Babol University of Medical Sciences
IJMCM
International Journal of Molecular and Cellular Medicine
p-ISSN: 2251-9637 o-ISSN: 2251-9645
Cellular and Molecular Biology Research Center

Stigmasterol- an Acetylcholinesterase Inhibitor from *Phormidium retzii* with relevance to Alzheimer's disease Therapy

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Article type: **Original Article**

Received: 2023.02.08
Revised: 2023.10.02
Accepted: 2023.11.12

ABSTRACT

This study observed *in vitro* screening, purification and identification of cholinesterase inhibitors from the microalgae *Phormidium retzii*. Mixed microalgal culture was screened from freshwater samples for *Phormidium* sp. Single colony was purified and authenticated as *P. retzii*. Acetylcholinesterase (AChE) enzyme was purified from hRBC ghost. Sequential extraction of *P. retzii* was performed using organic solvents. Cholinesterase enzyme activity and its inhibition by various extracts were then tested. The active fractions were then subjected to partial purification and characterization. Petroleum ether extract of *P. retzii* showed maximum inhibition of 68.6 % against AChE while other solvent extracts showed no inhibition. Seven fractions were obtained from the active extract using thin layer chromatography. Among which fraction no. 5 showed maximum inhibition of 86.37 % towards AChE. Fraction no. 5 when subjected to GC-MS led to determination of the active principle as stigmasterol. The maximum inhibition of stigmasterol (0.45µM) was 81.2±0.08% with IC50 value of 0.214. Stigmasterol from *P. retzii* inhibited AChE projecting itself as safer drug for Alzheimer's disease with minimal side effects.

Keywords: Acetylcholinesterase, Alzheimer's disease, inhibition, microalgae, *Phormidium retzii*, stigmasterol

Cite this article: Yesudas R, *et al*. Stigmasterol- an Acetylcholinesterase Inhibitor from *Phormidium retzii* with relevance to Alzheimer's Disease Therapy. *International Journal of Molecular and Cellular Medicine. 2023; 12(2):100-107*. **DOI:** 10.22088/IJMCM.BUMS.12.2.100

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[DOI: 10.22088/IJMCM.BUMS.12.2.100]

Introduction

The neurodegenerative characteristics of Alzheimer's disease (AD) comprises of the pathological alterations in the brain. Acetylcholinesterase (AChE) is the prime cholinesterase that splits acetylcholine (ACh), a neurotransmitter. The deficiency of ACh is the foremost reason that leads to the diseased condition of AD. These changes include the advancement of β-amyloid plaques and neurofibrillary tangles. Moreover, AD is connected with significant decreases in the functioning of choline acetyltransferase and dropped ACh levels in the brain (1). Cholinesterase inhibitors result in delaying the inactivity of ACh after synaptic release. It is the only significant treatment leading in noteworthy clinical benefit (2).

Anticholinesterases are prescribed for treating glaucoma, Myasthenia Gravis and AD (3). Phosphorylated admixtures binding to AChE's active site are commonly proven inhibitors (3). Therapeutics for AD comprises tacrine, memantine, galantamine, donepezil and rivastigmine. These medicines lead to liver damage, nausea, dizziness, vomiting, headache, confusion and loss of appetite. Due to this reason, researchers are keen to discover safe drugs for the disease.

Microalgae dwell in both marine and freshwater environs. They are proven as rich home of new bio compounds with immense activities. They tend to possess an extensive variety of different structural classes of compounds containing quite a lot of indole alkaloids. Over 15,000 new compounds are reported and extracted from microalgae as drugs either in its natural form or with minute modifications in them (4,5). Drugs that can help in treating diseases related to various viruses, fungi and other microbes can be obtained from the algae. Bioactive compounds that can play a vital role in health care including neuroprotective care are extracted from microalgae (6, 7, 8, 9).

