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Research Article

ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF *TERMINALIA CHEBULA, TERMINALIA BELLERICA* AND *EMBLICA OFFICINALIS* AND SOME PHENOLIC COMPOUNDS

GARGI NAG AND BRATATI DE*

Pharmacognosy Research Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India.
Email: bratatide@vsnl.net

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ABSTRACT

Acetylcholinesterase inhibitors have been extensively used for the symptomatic treatment of Alzheimer's disease. Methanolic extracts of Ayurvedic herbal drug Triphala and the ingredient fruits *Terminalia chebula, Terminalia bellirica,* and *Emblica officinalis* were assayed to study their acetylcholinesterase inhibitory properties. All the extracts inhibited the enzyme activity in a dose dependent manner. Gallic acid and ellagic acid, the phenolic acids present in all the fruits, also inhibited the enzyme acetylcholinesterase.

Keywords: Triphala, *Terminalia chebula, Terminalia bellirica, Emblica officinalis*, Gallic acid, Ellagic acid, Acetylcholinesterase inhibitor.

INTRODUCTION

Triphala is an Ayurvedic herbal formula consisting of a mixture of equal proportions of three ingredients e.g. Amalaki (*Emblica officinalis* Gaertn.), Bibhitaki (*Terminalia bellirica* (Gaertn.) Roxb.) and Haritaki (*Terminalia chebula* Retz.)[1,2,3]. The use of *T. chebula* and *E. officinalis* was mentioned in the ancient Ayurvedic text "Astangahradaya", an auto-commentary prepared by the author Vagbhata about 7th century A.D. A paste made with 34 herbs including *T. chebula* and *E. officinalis* was used for insanity, obstinate skin diseases (including leprosy) and fever [4]. Triphala is used as laxative and bowel tonic, astringent, in traditional Indian system of medicine, Ayurveda[5].It is used in polyurea, oedema, intermittent fever, leprosy, eye diseases. It is also used as carminative and tonic [6,7,8]. In Ayurvedic medicine *T. chebula* is used as rejuvenating tonic, astringent, nervine, expectorant, anthilmintic. *T. bellirica* is used as astringent, tonic, rejuvenator, expectorant, laxative, anthelmintic, antiseptic. *E. officinalis* is nutritive rejuvenating tonic, aphrodisiac, laxative, refrigerant, stomachic, astringent, hemostatic [5], *E. officinalis* is one of the ingredients of a drug MENTAT which facilitates learning and memory [9]. Triphala has been reported to possess anti-aging properties and improves the mental faculties [10].

Triphala is reported to have immunomodulatory [11], anticancer [12-14], antimicrobial [15], wound healing [16], hypolipidemic [17], anti-inflammatory [18], chondoprotective [19], radioprotective [10], antidiabetic [20] and antioxidant [3,21,22,1] properties. Acetylcholinesterase inhibitors have been most extensively used for the symptomatic treatment of Alzheimer's disease (AD) [23], a neurodegenerative disease. The most commonly recognized symptom of AD is memory loss. The memory impairment in the patients results from a deficiency in cholinergic function in the brain. Approaches to enhance cholinergic function in AD have included simulation of cholinergic receptors or prolonging the availability of acetylcholine (Ach) released into the neuronal synaptic cleft by inhibiting Ach hydrolysis by acetylcholinesterase (AchE) Inhibition of Ach hydrolysis may be achieved through the use of AchE inhibitors [24]. In this paper the acetylcholinesterase inhibitory property of triphala and its herbal and some chemical ingredients has been reported.

MATERIALS AND METHODS

Plant materials

Dried fruits of *E. officinalis*, *T. bellirica* and *T. chebula* were collected from Local market, Kolkata and were identified botanically. Voucher specimens are available in the Department of Botany, University of Calcutta. Fruits were dried in incubator (40^{0}C). Triphala was prepared in the laboratory by mixing powdered dry fruits (excluding the seeds) of these three medicinal plants in equal amounts. Triphala / powder of individual herbal ingredient were refluxed with methanol for 5 hours on hot plate with magnetic stirrer. The liquid extract was filtered and the filtrate was concentrated on a rotary evaporator under reduced pressure. Different concentrations of the methanolic solutions of the extracts were used for studying acetylcholinesterase inhibitory activity.

Chemicals

5,5' dithiobis (2 nitrobenzoic acid), acetylthiocholine iodide, gallic acid were obtained from Sisco Research Laboratories PVT. Ltd., India. Ellagic acid was purchased from Himedia. Acetylcholinesterase from *Electropus electricus* (electric eel) was prchased from Sigma. All other reagents were of analytical grade.

Acetylcholinesterase activity

Acetylcholinesterase inhibitory property was measured following the method of Ellman et al. [25] following Oh et al. [26] and Siqueira et al. [23]. Electric eel AchE was used for assay, the reaction mixture contained 0.02 ml AchE (19.93 unit/ml buffer, pH 8), 0.01ml plant extract, 1ml buffer, 0.01 ml 0.5mM DTNB and 0.02 ml. 0.6mM acetylthiocholine iodide solution. The reaction mixture was incubated at 37^{0}C for 20 min. The yellow anion produced by the reaction of thiocholine from the reaction with DTNB in the reaction mixture [27] was measured at 412 nm immediately. The percentage inhibition of AchE activity by plant extract was calculated.

