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Possible neuroprotective trait of *Dysophylla auricularia* methanol extract

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ABSTRACT

This study evaluated the inhibitory capacity of hydroxyl radical ($^{\bullet}$OH), nitric oxide radical (NO$^{\bullet}$), lipid peroxidation and acetylcholinesterase (AChE) activity of methanol extract of *Dysophylla auricularia* (MDA) whole plant. For this MDA at 50-200 µg/mL was tested by taking trolox (TRO: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard. The TRO was used at 50 µg/mL. The results suggest that, MDA concentration-dependently exhibited strong antioxidant and anti-AChE activities. Significant (p <0.05) activity was observed in the MDA and TRO co-treatment group. *D. auricularia* may be one of the good sources of neuroprotective phytoconstituents.

Keywords: acetylcholinesterase; antioxidant; *Dysophylla auricularia*; neurological activity.

Resumo

Este estudo avaliou a capacidade inibitória de radicais hidroxílicos (● OH), óxido nítrico (●NO), peroxidação lipídica e atividade de acetilcolinesterasica (AChE) do extrato de metanolico da espécie *Dysophylla auricularia* (MDA). Para isso, MDA a 50-200 µg/mL foi testado em comparação ao trolox como padrão. O TRO foi utilizado a 50 µg/mL. Os resultados sugerem que, dependendo da concentração de MDA, este exibe fortes atividades antioxidantes e anti-AChE. Foi observada atividade significativa (p <0,05) no grupo de co-tratamento MDA e TRO. Desta forma, este estudo apresentou a *D. auricularia* como uma boa fonte de fitoconstituintes neuroprotetores.

Palavras-chave: acetilcolinesterase; antioxidante; *Dysophylla auricularia*; atividade neurológica.

1. INTRODUCTION

The herb *Dysophylla auricularia* attributes some important biological activities in a number of non-clinical and pre-clinical studies (NUR et al., 2015). In a study, we have found that the *D. auricularia* methanolic extract possesses potent antioxidant capacity (ISLAM et al., 2017). To date *D. auricularia* is evident to have antimicrobial, antioxidant, anti-inflammatory, anti-diarrheal, membrane stabilization, alpha-glycosidase inhibitory and antipyretic activities (NUR et al., 2015; ISLAM et al., 2017). Moreover, auricularic acid, a spasmolytic cleistanthane type diterpenoid isolated from *Pogostemon auricularis* (synonym of *D. auricularia*) is known as a first plant-derived spasmolytic diterpenoid acid (ISLAM et al., 2016a).

Reactive oxygen and nitrogen species (ROS/RNS) are important for numerous physiological functions. However, a balance between their production and the body antioxidant molecules is crucial. Oxidative stress is the phenomenon, which refers to an augmented production ROS/RNS than the antioxidant molecules. Till date, over 100 neurological diseases (NDs) have been identified that are associated with the oxidative stress (ISLAM, 2016). Among them, Alzheimer's disease (AD) and Parkinson's disease (PD) are vastly studied (ISLAM et al., 2016b).

The enzyme, acetylcholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter acetylcholine to terminate signals across cholinergic synapses, including those at neuromuscular junctions. Thus, any substance having antioxidant and anti-AChE activity may be a good tool for the treatment of NDs (Ota et al., 2015).[6] Nevertheless, the rate of consumption and researches on plant-derived products is growing day by day. To date, a number of antioxidants from dietary supplements are considered as promising tools in the treatment of NDs (Ota et al., 2015; Islam et al., 2016b).[5,6]

The peroxynitrite radical ($ONOO^{\bullet}$), formed by the reaction between nitric oxide radical ($NO^{\bullet}$) and molecular oxygen (O_2) may oxidize cell macromolecules, including lipids, proteins, carbohydrates and genetic materials (e.g. - DNA/RNA), as well as increases aggregation of β-amyloid peptide (Aβ) (ISLAM et al., 2016b). The aggregation of Aβ is evident to accumulate in AD patients. However, excessive lipid peroxidation and an increased production of hydroxyl radical ($^{\bullet}OH$) may augment the levels of Aβ and result NDs (BARRERA, 2012; WARNER et al., 2015).