Nostoc sp., *Sargassum sagamianum* and various blue-green algae excrete toxins with effective pharmaceutical uses (10). There are many anticancer drugs extracted from blue green algae. Although there are some studies of the anticancer and antiplasmodial activities (11, 12) of substances isolated from various *Phormidium* strains, *Phormidium retzii* (*P. retzii*) remains not been well studied. *P. retzii* is described as having filaments that are straight, mainly not or occasionally constricted at the cross-walls, end cells that are neither capitate nor tapering, 4.5–12 m broad with a thin sheath, cells that are shorter than wide, 4–9 m long, and cross-walls that are not granular (13). It is also described as being found in flowing or still water on rocks.

To much surprise, the screening and working with algae and cyanobacteria in the field of drug discovery is very much confined. Microalgae are considered as better economical source and would be evident as safer drugs with minimal or no side effects. Objectives of this study are to isolate microalgae from the study area, identification of the potent microalgae and screening of AChE inhibitor from isolated microalgae and then characterize the inhibitor by using various chromatographic techniques.

Materials and methods

Collection, Cultivation and Authentication of Microalgae

Fresh water samples were collected from Wadakancherry, Kerala, India (10.6617°N, 76.2363°E). The sample was inoculated into BG11 media (HiMedia, India) and incubated at 28°C, 8 hrs/16 hrs dark/light cycles with white fluorescent light (1000 lux). After optimal growth, single colonies were obtained using

[DOI: 10.22088/IJMCM.BUMS.12.2.100]

pure culture technique (14). From the group of pure cultures, *Phormidium* sp. was obtained based on the colony morphology and microscopic observation and it was submitted to Botanical Survey of India, Coimbatore, TN, India for authentication. It was then subjected to mass cultivation (10 litres) at optimal culture condition.

Solvent Extraction of the Pure Microalgal Culture

The algal biomass was collected and washed thrice using cold neutral phosphate buffered saline (cPBS). The wet biomass was shade dried. Using organic solvents such as petroleum ether, chloroform, ethyl acetate and methanol (150 mL), 2 g dry weight biomass was led to sequential extraction by soxhlet apparatus.

Isolation of AChE from human blood sample

AChE was isolated from the outdated human blood sample collected from blood bank of a hospital at Coimbatore, TN, India (14). The enzyme was then stored at 4 ˚C and was used as enzyme source.

Screening of Cholinesterase Inhibitory Activity of Crude Extracts

Ellman's coupled enzyme assay was used to determine anti-AChE activity of crude extract of microalgae (15). Phosphate buffer (50 mM, pH 7.4) was used to prepare Ellman's reagent 5, 5′-Dithiobis (2-nitrobenzoicacid) (DTNB; Sigma), acetylthiocholine iodide solution. In a 96-well microtitre plate the wells were added with 65 µL of phosphate buffer and then 5 µL AChE. Duplicates of the samples were prepared; followed by the addition of 5 µL and 10 µL of different algal crude extracts leading to the onset of the reaction. The mixture was incubated at 37 ˚C for 10 min. After incubation, the mixture 20 µL of DTNB (0.38 mM) and 5 µL of acetylthiocholine iodide (0.50 mM) were added and kept for incubation at 37 ˚C for 10 min. To all the wells, 100 µL eserine (0.1 mM) was added. Bio-Rad® 680 microplate reader was used to monitor the reaction at 412 nm wavelength.

Inhibition for each sample was calculated as follows:

Inhibition (%) = 100 – (T/C) × 100

where T and C are the optical density for test sample and control at 412 nm, respectively.

Purification of the Active Principles

Thin Layer Chromatography with optimized resolving solvent system of petroleum ether, chloroform and ethyl acetate in the ratio 1:3:0.2 was used to separate the active principles with distinct UV fluorescent bands. R_f values of the different bands were calculated by,

R_f value = (Distance travelled by the solute / Distance travelled by the solvent)

Determination of AChE inhibitory activity

All the seven fractions obtained from TLC were tested for AChE inhibitory activity.

GC-MS analysis

The active fraction containing cholinesterase inhibitor was further characterized by GC (Model: clarus 680)-MS (Model: clarus 680) (Perkin Elmer).