High performance thin layer chropmatography

The dried methanol extracts were dissolved in methanol and the aliquots were chromatographed, along with the authentic samples gallic acid and ellagic acid on HPTLC plates (10 x 20 cm) precoated with silica gel 60 F_{254} (0.25 mm thickness). Samples (2-10 µl) and standard compounds (2 µl) were applied on plates by means of linomat 5 applicator (Camag, Switzerland) with the nitrogen flow providing a delivery speed of 150 nLsec^{-1} from the syringe. and the plates were run in the solvent system Toluene: Ethyl acetate: Formic acid: Methanol (9 : 9 : 2.4 : 0.6) [2]. The compounds were identified by superimposing the UV spectra of the samples and the standards within the same R_f values. Quantitative analyses of the compounds were done by scanning the plates using Camag TLC scanner model 3 equipped with Wincats software (Camag) (silt width condition 5.00x0.45 mm, at wavelength 280nm). Concentrations of gallic acid and ellagic acid were calculated from the peak areas in the sample extract and peak areas of known concentrations of authentic samples.

Statistical analysis

Each experiment was repeated three to five times. The differences in activity between two extracts were calculated by Welch's *t*-test. Differences were considered statistically significant if $p<0.05$. IC_{50} value (concentration of extract required to scavenge 50% enzyme activity) was calculated from the regression equation prepared from

the concentrations of the extracts and percentage inhibition of AchE activity.

RESULTS AND DISCUSSION

Among the possible strategies for enhancing brain cholinergic activity, acetylcholinesterase inhibitors have been the most extensively used for the symptomatic treatment of AD [23]. Physostigmine and tacrine are the only AchE inhibitors reasonably evaluated in AD patients, even though their use is limited by the short half-life and peripheral cholinergic side effects of physostigmine, and dose-dependent hepatotoxicity of tacrine [28-30]. The identification of new molecule(s) with less side effects in AD patients is required. Plant based medicines have long been used to treat the ailments in human being. Triphala and its herbal ingredients are reported to have effect on nerve and memory function. So we have studied the AchE inhibitory properties of this herbal medicine and its ingredients.

It was observed that the methanolic extract of triphala significantly inhibited AchE in a dose dependent manner. The methanolic extracts of the powdered fruits (excluding the seeds) of *E.officinalis, T.chebula and T. bellirica* also inhibited electric eel AchE. The percentage inhibitions were proportionate to the concentrations of the extracts. The IC50 values are shown in Table 1. Using eel AchE, highest activity was observed in *T. chebula* followed by triphala*, T. bellirica, E. officinalis.* Welch's T test showed that the differences in activity between Triphala and individual fruits and between the fruits are not statistically significant.

Table 1: Acetylcholinesterase inhibitory activity

Plant material (µg/ml)	Concentration ± sd	% Inhibition (r*)	Regression equation (µg/ml)	IC_{50} value
T. bellirica	1.89	3.27 ± 0.94	Y= 4.269x - 11.334	14.37
	4.72	12.27 ± 2.81	(0. 965)	
	9.43	16.16 ± 0.94		
	11.32	28.63 ± 1.55	(p < 0.001)	
	13.21	45.18 ± 1.90		
	15.09	57.26 ± 1.55		
	16.98	65.03 ± 0.61		
	18.87	72.19 ± 1.55		
T. chebula	1.89	9.41 ± 1.53	Y=4.6353x - 0.788	10.96
	4.72	13.90 ± 0.94	(0.99)	
	6.60	31.29 ± 1.84		
	9.43	45.40 ± 0.61	(p < 0.001)	
	11.32	56.03 ± 0.94		
	13.21	60.12 ± 1.23		
	15.09	70.35 ± 2.15		
	18.87	83.23 ± 1.28		
E. officinalis	4.71	4.91 ± 0.62	Y =1.780x - 2.086	29.26
	9.43	10.02 ± 0.94	(0.98)	
	13.21	22.90 ± 3.70		
	18.87	37.02 ± 5.36	(p < 0.001)	
	28.30	52.35 ± 1.55		
	37.74	60.12 ± 1.23		
Triphala	1.89	3.68 ± 2.68	Y=4.5494x - 9.5798	13.1
	4.72	14.88 ± 2.48	(0.98)	
	9.43	22.29 ± 2.83		
	11.32	36.40 ± 0.94	(p < 0.001)	
	13.21	50.72 ± 1.28		
	15.09	63.19 ± 1.23		
	16.98	71.37 ± 1.28		
	18.87	77.10 ± 1.28		
Gallic acid	0.53	30.65 ± 1.12	Y=23.71x + 23.59	1.1
	0.71	42.26 ± 1.12	(0.98)	(5.8465 µM)
	0.94	50.81 ± 0.81		
	1.42	57.26 ± 0.97	(p < 0.001)	
	1.89	68.71 ± 1.62		
	2.36	78.06 ± 1.05		
Ellagic acid	1.51	12.78 ± 1.01	Y=2.855x + 10.62	13.79
	1.89	22.98 ± 1.71	(0.994)	(45.63 µM)
	3.77	34.30 ± 2.02		
	7.55	43.69 ± 2.95	(p < 0.001)	
	11.32	54.06 ± 2.38		
	15.09	62.95 ± 1.01		
	18.87	67.48 ± 0.97		
Eserine	0.094	16.94 ± 2.99	Y=99.013x + 11.241	0.391
	0.14	22.98 ± 1.71	(0.98)	(1.42 µM)
	0.19	31.59 ± 0.92		
	0.236	35.62 ± 2.03	(p < 0.001)	
	0.28	41.53 ± 1.27		
	0.33	46.77 ± 0.44		
	0.38	46.51 ± 2.58		
	0.425	50.54 ± 0.98		
	0.472	58.33 ± 1.36		

