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Original Article

Screening of Anti-Alzheimer Potential of some Cyanobacteria (blue-green algae) Through in vitro Acetylcholinesterase Inhibition Assay

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Abstract

Alzheimer disease (AD) is the most common cause of memory impairment and dementia in the elderly. Inhibition of acetylcholinesterase (AChE), the key main enzyme in the breakdown of acetylcholine, is considered as a route for AD treatment. Cyanobacteria (blue-green algae) comprise a promising natural resources of potential a verities of neuro-chemicals including AChE inhibitors. Accordingly, ten different cyanobacteria species were isolated, identified and their growth on different standard nutrient media was evaluated. The modified Navicula medium supported relatively the highest growth of the test cyanobacteria. *In vitro* effects of methylene chloride/methanol crude extracts on the activity of AChE were tested and compared. Compared to control the organic solvent extracts from the isolate no. 1 *Anabaena anomala*, *Anabaena oryzae* and *Anabaena variabilis* inhibited AChE activity by 25%, 25% and 50%, respectively. The crude extract of *Anabaena variabilis* maintained the highest enzyme inhibition; this cyanobacterium may maintain a promising role for the treatment of Alzheimer disease.

1. Introduction

In the United States, Alzheimer's disease falls sixth on the list of leading causes of death (Campbell-Taylor *et al.,* 2014). It is the most common cause of dementia with elderly of some people. Dementia is the loss of cognitive, functioning, thinking, remembering, reasoning, and behavioral abilities. Dementia is caused due to the decrease of the level of acetylcholine (ACh) a neurotransmitter that enables the nerve cells to communicate with each other (Fisher *et al.,* 2003). The level of acetylcholine is governed in the human brain mainly by the activity of acetylcholinesterase (AChE) which increases under this condition (Ballard, 2002).

Several acetylcholinesterase inhibitors are approved as Alzheimer treatment drugs such as Donepezil (with trade name Aricept) which is approved to treat all stages of Alzheimer, in addition to Rivastigmine (with trade name Exelon) and Galanthamine (with trade name Raza-

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dyne) that treat mild to moderate Alzheimer (Schelterns and Feldman, 2003). It has become evident that the excessive usage of these drugs may cause liver damage, vomiting, nausea, and increase the frequency of bowel movements (Sugimoto, 2008). Apparently, exploring new drugs of natural origin without side effects is urgently needed and, in this context cyanoprokaryotes (blue-green algae) may constitute a promising renewable bio-resources of novel bioactive metabolites and neurochemicals that may be effectively used for Alzheimer therapy. Anatoxin-a(s), nostocarboline, and nostotrebin 6 are some of these compounds which are currently considered to be potent natural inhibitors against AChE. These compounds have been widely reported as biochemical constituents of biomass of certain cyanobacteria including different species of *Nostoc* and *Anabaena* (Becher *et al.,* 2009; Zelík *et al.,* 2010; Patocka *et al.,* 2011).

The current worldwide research interest to explore novel neurochemicals of natural origin and particularly the search for novel acetylcholinesterase inhibitors from cyanobacteria triggers the idea to conceive the present investigation. The present investigation aims primarily at monitoring and assessing the inhibitory effects of methylene chloride/ methanol crude extracts of some cyanobacteria against the acetylcholinesterase activity. The core objective was to identify and highlight any possible potential role of the test cyanobacteria against Alzheimer disease.

2. Materials and Methods

2.1. Collection of water samples for the isolation of cyanobacteria

Fresh water samples were collected from the River Nile system at Delta region in the front of Mansoura University (31° 2' 45.7620" N, 31° 21' 18.6444" E) (EPA, 1985).

Water samples were centrifuged at 4000 rpm for 10 minutes. Supernatant was discarded and the precipitated plankton pellets were picked up by sterile needle and streaked on solid agar of the nutrient medium BG11 (Andersen, 2005).The agar plates were incubated for one week at 25 ± 2 °C under continuous light of 2.789 W m^{-2}.

At the end of the incubation period a simple stereomicroscope was used to observe and locate cyanobacteria colonies growing on the surface of agar plates either those maintaining compact or diffuse growth habit. Under sterile conditions of a laminar cabinet, parts of clean cyanobacteria colonies were picked up by sterile needle and restreaked on the agar plates of BG11 medium (Stanier *et al.,* 1971).