GC conditions

Initial temp was 60 ˚C for 2 min, ramp was in 10 to 300 ˚C/min and held for 6 min. Total run time was 32.00 min and the sample injection volume was 1µL with split ratio of 10:1. Flow rate was maintained at 1 mL/min. The carrier gas used was helium and column used was Elite-5MS (30.0 m, 0.25 mmID, 250 µmdf).

Mass conditions

[DOI: 10.22088/IJMCM.BUMS.12.2.100]

Solvent delay was 2.00 min, transfer and source temperature of 230 ˚C and scanned from 50 to 600 Da. National Institute of Standards and Technology (NIST) standard reference database was used to identify the active principles.

AChE inhibitory activity of pure compound

The identified active compound was procured from HiMedia. A stock solution of the compound was prepared (6 mg/mL) and the AChE inhibitory activity of three concentrations (0.035, 0.07 and 0.14 µM) was checked.

Statistical Analysis

All analyses were performed in triplicates. SPSS® (v16) software for windows was used to calculate the descriptive statistical parameters i.e., mean and SD values.

Results

Collection, Cultivation and Authentication of Microalgae

The selected pure microalgal culture was identified and authenticated (Ref no: BSI/SRC/ 5/23/ 2017/ Tech/54) as *P. retzii* (Figure 1). *P. retzii* is the unbranched filamentous blue green algae with prominent granules spread throughout in cell volume. Three grams of dry biomass was obtained when the microalgae grew to 10 litres.

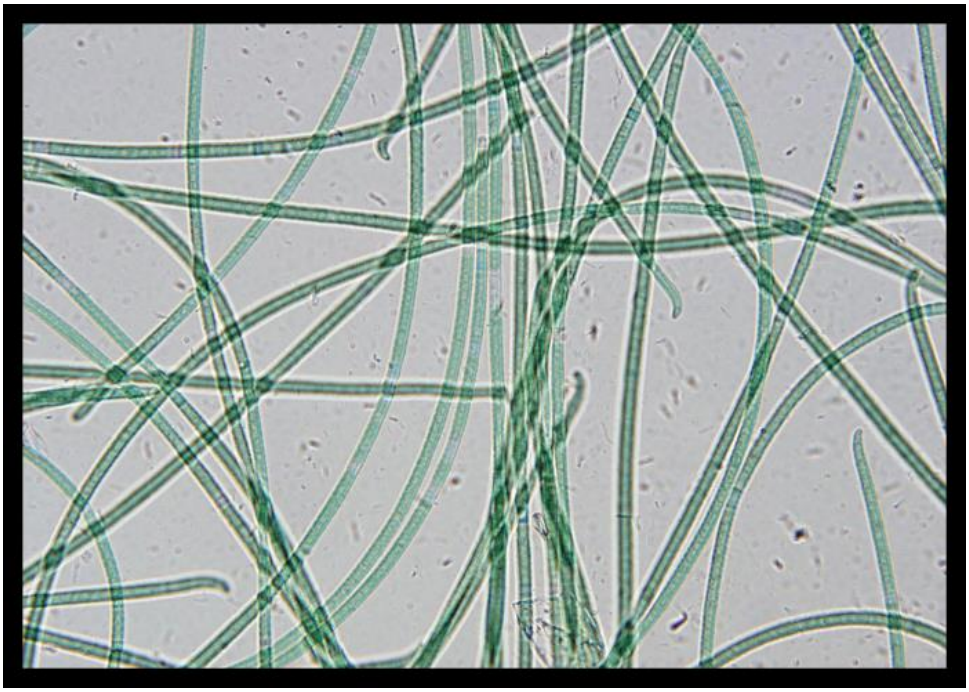


Fig.1. Morphology of isolated micro algal culture *P. retzii.*

Screening of AChE Inhibitor Activity of Crude Extracts

The petroleum ether extract of *P. retzii* showed a maximum inhibitory activity of 68.6±0.32% at 60 µg against AChE while the other solvent extracts showed minimal or no inhibition. Further study was focused on the petroleum ether extract of microalgae *P. retzii* with inhibitory activity against AChE (Table 1).

