Original Article

Anti-Alzheimer and Antioxidant Activity of
Celastrus paniculatus Seed

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Abstract

The crude methanolic extract of the seeds of *Celastrus paniculatus* along with its organic soluble fractions were tested for their possible antioxidant and anti-alzheirmer (AD) activity. The extracts showed prominent DPPH free radical scavenging activity, inhibiting activity of authentic peroxynitrite (ONOO-) and inhibition of total reactive oxygen species (ROS) generation. In DPPH radical scavenging assay, the EtOAc fraction showed the highest activity with a IC50 value of 25.92±1.02 µg/ml whereas aqueous fractions had no activity at all within the tested concentration. Scavenging of the authentic ONOO- system, all extract/fractions showed good activity and among them, EtOAc fraction had the highest activity with a IC50 value of 15.79±0.18 µg/ml. EtOAc fraction also showed significant ($p<0.001$) inhibitory activity against the total ROS generation which was almost similar with that of the positive control Trolox (IC50 16.79±0.19 µg/ml). All extract/fractions exhibited statistically significant ($p<0.001$) cholinesterases (ChEs) inhibitory effects with IC50 values ranging between 134.7-227.5 µg/ml for AChE and 209.6-562.1 µg/ml for BChE.

Keywords: Anti-alzheimer; Antioxidant; Cholinesterase, *Celastrus paniculatus*, ROS
Received: September 1, 2010; *Accepted:* November 8, 2010.

1. Introduction

Alzheimer's disease (AD) is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in activities of daily living [1-3]. Epidemiological data indicate a potentially considerable increase in the prevalence of the disease over the next two decades [4]. AD affects up to 5% of people over 65 years, rising to 20% of those over 80 years [5]. Until recently, two major hypotheses have been proposed regarding the molecular mechanism of AD pathogenesis: the cholinergic hypothesis and the amyloid cascade hypothesis [6]. Among them, the most promising approaches for treating this disease are to enhance the acetylcholine level in brain using acetyl-cholinesterase inhibitors [7]. Therefore, acetylcholinesterase (AChE) and butyryl-

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cholinesterase (BChE) inhibitors have become the remarkable alternatives in the treatment of AD. However, two major hypotheses are not sufficient to explain all the pathological pathways of AD. Recently, numerous studies have been performed supporting the correlation between AD, inflammation and oxidative stress and/or nitrosative stress [8-10]. In particular, AD has been reported to be highly associated with cellular oxidative stress, including augmentation of protein oxidation, protein nitration and lipid peroxidation as well as accumulation of Aβ [10]. Among cellular oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS) stand accused of the etiology of numerous human degenerative diseases. *In vivo* formation of peroxynitrite (ONOO-) has been implicated in Aβ formation and accumulation, with high levels of Aβ also augmenting ONOO- generation in the brain of AD patients [11, 12]. Therefore, the simultaneous studies on both cholinesterases (ChEs) inhibitory effects and antioxidant effects, including free radical (DPPH), ONOO- scavenging, reducing power assay and ROS inhibitory effects of the crude methanolic extract and its various organic soluble fractions of *Celastrus paniculatus* seeds, are worthy of development of promising anti-AD agents.

Pharmacological studies suggest that the oil obtained from the seed of *Celastrus paniculatus* possesses sedative and anticonvulsant properties [13]. Analgesic and anti-inflammatory effects of the methanolic extract of *C. paniculatus* seed have been reported in mice and rats [14]. *C. paniculatus* seed oil has been reported to improve memory processes in rats [15], beneficial to psychiatric patients [16], and it increased the intelligence quotient (IQ) of mentally retarded children [17]. More recently, rats treated with *C. paniculatus* seed oil for 15 days exhibited a significant decrease in the levels of norepinephrine, dopamine, serotonin and their

respective metabolites in both brain and urine [18]. Chronic treatment with *C. paniculatus* seed oil reversed scopolamine-induced deficits in navigational memory performance of rats [19]. A methanolic extract of *C. paniculatus* seed oil exhibited free radical scavenging effects [20].