The individual colonies were then spotted and carefully picked up by means of sterile loop and transferred separately into liquid BG11 medium, incubated at 25 ± 2 °C under continuous light of 2.789 W m^{-2} and left to grow for one week to get biomass sufficient for identification. These procedures result in isolation of a number of unialgal isolates ready for identification.

2.2. HeIdentification of cyanobacteria

Identification and nomenclature followed (Anagnostidis and Komárek, 1988 & Komárek and Anagnostidis, 1989). Classification and nomenclature of the isolated cyanobacteria were checked against the recent information posted at the webpage of alga base (http:// www.algaebase.org).

2.3. Isolated cyanobacteria

The isolated cyanobacteria include ten species of cyanobacteria, namely *Anabaena anomala* Fritsch (three isolates 1, 2, 3), *Anabaena khannae Skuja, Anabaena varibilis* var. *ellipsospora* Fritsch, *Anabaena variabilis Kützing ex* Bornet & Flahault, *Anabaena fertilisma Rao, Anabaena oryzae* Fritsch, *Nostoc entophytum Bornet & Flahault and Oscillatoria cortiana* Meneghini ex Gomont.

2.4. Effect of different nutrient media on growth of the isolated cyanobacteria

The The growth of test cyanobacteria was assessed using three different standard nutrient media including, BG11 medium (Stanier *et al.,* 1971), modified BG11 medium (Allen, 1968) and modified Navicula medium (Starr, 1978). The started inoculum of all tested cyanobacteria was equivalent to 0.005 g l^{-1} dry wt. The idea

was to select the medium supporting maximum growth of cyanobateria.

2.5. Growth assessment

Gravimetric dry weight biomass determination was used to assess the growth of the tested cyanobacteria. Cyanobacteria biomass was harvested at the beginning of growth stationary phase by filtering known volume of cyanobacteria culture through dry pre-weighed GF/C filter. Filters with cyanobacteria biomass were dried in a hot air oven at 105 ^{o}C for 12 hours, cooled down in a desiccator for an hour, and then reweighed to obtain the average dry weight (g $l^{-1)}$ of the tested cyanoprokaryotes (APHA, 2005).

2.6. Growth rate

Growth rates of cyanobacterial cultures were determined according to the following equations (Andersen, 2005):

Specific growth rate; $\mu = Ln (N_2 / N_1) / (t_2 - t_1)$

Where: N_1 and N_2 = biomass at time 1 (t_1) and time 2 (t_2), respectively.

Doublings per day; $(Dd\text{-}1) = \mu / Ln_2$

2.7. Biomass harvesting

known volumes of cyanobacteria cultures were collected after 15 days growth, centrifuged at 4000 rpm for 10 minutes then pellets were collected, washed several times with glass-distilled water and freezed at -20 ^{o}C over night. Frozen algal pellets were then lyophilized and the freeze-dried biomass was weighed using four decimal point balance (Stein, 1973).

2.8. Crude extraction with methylene chloride: methanol extraction (1: 1) v/v

A sample of 3 g freeze-dried powder of algal material was rolled in filter paper, gently pressed to make the biomass more fragile and porous then placed in the reservoir of a soxhlet extractor (Sadasivam and Manickam, 1996). Methylene chloride/methanol in a ratio of 1: 1 (v/v) was used as organic solvent for extraction. The extraction process continued for 4 hours and/or terminated when the solvent in the extraction reservoir became almost colourless. The extract was then evaporated under reduced pressure using rotary evaporator (SENCO. Model R206D). The dry extract was collected, weighed, and placed in clean dry glass vials. The solid crude extract was used for AChE activity assays.

2.9. Acetylcholinesterase activity assay

Reagent and chemicals:

The following chemicals used for acetylcholinesterase activity assay were obtained from Sigma;

(1) acetylcholinesterase (electric-eel AChE) (source for the enzyme) purchased from Sigma via invoice no. (#15569-2-93).

(2) acetylthiocholine iodide (ATCI) (substrate) purchased from Sigma via invoice no. (#15291-2-93).

(3) 5,5– Dithiobis (2-nitrobenzoic acid) (DTNB) (Ellman's reagent) purchased from Sigma via invoice no. (#15134-2-93).

