Damanhour Journal of Veterinary Sciences 9 (1), (2023) 29-34

Damanhour Journal of Veterinary Sciences

Journal homepage: https://djvs.journals.ekb.eg/

E-ISSN 2636-3003 | ISSN 2636-3011

Sea cucumber (*Holothuria atra*) ethyl acetate extract exerts anticholinesterase properties

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Abastract

Alzheimer's disease (AD) is characterized by hyperactivations of acetylcholinesterase (AChE) associated with neuroinflammation. Sea cucumber has anti-inflammatory, immunostimulant, and anticancer properties. It is considered a good nutraceutical candidate with low toxicity and high efficiency. This study assessed the activity of well-characterized sea cucumber- ethyl acetate crude extract toward AChE *in vitro*. Sea cucumber (*Holothuria atra*) samples collected from Hurghada were extracted by the maceration method using ethyl-acetate (99%), making a 0.12% yield. Furthermore, the extract phytochemical ingredients were identified using GC-MS. AChE enzyme was purified from male Egyptian Mediterranean bull brain tissue using ammonium sulfate precipitation and gel filtration (Sephadex G-100). The purification profile was assessed by polyacrylamide gel electrophoresis. The inhibitory effect of the *H. atra* crude extract toward AChE was assessed colorimetric, and the kinetics profile was studied to identify the inhibition type by calculating Km and Vmax. Finally, *in vitro* cytotoxicity profile of this extract toward WI38 cells was estimated. The results revealed that the AChE was properly purified as the purification fold was increased by 27 times. Ethyl acetate crude extract demonstrated an uncompetitive AChE inhibition profile with an IC_{50} value of 59.07 µg/ml toward WI38 cells**.** The results of the present investigation support the hypothesis that the crude ethyl-acetate extract of sea cucumber has anti-AChE properties.

Keywords: Anti-'Alzheimer's; Acetylcholinesterase inhibitors; lolly fish; cytotoxicity; GC-MS

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Email: dr_hazemshaheen3010@gmail.com
P ISSN: 2636-3003
EISSN: 2636-2996
DOI: 10.21608/djvs.2022.156975.1089
Received: August 18, 2022; Received in revised form: September 05, 2022; accepted: September 06, 2022
Editor-in-Chief:
Prof Dr/Ali H. El-Far (ali.elfar@damanhour.edu.eg)

1. Introduction

'Alzheimer's disease (AD) is a neurological illness that causes memory loss and cognitive deficits over time. It is characterized by low levels of acetylcholine (Ach) in 'patients' brains that indicate the high activity of acetylcholinesterase (AChE). This enzyme is responsible for AChE degradation, or low activity of acetylcholine transferase, which is responsible for AchE formation (Nasir et al. 2015, Abdel-Latif et al. 2019). According to the cholinergic hypothesis, suppression of AChE by using AChE inhibitors (AChEi) raises acetylcholine levels in the brain, increasing the cholinergic function in 'Alzheimer's disease patients (Murray et al. 2013).

To date, only four AChEi drugs have been approved by regulatory agencies for the treatment of AD: tacrine, rivastigmine, galantamine, and donepezil. Although these substances have a wide variety of negative effects, they are all suggested as viable treatments for people with mild to severe AD who are experiencing cognitive deterioration. For instance, 30% of those on these medications experience symptoms like diarrhea, vomiting, and upset stomach (Murray et al. 2013).

Over 70% of the 'Earth's surface is covered by the ocean, which is thought to be a source of organisms that can produce bioactive secondary metabolites with a distinctive structure, also known as marine natural products (MNPs). The sea cucumber is one of the most potential marine invertebrates consider rich sources of natural products and many biological activities, including anti-inflammatory effects. It belongs to the phylum 'Echinodermata's *Holothuroidea* and is a member of that group. The water is home to a kind of critters that resemble cucumbers. 66 species of this group of *Holothuroidea* that are designated sea cucumbers out of an estimated 1200 species worldwide (Abuzaid et al. 2020). The medical potency of sea cucumber saponins demonstrates a wide range of health benefits due to their cardiovascular, anti-diabetic, hypoglycemia, antioxidant, anti-asthma, anti-eczema, anti-inflammatory, anti-arthritic, anti-diabetic, cholesterol-lowering effect, immunomodulator, cytotoxic, anti-parasitic, anti-viral, antifungal, anticancer, anti-angiogenesis (Bahrami et al. 2018). The current study aimed to isolate and identify the *Holothuria atra* ethyl-acetate crude extract and estimate its activity toward the AChE enzyme.

2. Material and methods

2.1. Sample collection and identification

H. atra samples (Length 10 - 30 cm and total weight 300 - 700 g) were collected from Hurghada coastal along the Red Sea (27.28°N; 33.77°E), Egypt, in March 2021. The samples were identified under a microscope at the National Institute of Oceanography and Fisheries in Egypt based on their morphological features and taxonomic references. The acquired samples were cleaned from the sand, placed in an icebox, and then transferred to the laboratory, where they were maintained at -20°C until use (Dhinakaran and Lipton 2014).

2.2. Sample preparation and extraction

H. atra samples that had been frozen were cleaned with tap water and allowed to dry for three days at room temperature in the shade. The dried samples were cut into approximately 2-4 cm^3 and then pulverized in a mixer grinder. The extraction was conducted three times by 48 hrs. maceration using 99% Ethyl-acetate (1:4 w/v)

followed by filtration through Whatman filter paper (No.1) at room temperature. The extract was concentrated under reduced pressure at 40°C using a rotary evaporator to remove the ethyl-acetate. The residual sample was freeze-dried to remove water (Tagami et al. 2019), and the obtained yield was 0.12 %.

