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Screening of *Habenaria plantaginea* Lindl for anti-Alzheimer potential

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Abstract

The present work reported the anti-Alzheimer potential of various extracts of the plant *Habenaria plantaginea* (Hp) of the Orchidaceae family. Preliminary screening of the crude methanolic extract revealed the presence of different phytochemicals such as alkaloids, glycosides, flavonoids, saponins, anthraquinones, oils, tannins, and terpenoids by employing protocols mentioned in literature. To ascertain the anti-Alzheimer potential of the Hp extracts various pharmacological assays were conducted. In AchE and BchE assays, HP-CHF exhibited the highest activity against both AchE and BchE with IC_{50} (µg/ml) values of 0.94 and 0.86, respectively, followed by crude extract with IC_{50} (µg/ml) values of 2.02 and 1.72, and ethyl acetate with IC_{50} (µg/ml) values of 3 and 2.90, respectively as compared to the standard Galantamine 0.54 µg/ml and 0.51 µg/ml. In the beta-secretase (BACE1) inhibitory assay, evaluated on different fractions Hp.CHF exhibited the highest inhibitory activity against beta-secretase with IC_{50} value of 98 µg/ml. From the results of the study it can be concluded that the aforementioned plant has great potential to treat Alzheimer's disease through multiple mechanisms. Hence, further studies are recommended on this potentially valuable plant to develop safe and effective treatment for AD.

KEYWORDS
Anti-alzheimer's disease, Habenaria plantaginea, AchE, BACE1, β-secretase

1.0 INTRODUCTION

New drug from a natural source with a desirable profile is tremendously an inspiring task in the field of clinical research. There is extensive research in the domain of medical cure of Alzheimer's Disease (AD) around the world to find safer and effective remedies. AD is the most important cause of age-related dementia worldwide, and around 24 million individuals suffer from this debilitating condition. Every 20 years, the number of AD patients doubles owing to the ever-increasing population of the elderly [1-2]. The use of effective therapeutic agents could delay the onset and halt the progression of AD over the next five decades. AD is mainly a disease of the aging population. For the last decade, it has evolved into a serious public health concern, as evidenced by data indicating that 47 million people suffered in 2015, and it is estimated to rise up to 131 million by 2050 [3].

Alzheimer's disease that starts with imperceptible symptoms, including having trouble learning new information, misplacing things, loss of short-term memory and language impairment, is associated with a steady decrease in the cognitive functions . With the progression of the disease the patient faces more debilitating symptoms such as hostility, feelings of sadness, irritability, frustration hallucination and delusions. In the advanced stages of the disease the individual suffers from further severe symptoms including complete loss of memory, losing ability to articulate thoughts, loss of spatial awareness and incontinence becoming entirely dependent on others [4].

Alzheimer's is a multifaceted disease caused by

several risk factors including genetic and environmental factors. Owing to the dominant genetic mutations the occurrence of the disease is less, however, most of cases have found to occur sporadically with no single genetic causality. Higher risk of dementia is associated with both metabolic and environmental risk factors including, cerebrovascular, diabetes, inadequate diet, stress and head injury [5].

Accumulation of Aβ has been implicated in the death of neuronal cells through multiple mechanisms (Carter & Lippa, 2001), including mitochondrial dysfunction, oxidative stress, synaptic disruption, and excitotoxicity. NFTs, hyperphosphorylated tau protein, disseminate all over the brain like plaques [6].

Presently, there is no therapeutic entity available that effectively halt the progress of AD. Despite the fact that amyloid hypothesis is the dominant theory, drugs that lowers Aβ concentration are not very effective. Research strongly suggests that the presence of NFT and plaque have correlation with the progression of the disease but not causative. The complexity of the disease is suggested by the overlap of the environmental and genetic factors and the till known pathophysiology. The likelihood of finding the most effective therapeutic entity would be increase by understanding the underlying pathophysiology and interplay of the risk factors [7].

Genus Habenaria (Orchidaceae family), Orchids have a long history of folkloric use for different infirmities including, indigestion, piles, jaundice, dysentery, tuberculosis, eczema, diarrhea, hepatitis, pyrexia, boils, malaria, to expel worms, arthritis, inflammations and venereal diseases (Martha Pérez Gutiérrez, 2010).This research focusses on the evaluation of H. plantaginea plant for anti-alzheimer's potential based on the traditional use of the plant. Exploring the untapped plant sources can lead to the discovery of effective lead compounds and consequently better medicine.

2.0 MATERIALS AND METHODS

2.1 Chemicals and reagents

Potassium hydroxide, ethyl acetate, AchE, BchE, agarose, EDTA, Acetylthiocholine iodide, Butyrylthiocholine iodide, galantamine and boric acid.