The assay for AChE activity was slightly modified from the methods described by Ellman *et al.* (1961), Nguyen and Kim, (2012) and Mathew and Subramanian, (2014). The activity was measured in a 96-well microplate with a standard assay mixture (200 µl) containing 1.5 mM DTNB (This low concentration of DTNB used in this assay was recommended by (Komersova et al., 2007) to avoid its inhibitory effect on AChE activity), 50 mM Tris-HCl (pH = 8), 1 µl of AChE (0.08 µg) and the reaction was initiated by the addition of 20 µl ATCI (15 mM) (substrate) to the wells. After mixing, the development of yellow colour (TNB) was monitored continuously at 25 ^{o}C by the microplate reader at 405 nm and $\epsilon 405 = 14.05 \times 10^3\ M^{-1}\ cm^{-1}$. In order to test the inhibitory effect of the different crude extracts, 5 µl of each extract (with the same solid material strength 4µg μl^{-1}) was pre-incubated with the enzyme in the reaction mixture for a minute before adding the substrate and starting the measurements. The assay was also performed in presence of 5 µl of the used solvent (methylene chloride/methanol in a ratio of 1: 1 (v/v)) alone in order to monitor the effect of the solvent on the enzyme activity and this result was subtracted from the activity values obtained with the extracts as it should be considered as a negative control value.

The inhibition (%) was calculated as: Inhibition (%)= $[(A_0 - A_1)/A_0] * 100$.

Where, A_0 is the activity of the enzyme when the solvent only was used (control) and A_1 is the activity of the enzyme when the tested extract was used.

2.10. Statistical analysis of data

Values of each measurement represent the mean of three replicates ±SD (Standard Deviation).

3. Results

3.1. Growth of different cyanobacteria

3.1.1. Effect of nutrient media

Figure 1 illustrates the growth (expressed as gram dry weight per liter) of different cyanobacteria in different three standard growth media. It is clear cut that the modified Navicula medium supported relatively the highest growth of the tested cyanobacteria. The growth of different cyanobacteria fluctuated between 0.013 ± 0.001 and 0.087 ± 0.004 $g1^{-1}$ in BG11, 0.083 ± 0.015 and 0.265 ± 0.013 gl^{-1} in modified BG11 and 0.4 ± 0.045 and 0.697 ± 0.046 g l^{-1} in the modified Navicula media. Accordingly, the modified *Navicula* medium was chosen as best for growth of cyanobacteria for further research.

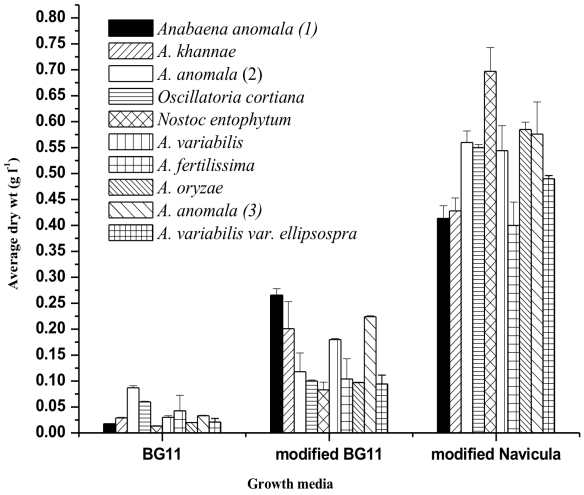

Fig. 1. Average dry weight (mean of three replicates ± SD) of different species of cyanobacteria grown in different nutrient media for 15 days at 25 ± 2 o C under continuous light of 2.789 w/m^2.

3.1.2. Growth assessment of different cyanobacteria on the modified Navicula medium

Growth curves of different ten species of cyanobacteria grown for 18 days in the selected modified *Navicula* medium are illustrated in the Figure (2) at the end of the incubation period (18 days). The cyanobacteria *Anabaena variabilis* maintained the highest growth (0.68 ± 0.08 gl^{-1}) and *Oscillatoria cortiana* exhibit the lowest growth (0.35±0.001 gl^{-1}). Growth curves were employed to calculate the specific growth rate and growth doublings per day (Table 1).