[DOI: 10.22088/IJMCM.BUMS.12.2.100]

Table 1. Inhibitory activity (%) of various extracts of *Phormidium retzii* towards acetylcholinesterase.

Solvent extracts	Inhibition percentage (%)
Petroleum ether	68.6±0.32
Chloroform	NA
Ethyl acetate	35.02±0.85
Methanol	NA

Partial Purification

The petroleum ether extract of *P. retzii* with the active principle accountable for enzyme inhibition was then projected to thin layer chromatography (TLC). Seven fractions identified in TLC were tested against AChE.

Determination of AChE Inhibitory Activity

The AChE inhibitory activity of petroleum ether extracted active principles of *P. retzii* is shown in Table 2. The maximum inhibitory activity of 86.37±0.24% was shown by the fraction no. 5 towards AChE, followed by fraction no. 3 with inhibition of 54.55±0.89% while fraction no. 7 showed minimal inhibition of 4.55±1.73%.

Table 2. Comparison of AChE inhibitory activity of compounds in TLC of petroleum ether fraction of *Phormidium retzii*.

Band No.	Inhibition %
	NA
1	NA
2	54.55±0.89
3	27.28±1.28
4	86.37±0.24
5	17.38±0.57
6	4.55±1.73

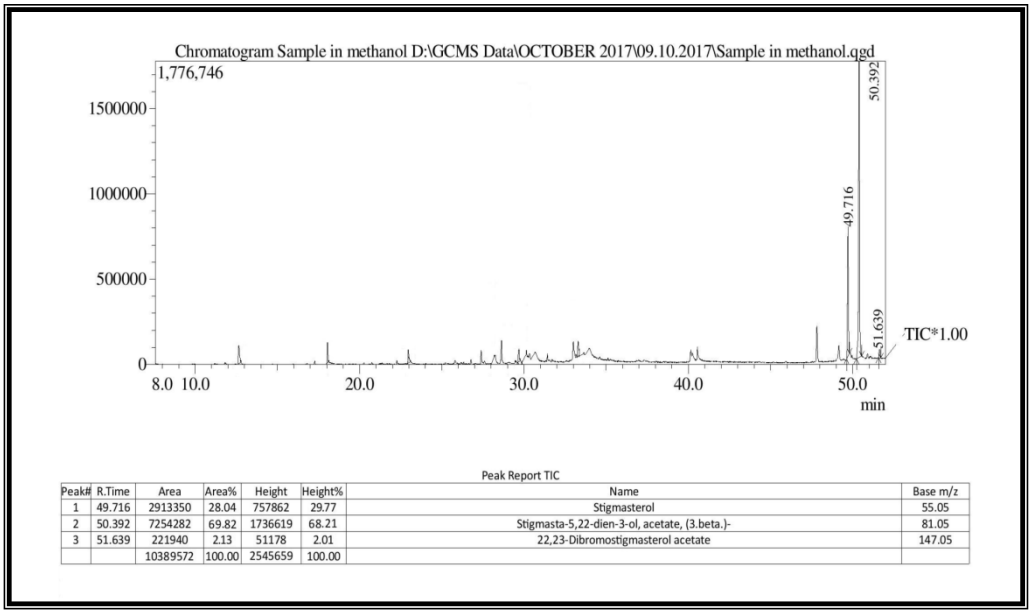
GC-MS

Fraction no. 5 from petroleum ether extract of *P. retzii* was subjected to GC-MS for further characterization of the active principle. The chromatograms of fractions no. 5 is shown in Figure 2. The respective peaks depending on retention time in the chromatogram were determined and compared with the database of NIST library. Identification was based on the molecular structure, molecular mass and calculated fragments (Retention time, peak length and width). Interpretation based on mass spectrum of active principle was also done using the NIST database with more than 62,000 patterns. The chromatogram of fraction no. 5 showed single active peak at the retention time of 19.610. This has been confirmed as Stigmasterol by NIST database library. Remaining peaks which appear cloudy are the GC-MS interference peaks of solvent and siloxane (column bed leak).

