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Isolation and Structure Elucidation of Acetyl Cholinesterase Inhibitor from *Gyrostoma helianthus* of the Red Sea, Egypt

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Abstract: The present study was carried out to isolate, purify and elucidate the structure of low molecular weight bioactive compounds from sea anemone, *Gyrostoma helianthus* of the Red Sea environment. The obtained results indicated that the ethanolic crude extract of the sea anemone, *Gyrostoma helianthus* showed inhibition activity against acetylcholinesterase (AchE). The 0.5kD fraction inhibited the activity of AchE that indicated the presence of low molecular weight active compound(s) of less than 0.5kD. Two active fractions were obtained after BioGel P2 fractionation of the 0.5kD of the *Gyrostoma helianthus.* The first active fraction inhibited the activity of the acetylcholinesterase, while the other fraction was not. HPLC technique aided by semipreparative columns was used to isolate the target compounds in a pure form for the structure elucidation, one dimensional NMR analysis (^{1}H and ^{13}C-NMR) and DEPT were carried out to elucidate the structure of the isolated compound which tentatively identified as N,N'-bis-(1-methyl-pyridin-2-yl)-hydrazine.

Key words: Sea anemone · *Gyrostoma helianthus* · Red Sea · Structure Elucidation · Acetylcholinesterase (AchE) inhibitor

INTRODUCTION

The study of marine organisms as a source of biologically active compounds is considered a very productive field, having already led to the discovery of various new pharmacological tools and medicines [1-3].

The work of Bergman and Feeney at the beginning of the 1950s initiated the study of marine natural products and in the last few decades, an appreciable number of new compounds have been isolated from marine organisms [1, 3,4]

Many authors explained this ecological success on the basis of the ability of marine invertebrates to synthesize secondary metabolites, which have an important defensive role against predation [5].

It is increasingly realized that many marine toxins are very site specific in their actions and hence are of value as biological tools. As a result, there is an increasing exploration of toxins from marine sources, especially those from invertebrates [6]. Moreover, the very high potencies of some marine toxins are attained through strong, highly selective interactions, frequently with specific sites on excitable membranes. Investigating the mechanism of toxin action has revealed a great deal about the physiology of affected tissues and systems [7].

The improvement in isolation and chemical identification techniques, the collaboration between chemists and pharmacologists and the interest of pharmaceutical industries have been important determinants in the development of marine natural products research [8].

The search for novel low molecular weight natural products from marine organisms is of crucial importance for different applications as drugs and pesticides. Of special interest is the Red Sea environment, which is unique in its life forms and considered as one of the richest marine areas in bio-diversity and richer than similar areas in the eastern Pacific and Atlantic [9]. Therefore, the objective of the present study was to isolate, purify and elucidate the structure of low molecular weight bioactive

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compounds from sea anemone, *Gyrostoma helianthus* of the Red Sea environment using the bioassay guided fractionation technique.

MATERIALS AND METHODS

Organism under Study: Sea anemone, *Gyrstoma helianthus* was collected from the vicinity of Hurghada, Red Sea, Egypt.

Sampling Locations: Samples of sea anemone, *Gyrstoma helianthus* were collected from Hurghada vicinity at latitude from 27°00'N to 27°45'N and longitude from 33°30'E to 34°00'E. The locations of the sampling sites were as follows:

Shaab AbuShaar, Shaab AbuGalawa, Shaab AbuSadaf, ElFanadir Island, Gafton Kabier Island, Gafton Saghier Island, Dishet AbuMinqar, Magawish Kabier Island, Magawish Saghier Island.

Sample Preparation: Sea anemone, *Gyrstoma helianthus* samples were collected by trained divers working in the areas known to harbor the specific species under study. The collected samples were kept in seawater during the sampling trip around the vicinity of Hurghada. Upon arrival at the laboratory of Hurghada Marine Station, National Institute of Oceanography and Fisheries, the samples were washed with distilled water followed by gentle centrifugation to remove excess water. Samples were weighed and counted and the wet weight was recorded. Samples were also extracted in the laboratories of the Hurghada Marine Station of the National Institute of Oceanography and Fisheries.

