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Full Length Research Paper

Exploring anti-acetylcholinesterase, antioxidant and metal chelating activities of extracts of *Moringa oleifera* L. for possible prevention and cure of Alzheimer's disease

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The aim of this research is to explore the natural flora of Kashmir for novel plant products for their possible applications in the prevention and treatment of Alzheimer's disease (AD). In the current study, *Moringa oleifera* L. was selected for its possible memory-enhancing potential. Extracts of various parts of the plant were screened for anti-acetylcholinesterase, antioxidant and metal chelating activities. The data obtained clearly establish that impure fraction (MSC) obtained from methanolic shoot extract by silica-gel chromatography showed significant anti-AChE (IC_{50}; 77.58 µg/ml), antioxidant (IC_{50}; 1.26 µg/ml) and metal chelating potential (80%). Factions MSC1 (pure compound) and MSC2 obtained from MSC by thin layer chromatography (TLC) separation showed significant action against AChE activity with IC_{50} values of 64.53 and 120.38 µg/ml, respectively. Methanolic leave extract has also shown significant anti-AChE activity (IC_{50}; 77.32 µg/ml) with weaker antioxidant potential (IC_{50}; 127.27 µg/ml). We conclude that methanolic shoot extracts of the plant with high anti-acetylcholiesterase and antioxidant activities may have AD therapeutic potential.

Key words: *Moringa oleifera* L., Alzheimer's disease, anti-acetylcholinesterase, antioxidant activity.

INTRODUCTION

Alzheimer's disease (AD), the most common type of dementia, is a neurodegenerative disorder that affects memory, understanding, behavior and the ability to work. An estimated 35.6 million people are living with dementia worldwide and the number is expected to increase up to 66 million by 2030. Only in Asia, 14 million people had Alzheimer's or other forms of dementia in 2005 and this is expected to grow to 24 million by 2020 (Anders and Martin, 2010). Currently, there is no cure for AD except the use of acetylcholinesterase inhibitors, the only most effective therapy that has shown consistent positive results (Wolf-Klein et al., 2007). However, pathophysiology of AD is quit complex which involves several different biochemical pathways (Bolognesi et al.,

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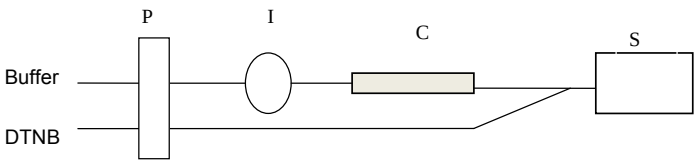

Figure 1. Flow Injection manifold used in this study. P = peristaltic pump, I = injection valve, C = enzyme column, S = spectrophotometer.

2009; Murali, 2002). There is increasing evidence that free radical-induced oxidative damage plays a vital role in the pathogenesis of AD (Foy et al., 1999).The multifactorial nature of AD suggests that a multi-targeted therapeutic approach might be more advantageous than single target drugs (Bolognesi et al., 2009). Natural products with antioxidant properties in addition to actylcholinesterase inhibitory activity could be regarded as highly desirable (Mathew and Subramanian, 2014). Effective AD therapeutic potential of currently approved drug galanthamine, a plant derived alkaloid, may also be attributed to its added antioxidant potential (Nordberg and Svensson, 1998). Until recently, natural plant products have gained much attention as AChE inhibitors and there is still a need for exploring nature for newer potent and long lasting AChE inhibitors with minimal side effects. A large number of plant species from different parts of the world have been screened for cholinesterase inhibitory activity (Mangialasche et al., 2010; Murray et al., 2013; Mehta et al., 2012).

In this study, we have selected *Moringa oleifera* L., a small medium-sized evergreen or deciduous tree, native to Northern India, Pakistan and Nepal (Gupta et al., 1989). Various parts of the plant are used for the treatment of nervous system disorders (Fahey; 2005). In this study, we have screened the crude extracts of various parts of the plant (leaves, shoot and stem-bark) for anti-AChE, antioxidant, and metal chelating activities for their possible role in memory-enhancing therapy.

EXPERIMENTAL

Chemicals

Acetylcholinesterase (E.C. 1.1.3.7) from electric eel, controlled porosity glass (CPG-24, 80-120 mesh, mean pore diameter of 22.6 nm), 2,2dithiobis (2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide were purchased from sigma-Aldrich. Potassium persulphate, 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and ferrozine (sodium3-(pyridin-2-yl)-1,2,4-triazine-5,6-diyl]bis(benzene-4,4'-sulphonate) were purchased from Merk, ferrous sulphate; and other reagents were of analytical grade. Distilled deionised water was used throughout the study.