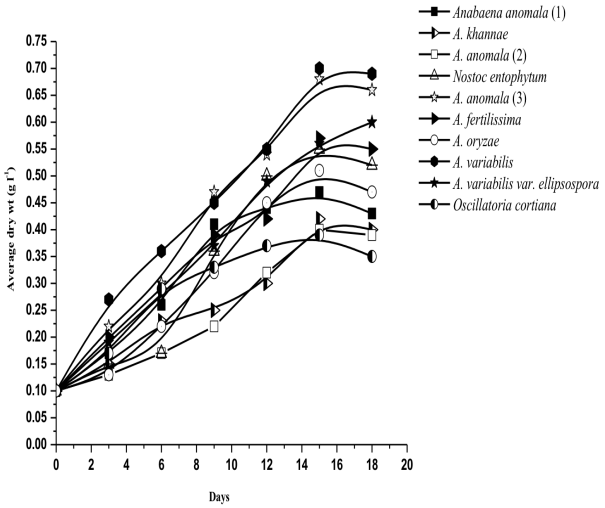

Fig. 2. Growth curves of different species of cyanobacteria grown in the modified Navicula medium

3.1.3. Growth rates of the tested cyanobacteria

In an attempt to get an accurate growth comparison of different tested cyanobacteria both the specific growth rate (μ) and growth doublings

(Dd^{-1}) were calculated. The results are listed in the Table 1.

It is evident that different tested cyanobacteria exhibit different specific growth rates and growth doublings. The specific growth rate ranged between a lowest value of 0.06 ± 0.003 (recorded for *Oscillatoria cortiana*) and a highest value of 0.11 ± 0.005 (recorded for *Anabaena variabilis*). The growth doublings (Dd^{-1}) fluctuated between 0.09 ± 0.002 (*Oscillatoria*) and 0.16 ± 0.007, (*Anabaena*).

Table 1. Specific growth rate (μ) and doublings per day (Dd^{-1}) of different cyanobacteria species grown on the modified Navicula growth medium

Cyanobacteria isolate	μ	Dd
Anabaena anomala (1)	0.081±0.01	0.117±0.014
Anabaena khannae	0.07±0.004	0.11±0.0061
Anabaena anomala (2)	0.075±0.003	0.11±0.005
Nostoc entophytum	0.1±0.003	0.13±0.0052
Anabaena variabilis	0.11±0.005	0.16±0.007
Anabaena fertilissima	0.094±0.009	0.136±0.013
Anabaen oryzae	0.085±0.014	0.124±0.016
Anabaena anomala (3)	0.104±0.003	0.15±0.005
Anabaena variabilis var. *ellipsospora*	0.099±0.005	0.143±0.007
Oscillatoria cortiana	0.06±0.003	0.09±0.002
Max.	0.11±0.005	0.16±0.007
Min.	0.06±0.003	0.09±0.002

3.2. Inhibition of acetylcholinesterase activity by tested cyanobacteria crude extracts

Effects of crude extracts of different tested cyanobacteria are listed in the Table 2. Only three extracts exhibited inhibitory effects on AChE. Crude extracts of *Anabaena anomala* (1), *Anabaena oryzae* and Anabaena variabilis inhibited AChE activity by 25%, 25% and 50%, respectively. Based on these results *Anabaena variabilis* methylene chloride: methanol crude extract maintained the highest percent of inhibition.

4. Discussion

The experimental results (Figure 1, 2 and Table 1) indicated that the modified *Navicula* nutrient medium supported the highest growth of the all tested cyanobacteria. It has been well documented (e.g Gong and Chen.,

Table 2. % of AChE inhibition in presence of (4 μg $μl^{-1}$) of tested cyanobacteria (methylene chloride/Methanol) crude extracts.

Sample	% of inhibition	Effect
Anabaena anomala (1)	25	Inhibitory
Anabaena khanne	--*	Stimulatory
Anabaena anomala (2)	--*	Stimulatory
Oscillatoria cortiana	--*	Stimulatory
Nostoc entophytum	--*	Stimulatory
Anabaena variabilis	50	Inhibitory
Anabaena fertilisma	--*	Stimulatory
Anabaen oryzae	25	Inhibitory
Anabaena anomala (3)	--*	Stimulatory
Anabaena varibilis var. *ellipsospora*	--*	Stimulatory

* These crude extracts increase the activity of AChE (stimulatory).