Extraction of Bioactive Material Form Marine Organism: Samples were extracted using a modified technique of Gomaa *et al*. [10]. Washed intact marine organism samples were weighed and equal amounts of absolute ethanol were added (1:1 w/v) and blended for 3 minutes. The extracted samples were centrifuged at 2570 g for 10 minutes and the supernatant was removed and kept for further extraction. The residues were subjected to a second extraction with absolute ethanol and to a third extraction with 50% aqueous ethanol.

For defatting and partial depigmentation of sea anemone, *Gyrostoma helianthus* ethanolic crude extract, as a step of purification, the supernatants were mixed with an equal volume with n-hexane. Defatting with n-hexane was repeated for 3 times. The residues were also blended with equal amounts (w/v) of n-hexane. Both aqueous ethanolic and hexane extracts were evaporated under reduced pressure at 40 °C.

Inhibitory effect on Acetylcholinesterase (AchE): The *in-vitro* AchE inhibition activity of the ethanolic crude extract and the other fractions was determined according to Ellman *et al.* [11] using reagent kits purchased from Qunica Clinica Aplicada S.A., Spain.

Isolation, Purification and Identification of *G. helianthus* AchE Inhibitor: Fractionation and purification of the *Gyrostoma helianthus* ethanolic crude extract was carried out using different chromatographic techniques, as described in the following sections, followed the AchE inhibition activity guided fractionation protocol.

Molecular Weight Exclusion Ultrafiltration: Crude extract of *Gyrostoma helianthus* was filtered through membrane filters with cut off 10 k, 5 k, 3 k, 1 k and 0.5 k Dalton (76 mm in diameter, Millipore Corporation, Bedford, MA, USA). Ultrafiltration was performed under pressure of nitrogen gas (40 $Kg.cm^{-2}$).

Biogel P2 Gel Filtration: Gel filtration column chromatography was prepared using a 3.5 X 80 cm Omni LC column, packed with BioGel P2 (BioRad Laboratories, Richmond, CA, USA), to reach a bed height of 75 cm and a bed volume of 728 ml [12-15]. Fifteen ml of Milli Q water was used to redissolve 5g of the less than 0.5 k Dalton freeze-dried filtrate and then applied on the top of the BioGel P2 column. The column was eluted with 2 bed volumes of milli Q water using a peristaltic pump to provide a flow rate of 48 ml/hr and 5ml fractions were collected.

Buckley spot plate technique [13] was used to detect fluorescence and quenching activity of the collected fractions along with their reaction with ninhydrin. Aliquots of all the collected fractions, 5 µl each, were spotted on 10x10 cm silica gel TLC plates (precoated, type 60 F254, with fluorescent indicator, aluminum backed, E. Merck, Germany). Fluorescent and quenching activity was observed under long wave (366nm) and short wave (254nm) UV. Spot plates were sprayed with 1% ninhydrin in ethanol to detect the presence of the free α-amino groups (purple color) or the substituted α-amino group (yellow color) in the active fractions.

Active fractions, detected by *in-vitro* AchE, were compared with any physical property that may appear by Buckley spot test. Percent bed volume was calculated for each fraction to correlate between the position(s) of the active fractions with the calculated percent bed volume.

Table 1: Mobile phase gradient program used for isolation of AchE inhibitor

Time (min)	% Water	% Methanol	% Acetonitrile
0	5	10	85
5	15	5	80
10	30	0	70
15	5	10	85

HPLC: Different HPLC techniques were tested to compare between the active and nonactive fractions to locate the peak that may account for the detected activity (AchE inhibition). The HPLC system used was Perkin-Elmer series 200 pump system equipped with a Perkin-Elmer series 200 UV absorbance detector set at wavelength of 203, 254 and 365 nm. Different mobile and stationary phases were used to find out the best method for the separation of the toxic compounds. Data were collected and integrated with a Total chrom Navigator Chromatography Manager.

The analytical column chromatography, Spheri 5 silica (100 x 4.6 mm; 5µm) (Applied Biosystems Inc. Hoster City, A 94404 USA, Brownlee columns) was used to separate the AchE inhibitor. The mobile phase system (H_2O: MeOH: Acetonitrile) gradient program as shown in the Table (1), UV detector wavelength at 254nm and the flow rate was 1ml/min. Also. The semi preparative column hyperprep HS/Silica (250 x 10 mm; 12µm) Thermo Hypersil was used to obtain enough materials for the structure elucidation.

Structure Elucidation: 1D NMR (^{1}H and ^{13}C) analysis and EI-MS was carried out to elucidate the structure of the isolated bioactive compound.