Extraction and purification

Fresh shoots, leave and bark of *M. oleifera* L. were collected and dried in gloom for several weeks. The dried material was crushed separately into powder form. 200 g dried powdered material of each part was soaked separately and progressively in organic solvents with increasing order of polarity (n-Haxan, ethylacetate, chloroform, acetone, ethanol and methanol) for 8 to 10 days along with successive extraction. Each extract was evaporated in a rotary evaporator and concentrates were collected and stored for further processing. In order to fractionate the crude extract and to purify different components, various chromatographic techniques were employed. Column chromatography was carried out using silica-gel G-F254 type 60 of E. Merck (Art. 7734, 70-230 mesh) as stationary phase and organic solvents were used as mobile phase. Preparative thin-layer chromatography was performed on silica-gel preparative plates (20" multiply 20", 0.5 mm thickness, Merck PF154, type 60).

Determination of anti-acetylcholinesterase activity

A simple flow injection technique with immobilized enzyme column was adopted for the study. The peristaltic pump for carrier stream propulsion was an Ismatech-Reglo, and Rheodyne RH 5020 (Anachem) injection valve was used for injection of standard and sample (extract). The manifold tubing was 0.5 mm i.d. PTFE. The volume of the sample and substrate mixture injection loop was 100 µl. Flow injection manifold used for the study is shown in Figure 1. Immobilized enzyme was packed in a mini column and its activity was measured by injecting standard substrate solutions into a carrier stream of the phosphate buffer (0.1 M, pH 8.5) after passing through the column. The reaction product was mixed with the chromogen (DTNB) in a second stream and absorbance (As) was measured at 405 nm in a flow cell (30 µl volume, 10 mm path length) using Shimdzu 1700 spectrophotometer. For inhibition study, each extract was mixed with standard solution of substrate and the mixture (sample+standard) was injected into a stream of the phosphate buffer (0.1 M, pH 8.5) passed through the immobilized enzyme column; then decrease in absorbance (Ai) was recorded. The activity of immobilized enzyme was studied both in the presence and absence of the inhibitor. Percent inhibition by the extracts was calculated by the formula; % inhibition= As–Ai/As*100. IC_{50} values were determined by log probit analysis.

Antioxidant activity

ABTS radical cation decolorization assay

ABTS radical cation decolorization assay was explored for the study. 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in water (3 mM final concentration) was oxidized using potassium persulfate (2.5 mM) for at least 12 h in the dark. The $ABTS^{\cdot+}$ solution was diluted with deionized water to an absorbance of 1.89 ± 0.3. Absorbance of $ABTS^{\cdot+}$(Ao) as a standard with 2 ml of deionized water was measured at 405 nm. For percent scavenging activity, extracts at final concentration of 10 to 200 µg/ml were mixed with 1 ml $ABTS^{\cdot+}$ and incubated at room temperature for 10 min. The reaction mixture was diluted to 3 ml by adding distilled deionized water. Absorbance (Ai) of the reaction mixture was recorded. Percent scavenging potential (PS) of the extracts was calculated by the equation; PS = (Ao – Ai)/Ao*100 and IC_{50} values were calculated as described above.

Metal chelation activity

Ferrizine assay was adopted for metal chelation activity study. 50 µg/ml of sample was added to a solution of ferrous sulphate (2 mM, 500 µl). The reaction was initiated by the addition of ferrozine (0.25 mM, 500 µl). The mixture was vigorously shaken and left standing for 10 min at room temperature. In controls, 200 µl of deionized water was used to quantify the reaction mixture. Ferrozine reacts

Table 1. Anti-AChE activity of various extracts of stem-bark, shoot and leaf of *M. oleifera* L.

S/No.	Fractions	Conc. tested (µg/ml)	M.I ± S.E.M[a]	(IC_{50} µg/ml)[b]
1	EAB	140	23.7±1.53	349.96.(290.34-452.32)
		280	42.40±1.65	
		500	61.43±2.10	
2	MB	140	20.10±1.32	441.11(357.53-629.19)
		280	32.53±1.48	
		500	55.90±2.16	
3	EB	27	14.20±1.90	401.39(267.69-765.34)
		266	38.50±1.1	
		366	52.80±2.40	
4	ML	17	17.23±1.30	77.34(60.91-100.32)
		86.66	50.36±0.81	
		150	68.06.±0.77	
5	EL	38	12.43±1.24	140.82(117.99-180.79)
		96	31.8±1.17	
		166	58.8±0.52	
6	EAS	43	16.0±0.70	254.04(192.85-376.98)
		172	40.3±1.17	
		354	57.2±1.57	
7	MS	16	14.4±1.41	120.59(88.10-190.22)
		33	20.46±1.95	
		167	58.20±2.56	
8	ES	28	12.40±2.00	121.70(100.42-150.55)
		140	50.50±1.6	
		180	65.70±1.55	
9	MSC	33	32.60±1.63	77.58(61.02-101.19)
		66	40.56±0.80	
		166	70.46±0.90	
10	MSC1	33	40.53±0.856	64.53(41.91-117.10)
		66	45.76±1.50	
		100	60.23±2.05	
11	MSC2	33	15.63±1.59	120.38(97.76-168.13)
		66	26.50±2.00	
		132	55.23±1.55	

M.I., mean %inhibition, [a]Each value represents the mean ±S.E.M. (n=3), [b]IC_{50} values determine by log probit analysis. 95% confidence intervals given in parentheses.

with the divalent iron to form stable magenta coloured complex which absorbs at 517 nm. Absorbance of the reaction mixture in the presence (Ai) and absence of the extracts (Ao) was recorded. Absorbance of all extracts was also recorded and omitted from the Ai where needed. The percentage chelating activity of the extracts was calculated by the formula,

(%) Chelation=(Ao – Ai)/Ao*100.