There is a lack of scientific data regarding the effect of crude extract and its organic fractions of *C. paniculatus* seed on cholinesterase enzymes inhibition as well as authentic peroxynitrite scavenging and inhibition of total ROS generation. Therefore, the present study was carried out to explore anti-AD and antioxidant effect of crude extract and its organic fractions of *Celastrus paniculatus* seed using *in vitro* cholinesterase enzyme inhibition assay and scavenging of DPPH, ONOO-, reducing power and inhibition of total ROS generation assay, respectively.

2. Materials and methods
2.1. Plant materials

The seeds of *C. paniculatus* were purchased in the month of April 2008 from the local herbal medicine market, Dhaka, Bangladesh and authenticated by Mrs. Mahmuda Begum, senior scientific officer, Bangladesh National Herbarium, Dhaka, where the voucher specimen has been deposited.

2.2. Chemicals

Electric-eel AChE (EC 3.1.1.7), horse serum BChE (EC 3.1.1.8), acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), serine, L-penicillamine (L-2-amino-3-mercapto-3-methylbutanoic acid), diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma Co. (St. Louis, MO, U.S.A.). 6-Hydroxy-2,5,7,8-tetram-ethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) The high quality

Table 1. Scavenging/inhibitory effects of the *C. paniculatus* seed extract/fractions against DPPH, ONOO⁻ and Total ROS generation.

Sample	[a]DPPH IC$_{50}$(μg/ml)	[b]ONOO⁻ IC$_{50}$(μg/ml)	[c]ROS IC$_{50}$(μg/ml)
MeOH	86.33 ± 1.02*	20.16 ± 0.61*	34.72 ± 0.48*
CH2Cl2	186.50 ± 1.02#	50.85 ± 0.28**	72.10 ± 0.68*
EtOAc	25.92 ± 1.02*	15.79 ± 0.18**	17.01 ± 0.32*
H2O	> 250	72.08 ± 2.41*	34.72 ± 0.48*
Ascorbic acid	12.50 ± 0.02		
L-penicillamine		8.20 ± 0.32	
Trolox			16.79 ± 0.19

IC$_{50}$ values are mean±SEM (n=3)*; *p*<0.001 by student's test for values between the sample and the control. **p*<0.05 by student's test for values between the sample and the control. #not significant.

2',7'-dichlorofluorescein diacetate (DCFH-DA) and dihydrorhoclamine 123 (DHR 123), and ONOO- were purchased from Molecular Probes (Eugene, Oregon, USA) and Cayman (Ann Arbor, MI, USA), respectively.

2.3. Preparation of plant extract

The plant materials were shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40 mesh, and stored in a air tight container. The dried powder material (1.5 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40 °C in a rotator evaporator to render the MeOH extract (260 g). This extract was suspended in H2O and then successively partitioned with CH_2Cl_2, EtOAC, and n-BuOH to afford the CH2Cl2 (160 g), EtOAC (10 g), and the H_2O residue (90 g), respectively.

2.4. In vitro tests for antioxidant activity
2.4.1. Free radical scavenging activity measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH)

The free radical scavenging activity of MeOH extract based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. [21]. Plant extract/fractions (0.1 ml) were added to 3 ml of a 0.004% MeOH solution of DPPH. Its absorbance at 517 nm was determined after 30 min, and the percentage of inhibition of the activity was calculated from $[(A_0-A_1)/A_0]\times100$, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/ standard. IC$_{50}$ value was calculated from the equation of line obtained by plotting a graph of concentration (μg/ml) versus % inhibition.

2.4.2. Measurement of the ONOO- scavenging activity

The ONOO- scavenging activity was measured by monitoring the oxidation of DHR 123, by modifying the method of Kooy *et al.* [22]. The DHR 123 (5 mM), in dimethylformamide, was purged with nitrogen, stored at -80 °C and used as a stock solution. This solution was then placed on ice, and kept from exposure to light, before study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, at pH 7.4, and 100 mM diethylenetriaminepentaacetic acid (DTPA), each of which were prepared with high quality deionized water, and purged with nitrogen. The final concentration of the DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment, both with and without the addition of authentic ONOO-. The DHR 123 was oxidized rapidly by authentic ONOO-, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.),

Table 2. Anticholinesterase activity of the plant extract and its organic soluble fractions against AChE and BChE.