2.2 Collection and Extraction of Plants

Midway through April, *H. plantaginea* (Hp) was acquired from the Dir (L) KPK, Pakistan and identified by Prof. Muhammads Ilyas, Department of Botany, University of Swabi, Swabi KPK, Pakistan. The plant specimen was preserved and documented in the herbarium under voucher number H.UOS.20-2.The aerial part of the plant (15Kg) was rinsed and shade dried for 21 days. The dried plant was then ground to coarse powder and subsequently macerated in 26 L of methanol (80 %) for 21 days. After maceration marc was removed by filtration through muslin cloth with subsequent filtration with Whatman filter paper. The resultant filtrate was dried at 40°C using rotavap. The methanolic extract thus obtained weighed 650 g with somber green hue [8].

2.3 Fractionation

The crude methanolic extract of H.P was reconstituted with distilled water and extracted with organic solvents viz *n*-hexane, ethyl acetate, chloroform, and butanol. First *n*-hexane fraction was formed by taking 200 mL of *n*-hexane in a separatory funnel (500 mL). Both *n*-hexane and aqueous layers of ethanolic extracts were shaken and swirled and left overnight. The upper layer of *n*-hexane was removed from the top of separatory funnel in a beaker. This process was repeated thrice each time pouring fresh 200ml of *n*-hexane in aqueous solution. At the end all fractions were mixed and dried by rotavap (Abubakar & Haque, 2020). The process was repeated for other solvents to obtain their respective fractions. The fractions thus obtained were stored at 4 °C for further investigation [9].

2.4 Preliminary Phytoconstituents analysis

Methanolic crude extract was analyzed for the presence of various phytochemicals includingalkaloids, glycosides, flavonoids, saponins, anthraquinones, oils, tannins, and terpenoids by employing protocols mentioned in literature [10].

2.5 Detection of alkaloids

The presence of alkaloids was detected in the sample by Dragendorff's test (Rishikesh Surve, 2023)**.** Orange-red ppts were observed upon mixing Dragendorff's reagent (1 mL) with extract (2mL), that confirmed the alkaloid presence in extract.

2.6 Test for Saponins

Approximately 10 ml of distilled water was mixed with 1 g of crude methanolic extract in test tube. The test tube was stoppered and heated for around 10 minutes 45 ºC in water bath with vigorous shaking. The test tube was set aside for 30 minutes and observed for the formation of froth, which indicated the presence of saponins.

2.7 Test for tannins (Ferric chloride test).

An aqueous solution was prepared by boiling 2 g of crude extract with 20 ml of distilled water in a water bath for 10 minutes at 45 ºC. Few drops of 10% Ferric chloride solution (light yellow) were added once the solution was cooled to room temperature. The color turned from yellow to back indicative of the presence of tannins.

2.8 Flavonoid test (Sodium hydroxide test)

About 5 mg of the compound was dissolved in water, warmed, and filtered. 10% aqueous sodium hydroxide was mixed with 2 ml of this solution which turned the solution yellow. Subsequent addition of dilute hydrochloric acid turned the solution colourless indicating the presence of flavonoids.

2.9 Terpenoid test (salkowski test)

In this test the appearance of a reddish-brown hue by mixing 5mg of the sample, 2ml of $CHCl_3$ and 3ml of the conc. H_2SO_4 signifies the presence of terpenoids.

2.10 Keller Killiani test.

Cardiac glycosides are detected by mixing 2 ml of the sample with 0.5 ml solution of 3 drops of $FeCl_3$and glacial acetic acid and adding 1 ml of Conc. H_2SO_4 afterwards. The test is considered positive if blue color appears at the interface of two liquids.

2.11 Anthraquinones test

In order to ascertain the presence of anthraquinones, dissolve 2 grams of the pulverized plant sample in ether within a container. Shake the solution vigorously for two to three minutes following combining. The identification of anthraquinones in an aqueous stratum is indicated by the appearance of a pink, red, or violet.

2.12 Evaluation of fixed oils

The presence of fixed oil was determined by conducting a stain test. Squeezed between two filter sheets was a little quantity of the unprocessed extract. A stain on the filter paper indicates the presence of fixed oil.

2.13 Pharmacological investigations.

Various fractions were subjected to the pharmacological assays described below.

