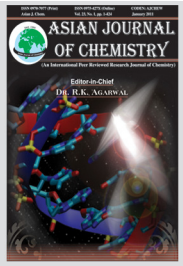
Asian Journal of Chemistry; Vol. 23, No. 4 (2011), 1783-1785

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in



Evaluation of Acetylcholine Esterase and Protease Inhibitory Activity of Scopolamine Extracted from *Datura innoxia*

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(*Received*: 9 August 2010; *Accepted*: 22 December 2010) AJC-9414

Scopolamine was extracted from aerial parts of *Datura innoxia* and its structure was confirmed by spectroscopic analysis. Acetylcholine esterase and protease inhibitory activity was determined. The results indicated that this compound has significant acetylcholine esterase potential (69.5 ± 0.5 % inhibition, IC_{50} = 0.205 ± 0.009 mM), while moderate protease inhibition activity (31.7 ± 0.8 % inhibition).

Key Words: Acetylcholine esterase, Protease, Natural products, *Datura innoxia*.

INTRODUCTION

Alzheimer's disease (AD) patients present a progressive loss of cholinergic synapses in the brain regions associated with higher mental functions, mainly the hippocampus and neocortex. In the Alzheimer's disease patients, a decrease in the acetylcholine (ACh), a neurotransmitter, appears to be critical element in the development of dementia. Hence, Alzheimer's disease and other form of dementia could be treated by the use of agents that restore the level of acetylcholine through the inhibition of both major form of cholinesterase: acetylcholinesterase (AChE) and butrylcholinesterase (BChE). Moreover, the inhibition of AChE plays a key role not only enhancing cholinergic transmission in the brain, but also reducing the aggregation of amyloid β peptide (Aβ) and the formation of the neurotoxic fibrils in Alzheimer's disease[1-3].

Cholinesterase inhibitors have also been used as antidotes to toxins such as the tropane alkaloid atropine, which are competitive inhibitors of AChE. Intoxication may occur through ingestion of plant material containing these compounds or from overdosing of medicinal plants which contain them as the active ingredients. The tropane alkaloid, cocaine was reported to inhibit BChE more than AChE[4]. Serine protease inhibitors play a vital role in the natural defense system of plants against insect predation by inhibiting insect proteinases. Trypsin, a serine protease which has recently attracted much more attention is found to be involved in the destruction of fibrous proteins[5]. Over activity of trypsin causes cancer, hepatitis, muscular dysentery and arthritis. Nature has provided some of drugs for various ailments. A variety of metabolites obtained from natural sources, posses moderate to good trypsin inhibitory activity[6]. Continuing our research on enzyme inhibition by natural products, we describe the acetylcholine esterase and protease inhibitory potential of scopolamine extracted from *Datura innoxia*.

Datura innoxia Mill. (thorn-apple) is a poisonous species of family Solanaceae, native to North America. It is an alkaloid rich species for which more than 50 tropane alkaloids have been reported[7-9]. *D. innoxia*, found to contain a number of extremely toxic alkaloids; namely atropine, hyoscyamine and scopolamine[10].

EXPERIMENTAL

IR spectra were recorded as KBr disks using a Perkin-Elmer 735B infrared spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 400 and 75 MHz instruments, respectively using a Bruker Avance spectrometer. NMR samples were prepared in CD_3OD containing tetramethylsilane as an internal standard. MS spectra were measured with a MAT 312 instrument. Silica gel 60 was used for column chromatography. Nα-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BApNA), trypsin from bovine serum and DMSO were purchased from Fluka. Acetylthiocholine iodide, 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB), galanthamine were purchased from Sigma (St. Louis, MO, USA) while, erythrocytes (acetylcholine esterase) obtained from the Biochemistry Lab, Mayo Hospital Lahore. Solvents of analytical grade were purchased from Panerac (Spain). All other chemicals and reagents of analytical grade were from Merck (Germany).