Determination of AChE Inhibitory Activity of Stigmasterol

Stigmasterol showed inhibition towards AChE in dose dependent manner. The maximum inhibition of stigmasterol at concentration 0.45µM was 81.2±0.08 % with IC_{50} value of 0.214 (Table 3).

[DOI: 10.22088/IJMCM.BUMS.12.2.100]



Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	Name	Base m/z
1	49.716	2913350	28.04	757862	29.77	Stigmasterol	55.05
2	50.392	7254282	69.82	1736619	68.21	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.)-	81.05
3	51.639	221940	2.13	51178	2.01	22,23-Dibromostigmasterol acetate	147.05
		10389572	100.00	2545659	100.00		

Fig.2. GC-MS analysis of the active principle of *P. retzii*.

Table 3. AChE inhibitory activity of stigmasterol at various concentrations.

	Stigmasterol (µM)		
	0.15	0.30	0.45
Inhibition (%)	32.4±0.12	78.4±0.21	81.2±0.08
IC_{50}		0.214	

Discussion

The most insistent dementia with progressive collapse of central nervous system is Alzheimer´s disease. The most common symptom that is related to the depletion of the neurons is directly connected to the memory loss and other cognitive disorders. AChE inhibitors are the most trending symptomatic medication which includes drugs such as galantamine, rivastigmine and donepezil. Research in the natural sources for the drug for AD is very much in need due the side effects that showed by the synthetic drugs mentioned earlier (16).

With reference to the studies carried out earlier (14), acetone extract of *Phormidium* sp. showed inhibition against butyrylcholinesterase. Hence, in this study *P. retzii* has been tested for AChE inhibition to find novel AChE inhibitors. The maximum inhibitory activity of 68.6±0.32% was shown by the petroleum ether extract of *P. retzii*.

According to study reports, the natural habitat of *P. retzii* is water. They are almost omnipresent showing their presence in freshwater, sea and hot springs as well. Some of the species secrete anatoxins and microcystins leading to interference with nerve signaling and liver bleeding respectively (4).

P. retzii has not yet been predominantly tested against AChE and this may be the first study in this trait. The petroleum ether extract of microalgae *P. retzii* found to contain the bioactive compound responsible for enzyme inhibition was then subjected to TLC and seven fractions were separated. Among the seven, the

[DOI: 10.22088/IJMCM.BUMS.12.2.100]

maximum inhibitory activity towards AChE was exhibited by fraction no. 5 with the inhibition of 86.37±0.24%. The need to obtain inhibitors against cholinesterases from algae has resulted in the isolation of nostocarboline from *Nostoc* 78-12A (17). In reports, sargaquinoic acid and sargachromenol isolated from *S. serratifolium* were reported to show inhibition against AChE (18). The main types of compounds that exhibit AChE inhibition are the glycosides, alkaloids, coumarins and terpenoids (19). The drugs that are prescribed for AD tend to be pseudo reversible and primarily comprises of esters and amines as the key functional groups (16).

P. retzii has never been reported before in AChE inhibition. In the present study, fraction no. 5 collected from petroleum ether extract of *P. retzii* showed inhibition against AChE and the active principle in the fraction responsible for inhibition was characterized as stigmasterol. Further work is in progress to determine enzyme kinetics of stigmasterol against AChE. It has been reported that one of the compounds, present in the bark extract of *Sarcocephalus latifolius,* that showed AChE inhibition and antioxidant property was stigmasterol and is used as traditional medicine in Sudan (20). In a recent *in vivo study*, it was found that the concentration of total deposition of amyloid β 42 (Aβ42) reduced in both cortex and hippocampus of stigmasterol treated mice (21). The active principle stigmasterol could develop as a natural substitute as anticholinesterase drug or as a basic part for the active analogue and supplemented as AChE inhibitors in the near future.

Acknowledgments

The authors are thankful to the authorities of CMS College of Science and Commerce, Dr. N.G.P. Arts and Science College and Botanical Survey of India, Coimbatore, Tamil Nadu, India for their facilities.

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