From the above viewpoints, this non-clinical study aims to evaluate the inhibition capacity of $^{\bullet}OH$, $NO^{\bullet}$, lipid peroxidation and AChE of MDA.

2. MATERIALS AND METHODS

2.1. *Research ethics*

This project was approved by the Department of Pharmacy, Southern University Bangladesh (SUB) with the deposition number #SUB (PH) 9991002/2014.

2.2. *Collection, identification and extraction of plant materials*

The herb *D. auricularia* was collected from the Chittagong division of Bangladesh and was identified by a taxonomist in Forest Research Institute, Bangladesh (FRIH, BD). A voucher specimen was deposited with the accession number BFRIH-4688.

After collection, *D. auricularia* (whole plant) was undergone shade drying (temperature not exceeding 50 °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 12.93%.

2.3. *Required chemicals and reagents*

Tween-80 (0.05%) dissolved in 0.9% saline was used as a vehicle for the MDA and TRO. Hydrogen peroxide (H_2O_2) (reconstituted with sterile distilled water) was used as a stressor (STR) in HL test. All the other necessary reagents and chemicals were purchased from (Sigma-Aldrich, St. Louis, MO; USA.).

2.4. *Sample preparation*

MDA was reconstituted in 50, 100 and

200 µg/mL, while TRO at 50 µg/mL with the above mentioned vehicle.

2.5. *Experimental animal*

For hemolysis test, a male Wister albino rat (*Rattus norvegicus*) of 220 g body weight (2 months old) was collected from the Bangladesh Council for Scientific Research and Institute, Chittagong, Bangladesh. The animal was allowed to free access to water and food (Purinas pellets) *ad libitum* and was kept under controlled lighting (12 h dark/light cycle) and temperature (24 ± 2 ^{O}C).

2.6. *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 Wistar 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 (MDA/ TRO/ MDA + 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:

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

2.7. *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.

2.8. *Lipid peroxidation test*

The TBARS (thiobarbituric acid substances) 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; 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.

2.9. *Anti-AChE activity test*

Ellman's method with the adaptation of POHANKA et al (2011) was used in this case. 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.

2.10. *Statistical analysis*

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

RESULTS

Table 1 suggests a concentration-dependent OH scavenging capacity of MDA, where highest %IHL was found at 200 µg/mL by 59.91 ± 2.33. The standard drug TRO at 50 µg/mL showed %IHL by 61.24 ± 1.93. Moreover, MDA at high concentration when co-treated with TRO µg/mL significantly increased the %IHL capacity than the MDA and TRO individual groups. The NC produced negligible %IHL (1.86 ± 0.58). The IC_{50} calculated for MDA was 58.02 ± 0.66 µg/mL.

Table 1 - •OH scavenging capacity in RRBCs of the test sample and controls

Treatment (conc.)	%IHL	IC_{50} [CI; R^2]
NC	1.86 ± 0.58	-
MDA 50 µg/mL	26.14 ± 2.18[a]	58.02 ± 0.66 [11.67 – 288.60; 0.96]
MDA 100 µg/mL	43.19 ± 1.86[ab]	
MDA 200 µg/mL	59.91 ± 2.33[abc]	
TRO 50 µg/mL	61.24 ± 1.93[abc]	-
MDA 200 µg/mL + TRO 50 µg/mL	67.13 ± 2.54[abcd]	-

Values are mean ± standard deviation (SD) (n = 5); p < 0.05 when compared to the NC[a], MDA 10[b], MDA 20[c] and MDA 50[d] (ANOVA and *t*-Student-Newman-Kewls as *post hoc* test); NC: negative control (vehicle: 0.05% Tween 80 dissolved in 0.9% saline); MDA: methanol extract of *D. auricularia*; TRO: trolox; RRBCs: rat erythrocytes; %IHL: percentage inhibition of hemolysis; IC_{50}: half-minimal inhibitory concentration; CI: confidence interval; R^2: co-efficient of determination.

