STPS)	28.40 ± 2.96	367 ± 2.19
NC (10 mL/kg) (NSTPS)	14.90 ± 3.45^{a}	468 ± 1.09^{abcdefgij}
DZP (2 mg/kg)	23.00 ± 2.55^{acd}	421 ± 3.08^{acdfghj}
FLU (2.5 mg/kg)	30.40 ± 3.53	354 ± 3.34
MSJ (50 mg/kg)	26.20 ± 1.82^{c}	359 ± 2.76
MSJ (100 mg/kg)	22.00 ± 2.55^{acd}	417 ± 3.51^{acd}
MSJ (200 mg/kg)	18.20 ± 3.21^{abcdegi}	456 ± 2.59^{abcdefi}
DZP 2 + FLU 2.5	23.60 ± 2.49^{acd}	431 ± 1.35^{abcdei}
DZP 2 + MSJ 200	15.40 ± 2.96^{abcdefgij}	466 ± 1.78^{abcdefgij}
FLU 2.5 + MSJ 200	24.40 ± 2.96^{ac}	421 ± 1.54^{acde}
DZP 2 + FLU 2.5 + MSJ 200	19.60 ± 1.33^{abcdeggi}	461 ± 3.51^{abcdefgi}

Values are mean ± SD (n = 5); STPS: short-term physical stress; NC (STPS): negative control (vehicle) with STPS; NC (NSTPS): negative

control (vehicle) with non-STPS; DZP: diazepam; FLU: flumazenil; MSJ: methanol extract of *S. japonica*; GABA: gamma amino butyric acid; p <0.05 compared to the NC, [b]DZP 2; [c]FLU 2.5; [d]FLU 2.5; [e]MSJ 50, [f]MSJ, and [g]MSJ 200; [h]DZP 2 + FLU 2.5; [i]FLU 2.5 + MSJ 400; [J]DZP 2 + FLU 2.5 + MSJ 200; (ANOVA followed by *t*-Student-Neuman-Keul's as a post hoc test).

4. Discussion

The reactive oxygen or nitrogen species (ROS/RNS) play important physiological roles in our body. However, an imbalance between the production of them and the physiological antioxidant molecules such as superoxide dismutase (SOD), catalase (CAT) and glutathione systems, produces oxidative stress. Therefore, we need external antioxidants to manage such detrimental situations (Islam et al. 2016b). In severe conditions, both $^{\bullet}OH$ and $NO^{\bullet}$ after reaction with O_2 forms peroxynitrite radical ($ONOO^{\bullet}$) which may oxidize cell macromolecules, including lipids, proteins, carbohydrates, and genetic materials (e.g. - DNA/RNA). Moreover, the $ONOO^{\bullet}$ can increase aggregation of β-amyloid peptide (Aβ) (Islam et al. 2016c), which is evident to accumulate in AD patients (Mestres et al. 2015). Thus, the scavenging capacity of $^{\bullet}OH$ and $NO^{\bullet}$, and an inhibition of lipid peroxidation by the MSJ in this study may relate to each other.

On the other hand, galanthamine, a potent AChE inhibitor, which is widely used in the treatment of AD has been evident for antioxidant capacity in some in vitro models with the reduction of ROS, especially the generation of $NO^{\bullet}$ in human neuroblastoma cells treated with H_2O_2 (Barrera 2012) and human lymphocytes (Triana-Vidal and Carvajal-Varona 2013). However, lipid peroxidation may augment the levels of Aβ in the brain. Furthermore, the hyper phosphorylation of tau protein (*P*-tau) is also known as an important cause of NDS, especially the AD. In a study, TRO has been reported to prevent hyper phosphorylation of *P*-tau (Warner et al. 2015). In this study, along with significant antioxidant and anti-inflammatory effects, MSJ showed a promising anti-AChE activity and in all cases, MSJ augmented the effects of standards used, including the activity of TRO. Thus, the MSJ-mediated antioxidant capacity by the inhibition of lipid peroxidation, reduction in Aβ and *P*-tau levels may be linked to each other.

5. Conclusion

This methanol leaf extracts of *S. japonica* (MSJ) showed antioxidant, anti-inflammatory, and anti-AChE activities, providing scientific relevance regarding to its ethnopharmacological usages, especially in inflammation. The MSJ also exhibited a GABAergic anxiolytic-like effect in Swiss mice, possibly via DZP-agonistic pathway. The antioxidative effect of MSJ may relate to its anti-inflammatory, thus the neuro protection capacity. Further studies are required to isolate the active phytochemicals and to elucidate the exact mechanism of actions underlying its neuro-protective effect.

6. Conflict of interest

None declared.

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