Sample	AChE $IC_{50}(\mu g/ml)$	BChE $IC_{50}(\mu g/ml)$	SI (BChE/AChE) $IC_{50}(\mu g/ml)$
MeOH	176.5±2.8*	218.6±3.5*	1.2
CH_2Cl_2	225.9±1.3*	456.2±5.6*	2.0
EtOAc	134.7±2.1*	209.6±2.4*	1.2
H_2O	227.5±3.9*	562.1±5.2*	1.6
Eserine	0.02 ± 0.01	0.04 ± 0.02	0.02

IC_{50} values aremean±SEM (n=3); *p<0.001 by student's test for values between the sample and the control.

with excitation and emission wavelengths of 480 and 530 nm, respectively. The results were expressed as the mean ± standard error (n=3) of the final fluorescence intensity minus the background fluorescence. The effects were expressed as the percentage of inhibition of the DHR 123 oxidation. IC_{50} was calculated from the equation of line obtained by plotting a graph of concentration (μg/ml) versus % inhibition.

2.4.3. Measurement of the inhibition of the total ROS generation

Rat kidney homogenates, prepared from the kidneys of freshly killed male Wistar rats, weighing 150-200 g, were mixed with or without a suspension of extracts, and then incubated with 12.5 μM DCFH-DA, at 37 °C for 30 min. Phosphate buffer (50 mM, pH 7.4) was used. DCFH-DA is a stable compound, which easily diffuses into cells, and is hydrolyzed by intracellular esterase to yield a reduced non-fluorescent compound, DCFH, which is trapped within the cells. The ROS produced by cells oxidize the DCFH to the highly fluorescent 2',7'-dichlorodihydro-fluorescein (DCF). The fluorescence intensity of the oxidized DCF was monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation and emission wavelengths of 460 and 530 nm, respectively [23]. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration versus % inhibition.

2.5. In vitro ChEs enzyme assay

The inhibitory activities of the ChEs were measured using the spectrophotometric method developed by Ellman et al. [24]. Ach and BCh were used as the substrates to assay the inhibitions of AChE and BChE, respectively. The reaction mixture contained: 140 μl of sodium phosphate buffer (pH 8.0); 20 μl of test sample solution, and 20 μl of either AChE or BChE solution, which were mixed and incubated for 15 min. at room temperature. All of the tested samples and the positive control (eserine) were dissolved in 10% DMSO. The reactions were nitrated with the addition of 10 μl of DTNB and 10 μl of either ACh or BCh, respectively. The hydrolysis of ACh or BCh was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min., which resulted from the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of either ACh or BCh. All reactions were performed in triplicate and recorded in 96-well microplates, using VERSA max (Molecular Devices, Sunnyvale, CA, U.S.A.). The percentage of inhibition was calculated from (1- S/E)×100, where E and S were the respective enzyme activities without and with the test sample, respectively. The ChEs inhibitory activity of each sample was expressed in terms of the IC_{50} value (μg/ml required to inhibit the hydrolysis of the substrate, ACh or BCh by 50%), as calculated from the log-dose inhibition curve.

2.6. Statistical analysis

The assays were conducted in triplicate and all tabulated results were expressed as means±SEM, and were compared using Student's t-test. A P value of less than 0.001,

0.05 were considered significant.