Both MDA 200 µg/mL and TRO 50 µg/mL showed almost similar NO• scavenging capacity. MDA when co-treated with TRO was also found to increase a significant (p <0.05) %I (69.01 ± 1.89) as compared to the TRO and MDA individual as well as NC treated groups. A concentration-dependent %I was also observed in this case. The IC_{50} calculated for MDA was 69.71 ± 0.78 µg/mL (**Table 2**).

Table 2 - NO• scavenging capacity of the test sample and controls

Treatment (conc.)	%I	IC_{50} [CI; R^2]
NC	2.01 ± 0.78	-
MDA 50 µg/mL	21.25 ± 1.21[a]	69.71 ± 0.78 [11.85 – 410.00; 0.95]
MDA 100 µg/mL	40.00 ± 1.01[ab]	
MDA 200 µg/mL	62.11 ± 1.11[abc]	
TRO 50 µg/mL	63.02 ± 2.10[abc]	-
MDA 200 µg/mL + TRO 50 µg/mL	69.01 ± 1.89[abcd]	-

Values are mean ± standard deviation (SD) (n = 5); p < 0.05 when compared to the NC[a], MDA 50[b], MDA 100[c] and MDA 200[d] (ANOVA and *t*-Student-Newman-Kewls as *post hoc* test); NC: negative control (vehicle: 0.05% Tween 80 dissolved in 0.9% saline); MDA: methanol extract of *D. auricularia*; TRO: trolox; RRBCs: rat erythrocytes; %I: percentage inhibition; IC_{50}: half-minimal inhibitory concentration; CI: confidence interval; R^2: co-efficient of determination.

In the TBARS test, MDA strongly inhibited the lipid peroxidation capacity, where MDA 200 µg/mL was found to augment %ILP by 18.95% than the TRO 50 µg/mL. An increased activity was observed in the MDA 200 µg/mL + TRO 50 µg/mL group, which was 2.28 and 21.67% better than the MDA 200 µg/mL and TRO 50 µg/mL treated groups, respectively. The NC produced negligible %ILP (1.53 ± 1.08). The IC_{50} calculated for MDA in this purpose was 60.23 ± 0.61 µg/mL. In this test, MDA also concentration-dependently inhibited TBARS formation (**Table 3**).

Table 3 - Lipid peroxidation inhibition capacity of the test sample and controls

Treatment (conc.)	%ILP	IC_{50} [CI; R^2]
NC	1.53 ± 1.08	-
MDA 50 µg/mL	33.50 ± 1.58[a]	60.23 ± 0.61 [15.55 – 233.40; 0.97]
MDA 100 µg/mL	59.35 ± 2.08[ab]	
MDA 200 µg/mL	81.91 ± 3.10[abce]	
TRO 50 µg/mL	68.86 ± 1.13[abc]	-
MDA 200 µg/mL + TRO 50 µg/mL	83.78 ± 2.58[abce]	-

Values are mean ± standard deviation (SD) (n = 5); $p < 0.05$ when compared to the NC[a], MDA 50[b], MDA 100[c], MDA 200[d], and TRO 50[e] (ANOVA and *t*-Student-Newman-Kewls as *post hoc* test); NC: negative control (vehicle: 0.05% Tween 80 dissolved in 0.9% saline); MDA: methanol extract of *D. auricularia*; TRO: trolox; RRBCs: rat erythrocytes; %ILP: percentage inhibition of lipid peroxidation; IC_{50}: half-minimal inhibitory concentration; CI: confidence interval; R^2: co-efficient of determination.

According to the **Table 4**, MDA showed a concentration-dependent AChE inhibitory activity, where MDA 200 µg/mL and TRO 50 µg/mL attributed almost similar %IAChE capacity. However, their co-treatment group significantly increased in %IAChE capacity than the other treatment groups in this assay. The IC_{50} of MDA was 62.29 ± 0.84 µg/mL. The NC exhibited a negligible %IAChE capacity (1.09 ± 0.33).