1997, Miller *et al.,* 1999 and Shay *et al.,* 1987) that the culture medium not only affects the microalgae growth but also affects their metabolic activities. Extensive studies (e.g Borowitzka, 2005, Domınguez-Bocanegra *et al.,* 2004 and Grobbelar, 2004) have proven that the composition of nutrient media affects not only biomass production but also the composition and yield of a specific metabolites. Accordingly, the marked increase in dry weight of all tested cyanobaceria grown in the modified Navicula medium could be largely attributed to the composition of this medium therefore, justified the selection of this medium for further growth experiments.

The effect of methylene chloride/ methanol extracts on AChE activity was either stimulatory or inhibitory (Table 2). The inhibition of AChE activity by three crude extracts may highlight the possible role of three tested cyanobacteria namely *Anabaena anomala* (1), *Anabaena oryzae* and *Anabaena variabilis* for the treatment of AD. Acetylcholinesterase inhibitors have been, and remain, the standard approach to the symptomatic treatment of AD. Ferreira et al. (2006), Konrath et *al*. (2012) and Mehta *et al*. (2012) stated that treatment of early and moderate Alzheimer disease has largely involved replacement of neurotransmitters that are known to be lacking, mostly based on AChE inhibition. In the United States, AChE inhibitors are the only approved pharmacologic approach shown to be effective in the management of AD (Grossberg, 2003).

Since the level of AChE is lower in certain regions of the AD brain due to the loss of presynaptic terminals, it is relatively higher within and around the infected regions (amyloid plaques) leads to break down of ACh (Siek *et al.,* 1990). Accordingly, AChE inhibition increase the concentration of acetylcholine in the synapse, this provides more acetylcholine to interact with the brain's cholinergic receptors (Kaduszkiewicz *et al.,* 2005). AChE inhibitors may be reversible (that block the enzyme for a short time), irreversible (long-time of action, they create long-lasting complexes with acetylcholinestrase) and quasi-irreversible (reversible but they show a very long time enzyme blockage) (Doucet-Personeni *et al.,* 2001).

It may be relevant to indicated that *Anabaena variabilis* maintained the highest AChE inhibition and, therefore may be a promising biomass resource of a variety of bioactive metabolites with possible role of Alzheimer treatment.

Accordingly, *Anabaena variabilis* will be in a focus of further research to reveal its potential as renewable bioresource of new neurochemicals with possible better management of AD.

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الملخص العربى

استبيان كفاءة بعض الطحالب الخضراء المزرقة فى معالجة مرض الزهايمر عن طريق تثبيط نشاط انزيم استيل كولين استريز معمليا

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مرض الزهايمر هو المسبب الأكثر شيوعا لضعف الذاكرة والنسيان في الشيخوخة. يعتبر تثبيط نشاط إنزيم استيل كولين استريز وهو الإنزيم المسئول عن تكسير الناقل العصبي استيل كولين ، أداه فاعلة فى علاج مرض الزهايمر. تعد الطحالب الخضراء المزرقة من المصادر الطبيعية الواعدة لمركبات كيمائية تشتمل على مثبطات لإنزيم استيل كولين استريز. تبعا لذلك تم عزل وتعريف وتنمية عشرة أنواع مختلفة من الطحالب الخضراء المزرقة على أوساط غذائية مختلفة، ولوحظ ان الوسط الغذائى *modified Navicula medium* حقق أعلى إنتاجية للطحالب المختاره. تم اختبار تاثير مستخلصات الميثيلين كلوريد /ميثانول المختلفة على تثبيط نشاط إنزيم استيل كولين استريز. وقد اتضح من النتائج ان خلاصة كلا من , *Anabaena variabilis, Anabaena oryza Anabaena anomala (1)* قد ثبطت نشاط انزيم استيل كولين استريز بنسب ٢٥٪، ٢٥٪ ، ٥٠٪ على التوالي. تبين من النتائج أن خلاصة طحلب *Anabaena variabilis* حققت أعلى نسبة تثبيط للإنزيم ولذلك يتعبر هذا الطحلب من الطحالب الواعده في علاج مرض الزهايمر.

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Screening of Anti-Alzheimer Potential of some Cyanobacteria (blue-green algae) Through in vitro Acetylcholinesterase Inhibition Assay

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