2.14Anticholinesterase assay (AChE & BChE)

Inhibition of AChE plays a crucial role in the management of AD. Aside from AD, AChE inhibitors are also important in the diagnosis as well as treatment of other ailments including, myasthenia gravis, glaucoma and distention of bladder. AchE Inhibitory assay is an Ellman method based colorimetric assay, where, thiocholine forms a yellow hue with DTNB (Ellman's reagent), after produced by the action of acetylcholinesterase on acetylthiocholine (substrate) [11]. The intensity of the product color, measured at 412nm, is proportionate to the enzyme activity in the sample.A compound that causes more inhibition will result in a weaker color and a lower measured absorbance.Briefly, various concentrations of fractions were prepared in methanol (1000, 500, 250, 125, 62.5) µg/ml. AchE and BchE were diluted to 0.03 U/mL and 0.01 U/mL in a buffer with a pH of 8.0. In test tube 250 µL of plant extracts at concentrations of (62.5 – 1000) µg/ mL, 10 µL of AchE (0.03 U/mL) and 20 µL of 10 mM of DTNB in buffer were added. The mixture was incubated for 15 min at 37 ° C. The blank was comprised of buffer, Acetylthiocholine iodide, DTNB and test sample but not enzyme. Postive control was galantamine, prepared in concentrations similar to test sample concentrations (1000-62.5) µg/ mL in 50 mM Tris–HCl buffer pH 8.0.

% inhibition = Absorbance of control – Absorbance of Sample (potential inhibitor) / Absorbance of control (absence of inhibitor) * 100. Each analysis was repeated three times and the half-maximal inhibitory concentration was determined via linear regression method by plotting % inhibition VS respective concentration in MS excel (Dhanasekaran et al., 2015).

2.15 β –secretase (BACE1) inhibition assay

The ability of different Hp extracts to inhibit β-secretase 1 (BACE1) enzyme, was evaluated using BACE1 inhibition assay, a key enzyme involved in the generation of amyloid-beta (Aβ) protein associated with Alzheimer's disease [12]. The tested compounds were assayed for BACE1 inhibition using a BACE1 Fluorogenic Assay. In this assay a synthetic peptide (labeled with donor and acceptor moieties) is used which is cleaved by the BACE1 enzyme to produce fluorescence. Fluorescent activity is proportional to BACE1 activity. A mixture of10 µl buffer (50 mM sodium acetate, pH 4.5), 10 µl substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM, ammonium bicarbonate), 10 µl BACE1 (1.0 U/mL) and 10 µl test samples (of each concentrations of various fractions was incubated for 60 min at 25 °C in the dark. Fluorescence was measured with a microplate spectrofluorometer (Gemini EM, Molecular Devices). To read the assay, mixtures were irradiated at 545 nm and the emission intensity at 585 nm was recorded. Percentage inhibition of the enzyme was then calculated from fluorescence of the control (control (enzyme, buffer, and substrate)) and the fluorescence of the tested samples (enzyme, sample solution, substrate). Quercetin was used as the positive controls.

2.16 Statistical Analysis

The data were analysed by ANOVA following multiple comparison Post-Hoc tests using GraphPad Prism-8. Results are presented in mean ± standard deviation (S.D.) with statistical significance $p < 0.05$.

3.0 RESULTS

3.1 Phytochemical Analysis

The methanolic extract of *H. plantaginea* was discovered to contain terpenoids,alkaloids, flavonoids, tannins, glycosides, saponins and anthraquinones. The fixed oil was not found in the extract of *H. plantaginea* Lindl.

Table 1:Phytochemical components of the crude extract of *H. plantaginea* Lindl**.**

S.No	Phytochemicals	Observations	Results
1	Fixed oils	Greasy Spot formation	-
2	Saponins	Frothing was observed	+
3	Flavonoids	At first pale color was observed that went colorless upon acid addition	+
4	Tannins	Blue-black color appeared	+
5	Anthraquinones	Violet-red hue	+
6	Glycosides	Red ppt	+
7	Terpenoids	Brown red ppt	+
8	Alkaloids	Turbidity/precipitates	+

The positive symbol (+) indicates the existence of phytocontituents, whereas the negative symbol (-) indicates their absence.

3.2 *In-vitro* AChE and BChE inhibitory activities of Hp extracts The table 2 provides a summary of the AChE and BChE inhibitory potentials of the fractions that were examined. HP-CHF exhibited the highest activity against both AchE and BchE with IC_{50} (µg/ml) values of 0.94 and 0.86, respectively, followed by crude extract with IC_{50} (µg/ml) values of 2.02 and 1.72, and ethyl acetate with IC_{50} (µg/ml) values of 3 and 2.90, respectively. Aqueous, *n*-hexane, and butanol extracts exhibited the lowest activities for both enzymes with IC_{50} values of (212 and 222), (152, 107), and (274 and 216), respectively, as compared to the standard Galantamine 0.54 and 0.51.