RESULTS AND DISCUSSION

Organism under Study: To identify the collected sea anemone samples from the Red Sea, specimens of the collected sea anemone species were sent to Prof. Dan Hartog of the National Museum of the Netherlands who was assigned by the British Museum of the Natural History as the best expert in this field. According to Prof. Dan Hartog the scientific name of the species under study was confirmed as *Gyrostoma helianthus or Entacmaea quadricolor*.

Crude Extract Bioactivity: According to Gomaa *et al.* [10], the crude aqueous ethanolic extract of *Gyrostoma helianthus* when intra peritoneally (i.p.) injected in the 20 g male mice showed symptoms of neurotoxicity.

Table 2: *In-vitro* AchE inhibition activity of the crude extract and different ultrafiltration fractions of *Gyrostoma helianthus*

Sample	AchE Concentration U/L	% Inhibition of AchE activity
Control	4201.35	---
Crude extract	340.65	91.9
10 kDa fraction	2573.8	38.7
5 kDa fraction	3255.1	22.5
3 kDa fraction	3180.4	24.3
1 kDa fraction	3146.8	25.1
0.5kDa fraction	2384.0	43.3

Gomaa *et al.* [10] Also showed that the part of the neurotoxicity observed in the mouth assay was due to reversible inhibition of the AchE in the brain and blood of the mice, which was confirmed by Gomaa and Aboul-Enien [9] after using the *in-vitro* AchE assay. The same assay was used in this study to track the bioactive compound during the bioassay-guided fractionation technique. AchE inhibition activity was confirmed in this study in the ethanolic crude extract of sea anemone *Gyrostoma helianthus*. Although different reports showed that sea anemones produce two types of protein toxins: neurotoxins, which act mainly on ion channels [16] and cytolysins (or actinoporins), which exhibit lytic activity on a variety of cells [17-20]. However, the presence of AchE inhibitors in marine invertebrate has not been reported in the different extracts even in those that exhibit high neuroactivity.

Molecular Weight Exclusion Ache Inhibition Activity: In this study *Gyrostoma helianthus* crude extract showed 91.9% inhibition in the AchE activity while the MW exclusion fractions showed lower percent inhibitions (Table 2).

This table shows the effect of purification steps of *Gyrostoma helianthus* on the activity of AchE. The more the purification, from the crude extract until the less than 5kD filtrate, the less the inhibition percent of AchE which means the presence of more than one AchE inhibitors in the crude extract. The only previous report detecting the AchE inhibitor in the extract of *Gyrostoma helianthus* was that of [10, 11]. However, Mebs *et al*. [21] did not detect any AchE inhibitors in *Gyrostoma helianthus* collected from the Red Sea, the AchE inhibition activity detected in the less than 0.5kD filtrate indicated the presence of low MW active compound (less than 0.5kD).

Biogel P2 Fractions Bioactivity: Using Biogel P2 fractionation and Buckley spot plate, the two distinct chemically active groups reported by Gomaa and

Aboul-Enien [9] were confirmed. The first group (65% to 72% bed volume) showed quenching under short wave (254 nm) while the second (72% to 77% bed volume) showed fluorescence activity under long wave (366 nm).

Only one of these two active groups showed *in-vitro* AchE inhibition activity which confirmed the reported results obtained by Gomaa *et al*. [10], while the second group showed no inhibition activity toward AchE. The AchE inhibition activity was detected along the purification steps from the crude to the 0.5 kD filtrate until the first active group of the Biogel P2 fractions, While AchE inhibition activity was not detected in the second active group.

HPLC Fractions Activity: Different HPLC methods were tested to compare between the toxic (AchE inhibitor) and nontoxic fractions to locate the peak that may account for the detected AchE inhibition activity. The best result was obtained when the 254-nm wavelength was used. The first active group, after Biogel P2 fractionation, that showed the inhibition of AchE was tested by the HPLC technique to define the peak responsible for such activity. AchE inhibition and non-inhibition fractions were injected in the HPLC using a spheri 5 silica column (100 x 4.6 mm; 5µm). Different mobile phase systems were used and the best resolution was achieved using a mixture of H_2O: Methanol: Acetonitrile in a gradient program. The peak responsible for AchE inhibition was observed at 8.5 minutes retention time. The active fractions of the Biogel P2 have a higher peak area at this retention time while the non-active fractions before and after these active ones showed no peaks or a very small one. Also the peak areas were proportionally related to the activity level.