Table 4 - Anti-AChE activity of the test sample and controls

Treatment (conc.)	%IAChE	IC_{50} [CI; R^2]
NC	1.09 ± 0.33	-
MDA 50 µg/mL	29.03 ± 1.81[a]	62.29 ± 0.84 [5.99 – 647.50; 0.92]
MDA 100 µg/mL	45.26 ± 2.61[ab]	
MDA 200 µg/mL	69.19 ± 1.93[abc]	
TRO 50 µg/mL	69.78 ± 2.78[abc]	-
NS 200 µg/mL + TRO 50 µg/mL	79.13 ± 2.51[abcde]	-

Values are mean ± standard deviation (SD) (n = 5); $p < 0.05$ when compared to the NC[a], MDA 50[b], MDA 100[c], MDA 200[d] and TRO 50[e] (ANOVA and *t*-Student-Newman-Kewls as *post hoc* test); NC: negative control (vehicle: 0.05% Tween 80 dissolved in 0.9% saline); MDA: methanol extract of *D. auricularia*; TRO: trolox; RRBCs: rat erythrocytes; %IAChE: percentage inhibition of acetylcholinesterase; IC_{50}: half-minimal inhibitory concentration; CI: confidence interval; R^2: co-efficient of determination.

DISCUSSION

The nitric oxide radical ($NO^{\bullet}$) reacts with O_2 and forms $ONOO^{\bullet}$, which is evident to oxidize cell macromolecules and increase in aggregation of Aβ (ISLAM et al., 2016b). On the other hand, $^{\bullet}OH$ and H_2O_2 can directly cause destruction of cell membranes, cell macromolecules and genetic materials (BARRERA, 2012), while lipid peroxidation resulted from oxidative stress may augment the levels of Aβ. Moreover, the hyperphosphorylation of *tau* protein is also known as another important cause of NDs, especially AD (WARNER et al., 2015). Galanthamine, a potent AChE inhibitor, which is widely used in the treatment of AD has shown significant antioxidant properties in some *in vitro* models with the reduction of ROS, especially the generation of $NO^{\bullet}$ in human neuroblatoma cells treated with H_2O_2 (BARRERA, 2012). This drug is also evident to protect human lymphocytes from H_2O_2-induced oxidative (TRIANA-VIDAL; CARVAJAL-VARONA, 2013).

This study suggests that, MDA alone or in combination with the TRO significantly (p <0.05) scavenged $^{\bullet}OH$ and $NO^{\bullet}$ along with a strong inhibitory capacity of TBARS formation and AChE activity. Mestres et al (2015) suggested that, the substance having $NO^{\bullet}$ scavenging capacity may be helpful to treat AD. Otherwise, in a study, TRO has been reported to prevent hyperphosphorylation of *tau* protein (WARNER et al., 2015). The overall findings demonstrate that, MDA exerted a synergistic antioxidant and anti-AChE activity. In general MDA and/or TRO mediated neurological pathways are shown in **Figure 1**.