Collection of plant materials: *Datura innoxia* was collected from Botanical Garden of Government College University,

Lahore. The plant material was identified at the Department of Botany (GC University, Lahore) where a voucher specimen was submitted.

Extraction: Aerial parts of *D. innoxia* were extracted in ethanol at room temperature. Crude extract was filtered and concentrated at reduced pressure using rotary evaporator. Ethanolic crude extract was fractionated with *n*-hexane, chloroform pH 9.0 and *n*-butanol successively.

Acetylcholine esterase assay: Acetylcholine esterase inhibitory activity was measured by the spectrophotometric method developed by Ellman *et al.*[11]. Acetylthiocholine iodide was used as substrate in the assay. The reaction mixture contained 1500 µL of (100 mM) tris buffer (pH 7.8), 1000 µL of DTNB, 200 µL (50, 100, 150, 200, 250 µg/mL) of test compound solution and 200 µL of acetyl cholinesterase solution (erythrocytes), which were mixed and incubated for 15 min (25 ºC). The reaction was initiated by the addition of 200 µL acetylthiocholine. The hydrolysis of acetylthiocholine was monitored at 412 nm after 0.5 h. Galanthamine was used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

$$\text{Inhibition } (\%) = \frac{(E - S)}{E} \times 100$$

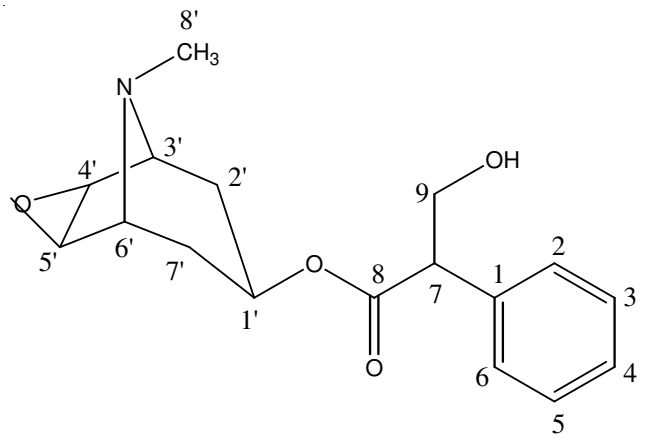
where; E = activity of the enzyme without test compound and S = activity of enzyme with test compound.

Protease inhibition assay: Protease inhibition assay was carried out according to the method of Jedinak *et al.*[6] with some modification. Tris buffer (100 mM) of pH 7.5 was prepared by dissolving 12.1 g of tris (hydroxymethyl)aminomethane in distilled water and adjusted pH 7.5 with HCl (5 M). The stock solution of trypsin was prepared[6] by dissolving 2 mg of trypsin in 10 mL of 1.0 mM HCl. N-α-benzoyl-DL-arginine-*para*nitroanilide hydrochloride (BA*p*NA) was dissolved in DMSO (20 mg/mL). Enzyme (0.3 mL) and inhibitor (100 µL) was incubated at 37 ºC for 15 min then 0.6 mM substrate was added and final volume was made 2.5 mL with tris buffer. The reaction mixture was incubated at 37 ºC for 0.5 h. The reaction was quenched by adding 30 % acetic acid and read the absorbance at 410 nm using UV/vis spectrophotometer. Phenylmethanesulfonylfluoride (PMSF) was used as positive reference. The percentage inhibition was calculated by using the following formula;

$$\text{Inhibition } (\%) = \frac{\text{Absorbance (blank)} - \text{Absorbance (test)}}{\text{Absorbance (blank)}} \times 100$$

Isolation of scopolamine: The chloroform fraction at pH 9.0 (0.74 g) of *D. innoxia* was loaded on a glass column already packed with silica and eluted successively with *n*-hexane, chloroform and ethyl acetate and methanol with increasing gradient polarity and collected 29 fractions. The fractions obtained in chloroform-ethyl acetate (10:90) were combined which resulted in a white solid, recrystallized in ethyl acetate-methanol to yield scopolamine (115 mg).