In the fruits of Triphala, gallic acid is present either in free form or bound tannin form (gallotannins and ellagotannins) [3]. Other constituents present are ellagic acid [2], tannic acid, syringic acid and epicatechin along with ascorbic acid [31]. Components of hydrolyzable tannins of *T. chebula* are gallic acid, chebulic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulegic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl-H-D-glucose, 1,6,-di-O-galloyl-D-glucose, casuarinin, 3,4,6-tri-O-galloyl-D- glucose, terchebulin [32]. *T. bellirica* is reported to contain gallic acid [33]. Ascorbic acid and tannoids (emblicanin A and B, punigluconin and pedunculagin) are reported from *E. officinalis* [34,35]. Present study of the extracts by HPTLC (Fig. 1) revealed that gallic acid was one of the major constituents of *E. officinalis, T. chebula and T. bellirica.* So the AchE inhibitory activity of gallic acid was also studied.

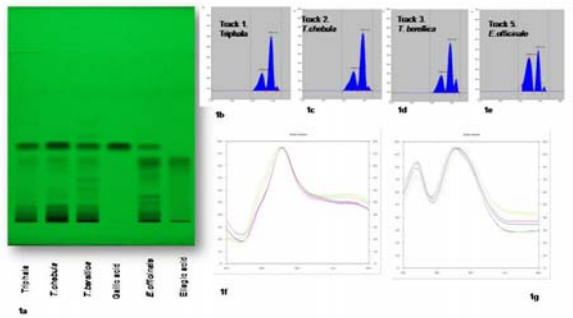

Fig. 1 HPTLC of Triphala and ingredients

1a: HPTLC Plate; 1b – 1e: Chromatogram;
1f: Superimposed UV absorbance of ellagic acid in plant extracts and authentic sample
1g: Superimposed UV absorbance of gallic acid in plant extracts and authentic sample

The IC_{50} value of Gallic acid was 1.1 µg/ml (5.8465 µM). AchE inhibitory activity of ellagic aid was also studied. IC_{50} value of ellagic acid was 13.79 µg/ml (45.63 µM). Activity of gallic acid was much higher than that of ellagic acid. Gallic acid has been reported to have antioxidant properties [36,37], antiallergic [38], anti-inflammatory [39], anticarcinogenic [40-43]. Gallic acid and ellagic acid showed very little α-amylase and α- glucosidase inhibitory activity [44]. Gelatin-gallic acid conjugate inhibited acetylcholinesterase activity [45]. We report AchE inhibitory properties of gallic acid and ellagic acid. Total phenol content (gallic acid equivalent) in triphala, *E. officinalis, T. chebula and T. bellirica* was measured (Table 2). The correlation coefficient (r) of gallic acid content and activity was - 0.877398 indicationg that the activity in plant extract increased (IC_{50} value decreased) with increase in gallic acid content. The correlation coefficient (r) of ellagic acid content and activity was 0.925373 indicationg that the activity in plant extract decreased (IC_{50} value increased) with increase in ellagic acid content. It has recently been reported that brain cholinesterase activity reduced after oral administration of *E. officinalis* to young and aged mice [46]. This work is further supported by our in vitro study of *E. officinalis.*

Table 2: Gallic acid and ellagic acid content

Plant material	Amount of gallic acid (µg/µg crude extract ± sd)	Amount of ellagic acid (µg /µg crude extract ± sd)
T. bellirica	0.131 ± 0.006	0.0591 ± 0.0008
T. chebula	0.249 ± 0.015	0.0797 ± 0.0013
E. officinalis	0.042 ± 0.018	0.1451 ± 0.0043
Triphala	0.147 ± 0.014	0.0665 ± 0.0021

Oxidative stress has been implicated in the cognitive impairment and may be responsible for the development of AD in elderly persons [47-49]. So antioxidants having acetylcholinesterase inhibitory properties may have beneficial effects in AD. Antioxidant activity of triphala [1,3,21,22] and gallic acid [50,51] is well known. Vitamin C, present in *E. officinalis*, is also a good antioxidant [52-54]. The neuroprotective effect of triphala may be due to antioxidant and acetylcholiesterase inhibitory property. Further in vivo study is required in this regard.

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