HPLC Purification of AchE inhibitor: The HPLC technique described in the current study aided by semipreparative columns was used to isolate the target compounds in a pure form for the structure elucidation. Thus, the present study may be a step towards the discovery of novel compounds from the sea anemone *Gyrostoma helianthus.*

Figs (1-4) illustrate the HPLC chromatograms of the different purification steps of *Gyrostoma helianthus* ethanolic crude extract to isolate the AchE inhibitor compound through ultrafiltration steps reaching less than 0.5kDa filtrate to the Biogel filtration and finally to obtain the pure bioactive compound from the HPLC aided by semi-preparative column. The percent of the peak area of the desired AchE inhibitor bioactive compound in the HPLC chromatograms were 29.9, 51.6, 75.8 and 98% in the crude extract, 0.5kDa filtrate, AchE inhibition fraction after Biogel P2 and the isolated pure toxin after HPLC, respectively.

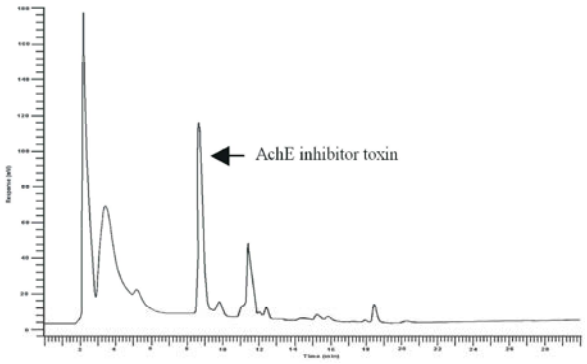

Fig. 1: HPLC chromatogram of the sea anemone, *Gyrostoma helianthus* ethanolic crude extract.

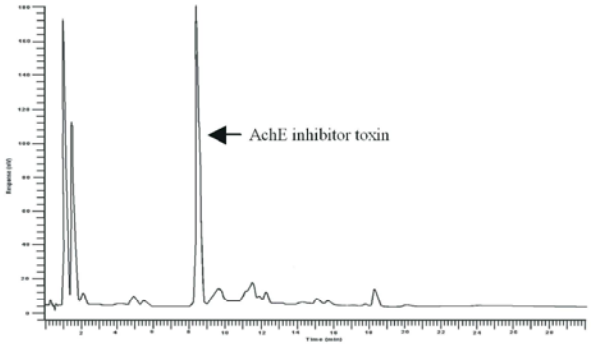

Fig. 2: HPLC chromatogram of the less than 0.5kD ultrafiltration fraction of sea anemone*, Gyrostoma helianthus* ethanolic crude extract.

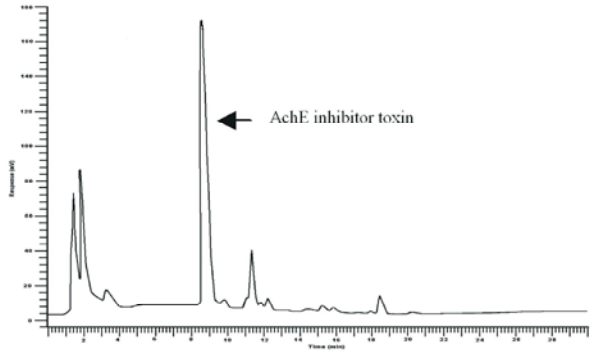

Fig. 3: HPLC chromatogram of toxic fraction after BioGel P2 of the less than 0.5kD ultrafiltration fraction of sea anemone, *Gyrostoma helianthus.*

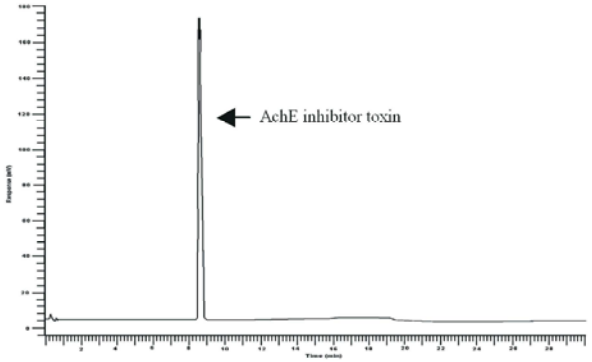

Fig. 4: HPLC chromatogarm of the pure isolated sea anemone, *Gyrostoma helianthus*, AchE inhibitor