Scopolamine: m.p. 143 ºC, $C_{17}H_{21}NO_4$, m.w. 303.3, UV (MeOH) λ_{max}: 235 nm, FTIR (KBr, ν_{max}, cm^{-1}): 3323, 2957, 1727, 1490, 1032. EIMS m/z (Int. rel., %): 303.1 (M^+, 5.49), 154.1 (16.51), 138.1 (73.56), 108.1 (50.72), 94.1 (100.0), 57.0 (15.70). ^{1}H NMR (400 MHz, CD_3OD): δ: 1.82 (2H, m, H-2'a, H-7'a), 2.05 (2H, m, H-2'b, H-7'b), 2.83 (3H, s, H-8'), 3.31 (4H, d, *J* = 6 Hz, H-3', H-4', H-5', H-6'), 3.82 (2H, m, H-9a, H-7), 4.15 (1H, q, H-9b), 5.03 (1H, m, H-1'), 7.30-7.37 (5H, m, H-2, H-3, H-4, H-5, H-6). ^{13}C NMR (100 MHz, CD_3OD): δ: 29.1 (C-2'), 29.4 (C-7'), 41.1 (C-8'), 53.8 (C-7), 55.7 (C-6'), 55.9 (C-3'), 58.1 (C-5'), 58.4 (C-4'), 64.0 (C-9), 64.5 (C-1'), 127.2 (C-4), 129.2 (C-3), 129.4 (C-5), 129.6 (C-6), 130.0 (C-2), 137.3 (C-1), 172.5 (C-8).



Scopolamine

RESULTS AND DISCUSSION

The aerial parts of *D. innoxia* were extracted in ethanol, crude extract was defatted with *n*-hexane then fractionated with chloroform at pH 9.0, further subjected to column chromatography, which yielded scopolamine the structure was confirmed by spectral analyses using UV, IR, EIMS, ^{1}H and ^{13}C NMR spectrometry.

Acetylcholine esterase and protease inhibition potential of scopolamine was determined by Ellman *et al.*[11] and Jedinak's method[6], respectively. The results indicated that purified compound has significant acetylcholine esterase activity, while moderate protease activity (Table-1). Enzyme inhibition is an important area of pharmaceutical research and studies in this field have already resulted in the discovery of a wide variety of drugs, useful in a number of diseases. Specific inhibitors interact with enzymes and block their activity towards corresponding physiological substrates.

TABLE-1
ACETYLCHOLINE ESTERASE AND PROTEASE INHIBITORY POTENTIAL OF HYOSCINE/REFERENCE

Compound/ standard	Inhibition		IC_{50} (mM)	
	AChE	Protease	AChE	Protease
Scopolamine	69.5 ± 0.5	31.7 ± 0.8	0.0205 ± 0.009	–
PMSF	–	87.0 ± 1.2	–	0.11 ± 0.02

All experiments were performed three times and SD was calculated using MS Excel 2003, –: not calculated.
PMSF = Phenylmethane sulfonylfluoride.

Acetylcholine (ACh), a neurotransmitter, is widely distributed in the nervous system and has been implicated to play an important role in cerebral cortical development, cortical activity, cerebral blood flow control, modulation of cognitive performance and a signal transfer in the synapses. Loss of cholinergic innervations, as demonstrated by reduced choline

acetyltransferase (ChAT) and increased AChE activity, is correlated with the degree of dementia and the severity of the neuropathological hallmarks of Alzheimer's disease[12-14]. Proteases are amongst the most studied group of enzymes. Trypsin, a serine protease plays important role in the regulation of many physiological functions in the human body[15]. The results shown in Table-1 indicated a highly selective activity of scopolamine towards acetylecholine esterase, which is consistent with the literature. Tropane alkaloids have already been reported as effective inhibitors of acetylcholine esterase[16]. These results suggested the possibility of developing derivatives of tropane alkaloids as drugs with selective effect, against the diseases associated with the over activity of acetylecholine esterases.

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