3. Results

3.1 In vitro antioxidant activity

As illustrated in Table 1, EtOAc fraction showed highest free radical (DPPH) scavenging activity with IC_{50} value of 25.92±1.02 µg/ml whereas the IC_{50} value of standard Ascorbic acid was 12.50±0.02 µg/ml. The aqueous fractions showed no activity within the tested concentration against DPPH scavenging assay. Methanolic extract and CH_2C_{12} fraction showed moderate activity with IC_{50} value of 86.33±1.02 and 186.50±1.02 µg/ml, respectively. In the scavenging of authentic ONOO- system, all extract/fractions showed good activity. Among them, EtOAc fractions had the highest activity with IC_{50} value of 15.79±0.18 µg/ml whereas the IC_{50} value of L-penicillamine, a well known $ONOO^-$ scavenger, was 8.20±0.32 µg/ml (Table 1). In the total ROS system, the IC_{50} values of MeOH extract and its consequent CH_2C_{12}, EtOAc, and H2O fractions were 34.72±0.48, 72.10±0.68, 17.01±0.32 and 34.72±0.48 µg/ml, respectively. EtOAc fraction especially exhibiting almost similar inhibitory activity of the positive control Trolox (IC_{50} 16.79 ± 0.19 µg/mL). These results suggest that there are likely to be many antioxidants in the EtOAc soluble fraction, so much attention should be given to the isolation of the natural $ONOO^-$ scavenger from this fraction.

3.1. In vitro ChEs enzyme activity

To evaluate the potential of the *C. paniculatus* seed extract and its organic soluble fractions as anti-AD drug, its AChE and BChE inhibitory activities were measured using the modified method of Ellman *et al.* [24]. As shown in Table 2, the methanolic extract of *C. paniculatus* seed and its soluble organic fractions exerted statistically significant ($p<0.001$) AChE and BChE inhibitory effects. In both cases, EtOAc fractions showed highest activity than the other extract/fractions with IC_{50} value of 134.7±2.1 µg/ml for AChE and 209.6±2.4 µg/ml for BChE, respectively. The aqueous extract showed the lowest activity in both the cases (IC_{50} value 227.5±3.9 and 562.1±5.2 µg/ml for AChE and BChE, respectively).

4. Discussion

The major hurdle in understanding Alzheimer's disease (AD) is a lack of knowledge about the etiology and pathogenesis of selective neuron death. In recent years, considerable data have been accrued, indicating that the brain in AD is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death due to this disorder. The direct evidence is supporting the increased oxidative stress in AD which increased brain Fe, Al, and Hg in AD and capability of stimulating free radical generation [10]. Thus, protection and inhibition against oxidative stress may play an important role in the development of anti-AD agents. However, $ONOO^-$, formed from $NO^•$ and $^•O_2^-$, is a highly reactive oxidizing and nitrating agent, leading to oxidize cellular components, including proteins, lipids, carbohydrates, and DNA, increased aggregate of Aβ, and stimulated inflammatory response [9-10]. Since there are no endogenous antioxidant enzymes to scavenge $ONOO^-$ and a variety of ROS and/or RNS is partly involved in the Aβ pathway, it might be important to evaluate *C. paniculatus* seed extract/fractions, harboring scavenging of free radical DPPH, $ONOO^-$ and ROS inhibitory effects, as potential anti-AD candidates.

Acetylcholine is a neurotransmitter primarily by acetylcholinesterase and secondly by butyrylcholinesterase, considered

to play a role in the pathology of AD [25]. Both enzymes are present in the brain and are detected among neurofibrillary tangles and neutitic plaques [26]. So the elevation of acetylcholine amount through AChE enzyme inhibition has been accepted as the most effective treatment strategy against AD [27]. All of the known acetylcholinesterase inhibiting drugs used in the therapy of AD suffers from several side effects such as high toxicity, short duration of biological action, low bioavailability and narrow therapeutic effects. Consequently, development of new acetylcholinesterase inhibitors with less toxicity and more potent activity is compulsory. However, acetylcholinesterase inhibitors have been accepted to be the most effective for the treatment of AD, to date. These observations indicate that, the available biodiversity of natural sources and the isolated bioactive compounds may act as potential leads for the development of clinically useful pharmaceuticals.

5. Conclusion

From the results of the present study, it can be concluded that *C. paniculatus* seed extract and its organic fractions possesses antioxidant and moderate anticholinesterase activity. There is a need to isolate and characterize the compounds responsible for the anticholinesterase activity for their effective utilization in the treatment of Alzheimer's disease and other stress related disorders. Studies in this direction are in progress in our laboratory.

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