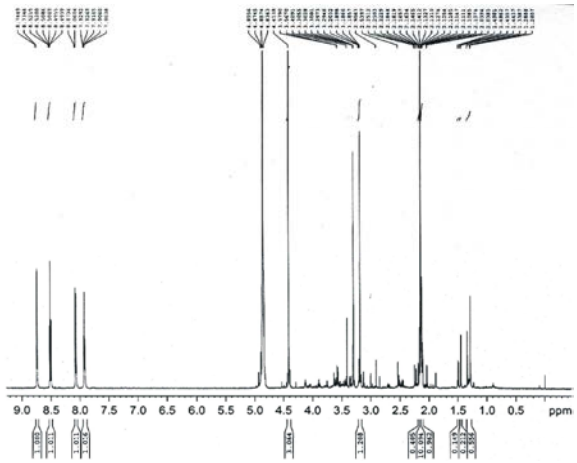

Fig. 5: ^{1}H-NMR spectrum of the pure isolated sea anemone, *Gyrostoma helianthus*, AchE inhibitor

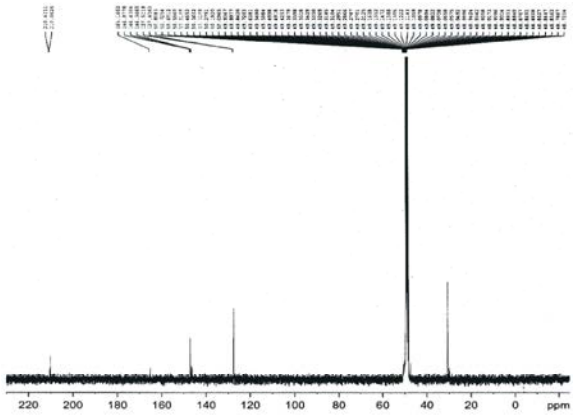

Fig 6: ^{13}C-NMR spectrum of the pure isolated sea anemone, *Gyrostoma helianthus*, AchE inhibitor

Structure Elucidation of AchE inhibitor: Further purification on a semi-preparative HPLC led to the isolation of one major constituent in a pure form

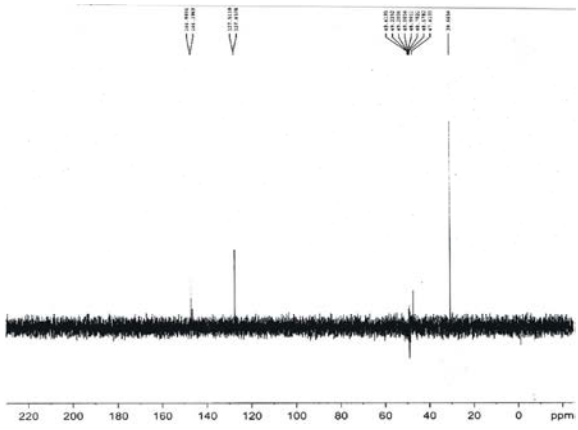

Fig. 7: DEPT spectrum of the pure isolated sea anemone, *Gyrostoma helianthus*, AchE inhibitor

the first active fraction after Biogel P2. The purity of the isolated bioactive compound was examined by TLC and HPLC where it gave only one spot on TLC and one peak in the HPLC chromatogram (Figs. 4). One dimensional NMR analysis ^{1}H, ^{13}C-NMR and DEPT were carried out to elucidate the structure of the AchE inhibitor isolated from the sea anemone, *Gyrostoma helianthus*, (Fig. 5-7).

^{1}H NMR (600MHz, MeOH-d_4/Acetone-d_6) (Fig 5): δ ppm

8.74 (2H, *br.*, J = 6.2 Hz, H-6/6'), 8.51 (2H, *ddd=td*, J = 7.8, 1.0Hz, H-4/4'), 8.07 (2H, *br.*, J = 7.8 Hz, H-3/3'), 7.92 (2H, *ddd*=td, J = 6.2, 1.3 Hz, H-5/5'), 4.41 (6H, *s*, 2xN-Me)

^{13}C NMR (150 MHz, MeOH-d_4/ Acetone-d_6) (Fig 6): δ ppm 165.16 (C-2/2'), 146.98 (C-4/4'), 146.43 (C-6/6'), 127.57 (C-5/5'), 127.46 (C-3/3'), 47.41 (2xN-CH_3).