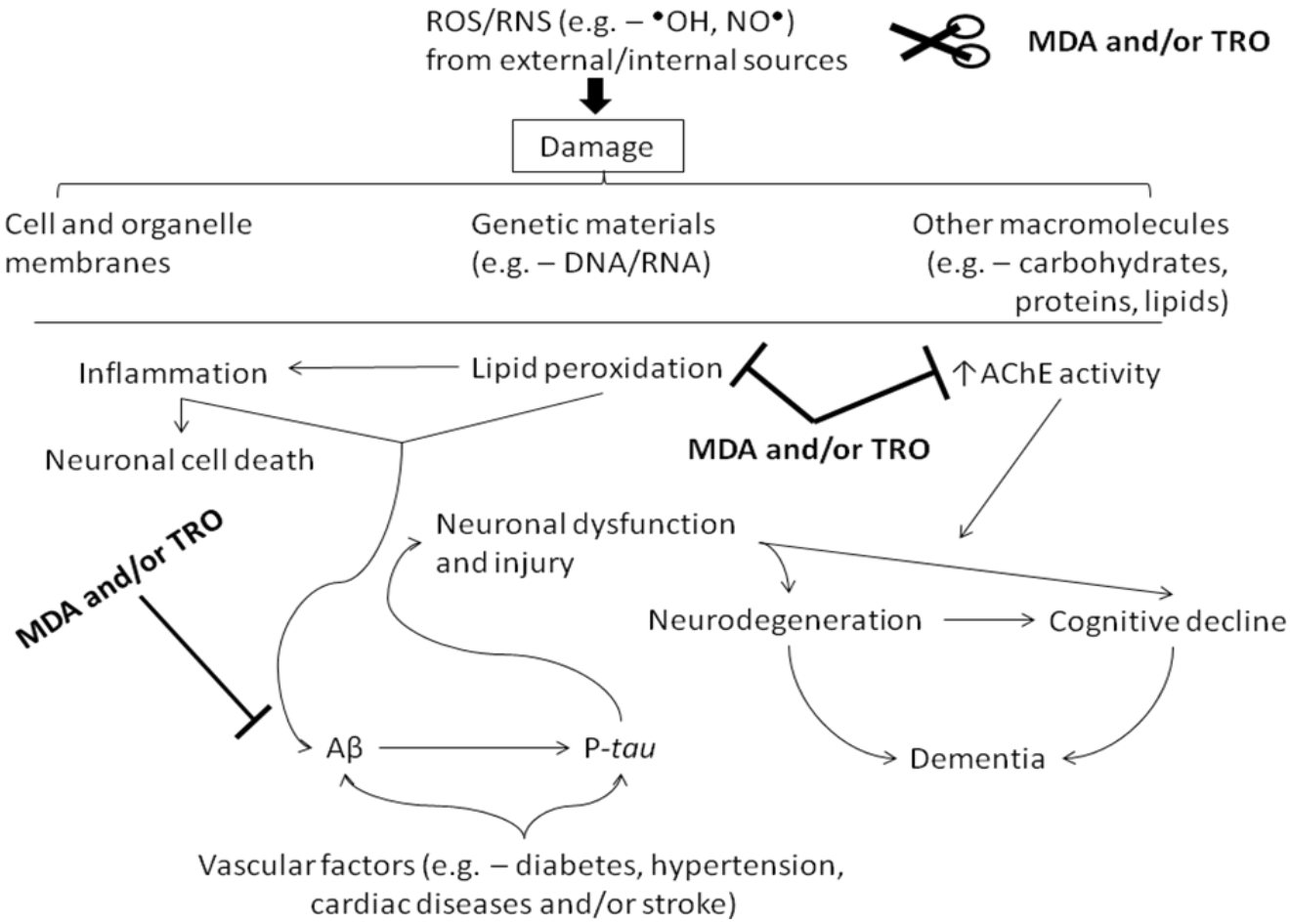

Figure 1 - Possible MDA and/or TRO mediated neuroprotection pathways. [MDA and/or TRO can directly scavenge the reactive oxygen/nitrogen species (ROS/RNS) and reduce neuroinflammation, thus the reduction in neuronal cell death. However, by inhibiting lipid peroxidation and acetylcholinesterase (AChE) activity as well as directly interfering beta-amyloid peptide (Aβ) production, they can inhibit tau protein (P-*tau*)-mediated neuronal dysfunction and injury and related other neuronal diseases such as neurodegeneration, cognitive decline and dementia. •OH: hydroxyl radical; NO•: nitric oxide radical; MDA: methanol extract of *D. auricularia*; TRO: trolox]

CONCLUSION

The MDA exhibited a potent antioxidant and anti-AChE capacity in a concentration-dependent manner. Potential •OH scavenging capacity may be linked to its hemolysis inhibitory power in rat erythrocytes. The NO• scavenging, inhibition of lipid peroxidation and anti-AChE capacity of MDA may be helpful manage the aggregation of Aβ and other related events in the NDs patients. Further researches are necessary to isolate the neuroactive chemicals from this hopeful herb.

CONFLICT OF INTEREST

None declared.

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