RESULTS AND DISCUSSION

Our results demonstrate that ethyl acetate (EAB), methanolic (MB) and ethanolic (EB) extracts of bark and ethyl acetate extract of shoot (EAS) have weaker action against AChE activity with IC_{50} values of 349.96. 441.11 and 401.39 µg/ml, respectively. Furthermore, methanolic (MS), ethanolic shoot (ES), and ethanolic leave (EL) extracts showed moderate action (IC_{50}120.59, 121.70 and 140.82 µg/ml) while methanolic (ML) extract showed stronger action (IC_{50} 77.34 µg/ml) (Tables 1 and 2).

Appearance of fine needle like crystals in MS and significant anti-AChE activity led to the isolation and further study of these crystals. The extract was subjected to silica-gel chromatography and three main fractions, MSA (1% n-haxane/Acetone), MSB (2%Methanol/Chloroform) and MSC (5% Methanol/Chloroforms) were collected. All impure fractions were studied for their anti-AChE activity and only MSC (IC_{50} 77.58 µg/ml) was found active.

Table 2. Antioxidant and metal chelating activity of various extracts of stem-bark, shoot and leave of *M. oleifera* L.

S/No.	Fractions	Conc. tested (µg/ml)	M.I ± S.E.M[a]	(IC_{50} µg/ml)[b]	% inhibition 50 µg/ml
1	EAB	33	27.84±0.65	146.13(95.98-446.48)	54.4
		66	40.34±0.55		
		166	51.03±1.6		
2	MB	3	23.30±.0.56	23.43(15.54-36.32)	38.6
		33	50.56±1.2		
		66	68.32±3.4		
3	EB	3	15.34±0.66	27.61(19.96-39.28)	68.6
		33	50.24±1.3		
		66	65.41±3.4		
4	ML	33	13.19±0.43	127.27(104.97-166.36)	NA
		66	25.21±0.8		
		166	60.56±2.5		
5	EL	33	15.56±0.45	110.50(92.26-139.85)	NA
		66	29.85±0.98		
		166	65.17±2.6		
6	EAS	33	13.9±0.15	201.33(175.72-242.63)	32.4
		166	40.23±0.45		
		200	50.32±0.99		
7	MS	3	28.12.±1.2	20.64(12.47-34.65)	NA
		33	53.54±2.3		
		66	65.64±3.5		
8	ES	3	29.35±0.89	13.22((8.33-19.60)	NA
		33	60.56±1.6		
		66	75.12±2.6		
9	MSC	3	61.99±1.6	1.26(0.29-2.69)	80.0
		33	85.21±2.6		
		66	92.34±1.8		

M.I., mean %inhibition, [a]Each value represents the mean ±S.E.M. (n=3), [b]IC_{50}values determine by log probit analysis. 95% confidence interval given in parentheses.

Active fraction, MSC, was further subjected to thin layer chromatography (TLC) based separation for purification. Two fractions, MSC1 and MSC2 (8:2 n-Hexane/Acetones) were collected. Fraction MSC1 was identified as a pure compound. Fraction MSC2 showed moderate while MSC1 showed stronger action against AChE activity with IC_{50} values of 120.38 and 64.53 µg/ml, respectively (Table 1).

Results of the study further revealed that both shoot and bark extracts possess stronger $ABTS^{\cdot+}$ scavenging potential with IC_{50} values of 23.43, 27.61, 20.64, 13.22 and 1.26 µg/ml for MB, EB, MS, ES and MSC, respectively. ML and EL extracts showed reasonable activity, whereas EAS and EAB showed comparatively weaker action. Results are presented in Table 2. The percentage metal chelating potential for 50 µg/ ml of extract in a reaction mixture was determined for all of the above mentioned extracts. In the study, leaf and shoot extracts (ML, EL, MS and ES) were found inactive. Only ethanolic and ethyl acetate extracts of bark exhibited good activity (68 and 54%, respectively). MB and EAS showed weaker potential (38 and 32%). However, MSC showed excellent metal chelating potential (80%) (Table 2).

Conclusion

We conclude that methanolic shoot extracts of the plant with high anti-acetylcholiesterase and antioxidant activities may have an AD therapeutic potential. Further evaluation is required to identify the active ingredients, access their bio-safety in *in vivo* animal models.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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