Table 2: Acetylcholinesterase (AchE) and Butyrylcholinesterase activity of tested samples

Fractions	Conc (µg/mL)	Acetylcholinesterase (AchE)		Butyrylcholinesterase (BchE)	
		% inhibition	IC_{50} (µg/mL)	% inhibition	IC_{50} (µg/mL)
Hp.Crude	1000	94.12±0.92^{ns}		96.91±0.97^{ns}	
	500	89.24±1.84^{ns}		91.45±0.57***	
	250	85.66±1.34^{ns}	2.02	88.57±1.79**	1.72
	125	80.78±0.76^{ns}		83.66±0.98*	
	62.5	75.77±1.26**		78.94±0.62*	
Hp.Chf	1000	97.04±1.36^{ns}		99.58±0.24^{ns}	
	500	93.70±0.34^{ns}		94.70±1.22^{ns}	
	250	88.15±0.61^{ns}	0.94	90.74±0.58^{ns}	0.86
	125	84.45±0.88^{ns}		85.44±0.82^{ns}	
	62.5	79.73±0.46^{ns}		80.61±1.64^{ns}	
Hp.EtAc	1000	96.32±1.22^{ns}		97.31±0.72^{ns}	
	500	90.74±1.02^{ns}		92.74±0.82^{ns}	
	250	85.58±1.08*	3	86.93±0.89***	2.90
	125	80.93±0.89^{ns}		82.82±0.94^{ns}	
	62.5	75.91±1.01**		76.02±0.62***	
Hp.Hex	1000	69.92±0.34***		78.39±0.27***	
	500	64.72±1.17***		73.54±0.98***	
	250	58.34±0.31***	152	66.92±0.48***	107
	125	54.14±0.08***		62.04±0.20***	
	62.5	49.69±0.09***		51.05±0.17***	
Hp.Bt	1000	68.60±1.14***		72.38±0.52***	
	500	64.68±1.36***		68.06±0.22***	
	250	62.92±0.98***	274	63.03±0.58***	216
	125	56.05±0.52***		52.69±0.91***	
	62.5	33.93±0.50***		43.03±1.13***	
Hp.Aq	1000	71.59±0.53***		69.44±0.48***	
	500	65.57±0.69***		65.57±0.43***	
	250	59.43±0.47***	212	58.66±0.66***	222
	125	55.69±0.57***		53.47±0.49***	
	62.5	42.34±0.64***		43.53±0.63***	
Galantamine	1000	99.10±0.34	0.54	98.44±0.65	0.51
	500	97.30±0.22		95.32±0.44	
	250	91.20±1.76		91.57±1.11	
	125	84.42±1.72		85.42±1.88	
	62.5	81.22±0.92		81.11±0.33	

*: p < 0.05, **: p < 0.01, ***: p < 0.001, ns: values that do not differ significantly from the +ve control.

3.3 Assay for beta-secretase (BACE1) enzyme inhibition

The inhibitory assay for beta-secretase (BACE1) enzyme activity was evaluated on different fractions of H.P. Among various fractions, Hp.CHF exhibited the highest inhibitory activity against beta-secretase with IC_{50} value of 98 µg/ml followed by Hp.EtAc (301 µg/ml) , Crude (518 µg/ml), HP.Aq (542 µg/ml), Hp.Butanol (545 µg/ml) and Hp.Hex (728 µg/ml) respectively against standard Quercetin with IC_{50} value of 18.88 µg/ml. Percent inhibition and corresponding IC_{50} values of each fraction is given in table 3.

Table 3: Beta-secratase(BACE1) activity of fractions of *H. plantaginea* lindl.

Fraction	Conc. (µg/mL)	% β Secratase inhibition	IC_{50} (µg/mL)
Crude	1000	63.44±2.02***	518
	500	57.22±2.01***	
	250	49.53±1.53***	
	125	46.14±2.32***	
	62.5	38.32±1.30***	
Hp.Chf	1000	76.98±2.02*	98
	500	70.52±1.50**	
	250	63.13±1.17***	
	125	56.39±1.12***	
	62.5	52.68±1.44***	
Hp.EtAc	1000	68.29±1.45***	301
	500	65.28±2.86***	
	250	56.90±1.90***	
	125	46.68±2.12***	
	62.5	41.92±2.22***	
Hp.Hex	1000	61.22±1.20***	728
	500	53.82±1.80***	
	250	47.63±1.37***	
	125	41.70±1.12***	
	62.5	34.43±0.87***	
Hp.Bt	1000	63.12±1.10***	545
	500	56.19±2.03***	
	250	50.30±1.11***	
	125	45.22±1.44***	
	62.5	36.15±1.30***	
Hp.Aq	1000	60.12±2.02***	542
	500	56.29±1.13***	
	250	52.14±1.02***	
	125	44.32±2.04***	
	62.5	32.42±2.44***	
Quercetin (std)	1000	84.20±0.74	18.88
	500	78.55±0.97	
	250	72.94±0.12	
	125	66.73±0.47	
	62.5	62.68±0.92	