EI. MS, *m/z* (%) (Fig. 8): 186.0 [M-C_2H_6]$^+$, (40%); 171.0 [M-C_2H_7N]$^+$, (90%); 94.0 [M-$C_7H_{10}N_2$]$^+$, (100%); 79.0 [M-$C_7H_{11}N_3$]$^+$, (23%); 52.0 [M-$C_9H_{14}N_3$]$^+$, (23%)

As it was interpreted above the isolated compound was expected to be 2-subsitituted pyridine-like structure on the basis of its splitting pattern, δ- and J-values in the ^{1}H NMR data (Fig. 5). The presence of N-CH_3 functionality was deduced from the singlet at 4.41 ppm. Depending on its M.S fragments (Fig. 8), the isolated bioactive compound was tentatively identified as a symmetric dimer of 2-amino-N-methylpyridine. The base peak (100%) at *m/z* 94.0 mu was confirmative evidence for the homolytic cleavage of N-N bond.

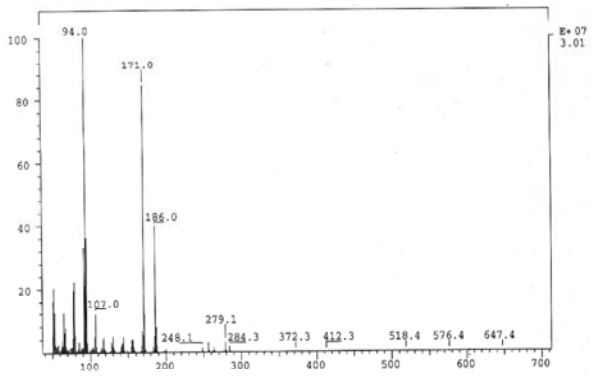

Fig. 8: Mass spectrum of the pure isolated sea anemone, *Gyrostoma helianthus*, AchE inhibitor

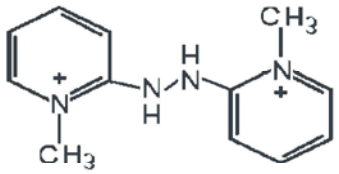

Fig. 9: Chemical structure of the AchE inhibitor compound isolated from Sea anemone, *Gyrostoma helianthus*

The fragment was diagnostic for the aminopyridine structure, which was followed by loss of 15 mu of NH to give a relatively weak abundant ion at *m/z* 79.0 (23%). In its ^{13}C-NMR spectrum (Fig. 6), five characteristic carbon resonances were assigned to two pyridine moieties together with an aliphatic C-resonance at 47.41 of two N-CH_3 groups. Large down field shift (+16 ppm) of the C-2 to 165.16 ($\simeq$149 in pyridine) was indicative for the attachment of amino group to C-2 and accumulation of positive charge on the ring nitrogen.

All ^{13}C-resonances were assigned by aid of increment subsistent additive rule and comparison with structure related compounds [22]. The appearance of only four ^{1}H-resonances in the aromatic together with the aliphatic (CH_3) regions, respectively and six ^{13}C-resonances (each of two equivalent carbons), was indicative of an asymmetric bis-(2-amino-N-methylpyridine) structure. Finally, DEPT spectrum (Fig. 7) confirmed the suggested structure of the AchE inhibitor toxin as N,N'-bis-(1-methyl-pyridin-2-yl)-hydrazine (Fig. 9) through the differentiation among C, CH and CH_3 resonances.

CONCLUSION

The study of marine organisms as a source of biologically active compounds is considered a very productive field, having already led to the discovery of various new pharmacological tools and medicines. The inhibition activity toward acetylcholinesterase (AchE) that detected in crude extract of the sea anemone, *Gyrostoma helianthus* and in the 0.5kD fraction indicates a presence of low molecular weight active compound of less than 0.5kD, this hypothesis coincided with the calculated molecular weight (216 D) after the structure elucidation of the isolated compound using mass spectrometer, NMR analysis (^{1}H and ^{13}C-NMR) and DEPT. The isolation of a low molecular weight AchE inhibitor compound in a pure form with known chemical structure will be a step forward to discover a new drug.

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