To find out whether there were any significant differences between the test samples and the standard drugs, we used two-way ANOVA with the Bonferroni post-test. The corresponding p-values are $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4. DISCUSSION

Alzheimer's disease involves a persistent decrease in cognitive functions of the brain. The onset of the disease consists of short-term memory loss, trouble learning new information, mood swings, language difficulties, and misplacing objects [13]. However, progression of the disease leads to experience frustration, hostility, and irritability, feelings of sadness, delusions, and hallucinations (Matsubara et al., 2024).AD is a multifactorial disease including genetic and environmental risk factors. Amyloid-β plaques (senile plaques) and neurofibrillary tau tangles (NFT's) are the hallmarks of Alzheimer's disease. The exact cause of this disease has still not been figured out, though the role of various factors has been well-established such as aggregates of τ-protein, senile plaques and drop in the levels of Ach. Presently, there is no therapeutic agent available that checks the progress of AD effectively [14-15].

Herbal drugs decrease the symptoms via multiple mechanisms such as decreasing inflammation, reducing production of amyloid beta proteins and decreasing free radical production [16]. This research work was undertaken to provide a scientific basis for the folkloric use of *H. plantaginea* for Alzheimer's disease. Evaluation of the untapped sources increases the likelihood of finding a promising lead compound and consequently safe and effective therapy of AD. Hp was extracted with methanol to form crude methanolic extract and subsequent extracts including, *n*-hexane, chloroform, ethyl acetate and aqueous extracts were prepared from the crude extract. Phytochemical evaluation indicated positive results for phytoconstituents such as, glycosides, terpenoids, alkaloids, anthraquinones, flavonoids and tannins and negative for fixed oils in crude methanolic extract [17]. In AD ACh level is severely impacted due to loss of neurons in the forebrain which is the main perpetrator involved in cognitive decline. This abnormality can be partially managed via using therapeutic agents that can either affect ACh receptors or inhibit AChE enzyme. AChE's are serine hydrolases, involved in the breakdown of ACh, found in the cholinergic synapses. ACh inhibiting agents increase the level of ACh in the synaptic area, which possibly improves cholinergic transmission thereby improving the symptoms of disease . In the present study HP-CHF exhibited the highest activity against both AchE and BchE with IC_{50} (µg/ml) values of 0.94 and 0.86, respectively, followed by crude extract with IC_{50} (µg/ml) values of 2.02 and 1.72, and ethyl acetate with IC_{50} (µg/ml) values of 3 and 2.90, respectively. as compared to the standard Galantamine 0.54 and 0.51.The breaking of amyloid precursor protein into amyloid-beta (Aβ) peptide, which form amyloid plaques, is mediated by the enzyme named Beta secretase (BACE1). Therefore, BACE1 enzyme provides a major target for anti-Alzheimer's drug development (Pandey et al., 2024). The inhibitory assay for

beta-secretase (BACE1) enzyme activity was evaluated on different fractions of H.P. Among various fractions, Hp.CHF exhibited the highest inhibitory activity against beta-secretase with IC_{50} value of 98 µg/ml followed by Hp.EtAc (301 µg/ml) and Crude (518 µg/ml), respectively against standard Quercetin (IC_{50} : 18.88 µg/ml). Persistent decline in the cognitive capabilities is the characteristic feature of AD, owing to the widespread death of neuronal cells. Translational therapeutic interventions can be developed by investigating the effect of agents on the behavioral phenotypes. Extensive research has shown mice an excellent resource for the conduction of behavioral assays for the evaluation of new therapeutic compounds. Using mouse model molecular targets can be easily identified and screening compounds with potential therapeutic effects utilizing mice paves the way for translational medicine (Puzzo et al., 2014).

5.0 CONCLUSION

The results of both the *in vitro* investigations conducted on various fractions of the plant *Habenaria plantaginea* strongly suggest that the aforementioned plant has excellent potential of treating Alzheimer's disease through multiple mechanisms. Hence, further *invivo* studies are recommended on this potentially valuable plant to develop safe and effective treatment for AD.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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