2.3. Screening of compounds by thin layer chromatography (TLC)

TLC was performed using aluminum plates impregnated with silica gel 60 F254(20x20 cm) for the crude extract of *H. atra*. The formed spots were discovered with the help of an ultraviolet light detector lamp operating at 254 nm. This lamp was used to find ultraviolet-active chemicals that have an unsaturation system (double bonds or benzene ring). To detect additional chemicals, sulfuric acid (10%) was poured onto the TLC and heated on a hot plate (Hoque 2019).. Retardation Factor (Rf) value, which is the ratio of the 'solute's distance traveled to the 'solvent's distance, was calculated as the following equation:

$$RF = \frac{Distance\ of\ the\ sample\ (solute) from\ the\ origin}{Distance\ of\ the\ solvent\ from\ origin}$$

2.4. GC-MS analysis of ethyl-acetate crude extract

GC-MS (Make: Fisons GC8000 series and MS:md800) was used to examine the *H. atra* ethyl-acetate extract. 30 mm, 0.25 mm, and 0.5 mm AB-35MS fused silica capillary columns made up the GC 'column's dimensions. The GC conditions were as follows: Column temperature is expected to be isothermal at 100 °C, rise to 250 °C at 6 °C/min, and remain there for 10 min. 250 °C is the injector temperature. The ion source was 200 °C warmer than the contact, which was 250 °C. The carrier gas was helium gas at a rate of one milliliter per minute. The ionization energy of 70 eV in the EI mode was used to acquire the spectra. The compounds were discovered by comparing the mass spectra with a NIST internal library (Stonik et al. 1998).

2.5. AChE enzyme purification

A male Egyptian Mediterranean Bull 'calf's fresh brain tissue was harvested and rinsed in ice-cold normal saline immediately after scarification. The brain tissue was cut into smaller pieces and stored in the refrigerator for three days under ice-cold hexane. The brain tissue was washed again with ice-cold phosphate buffer (pH 7.6, 0.1 M), containing NaCl (0.2M) and Na_2EDTA (0.001M). Four volumes of cold, 0.1 M phosphate buffer (pH 7.6) containing NaCl (0.2 M), Na2 EDTA (0.001 M), a 5 mM protease inhibitor cocktail, and 0.5% triton X-100 were used to homogenize the 380 g of brain tissue using a homogenizer, followed by centrifugation at 4000 rpm for 30 minutes at 4 °C. (Thermo Fisher Scientific, Germany). Finally, The clear supernatant was collected and used to make a crude version of AChE (F2)(Rao and Dasgupta 1991, Ghareeb et al. 2010).

This 'supernatant's total volume was measured, and the Protein content was determined according to the Protein biuret method kit Biodiagnostic Co. (Dokki, Giza, Egypt). The partial enzyme purification by using ammonium sulfate precipitation at 50% saturation (252 g), gel filtration by using Sephadex G50, and finally, the measurement of purity and approximate molecular weight by using sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) were performed as described by Ghareeb et al (2010).

2.6. Determination of acetylcholinesterase activity

130 mL of phosphate buffer (pH 8.0) was poured directly into the ELISA activity wells, and 140 mL was put into the ELISA blank well of an ELISA plate (Bio Tec. USA). 5 ml of the substrate ACTI (75 mM) was applied to the blank and activity wells, and 10 ml of the enzyme was then administered to the activity ELISA wells only. Before adding the second substrate, 0.32 mM DTNB (10 L), to the blank and active wells, the plate was preincubated for 15 min at 37 ºC. The ELISA reader recorded absorbance at 450 nm (Bio-Tek instruments). Analysis of the values obtained involved subtracting blank readings from sample readings (Ellman et al. 1961). The enzyme-specific activity in moles of substrate hydrolyzed / minute/mg of protein was calculated according to the following equation:

$$\frac{(\text{change in O.D. per minute}) \times Total\ volume\ in\ cuvette(\mu L)}{molar\ extinction\ coefficient\ of\ DTNB\ (1.36\ X\ 10e4) \times volume\ of\ homogenate(\mu L) \times \text{protien concentration}}$$

2.7. The in-vitro effect of different concentrations of H. atra ethyl-acetate extract on purified AChE enzyme activity

Eight concentrations of previously prepared *H. atra* ethyl-acetate extract (10, 5, 2.5, 1.5, 1, 0.75, 0.5, and 0.1 mg/mL of DMSO) and seven concentrations of reference compound (Donepezil hydrochloride) 5, 2.5, 1.5, 1, 0.75, 0.5, and 0.1 mg/ml in $d.H_2O$ were prepared. Then 10 µL of each concentration was separately incubated with 10 µL purified enzyme and incubated for 45 minutes then the enzyme activity was carried out as described in section 2.8 (Liu et al. 2021). The inhibition rate was calculated by subtracting the activity in the presence of the tested compound (At) from the total enzyme activity (Ac) then the result was divided over Ac and multiplied by 100.

2.8. The Kinetic Parameters of AChE Determination

Km and Vmax of the purified AChE were estimated at five different substrates (ACTI) concentrations (1, 2, 3, 4, and 5 µM) the presence and absence of certain concentrations of tested compounds 0.1, 0.5, and 0.75 mg/ml (ethyl acetate crude extract and donepezil hydrochloride), using a Lineweaver-Burk plot (Lineweaver and Burk 1934). All kinetic studies were performed at 25°C and optimal pH 8.0.

2.9. Cytotoxicity effect of H. atra ethyl acetate crude extract on Human fetal lung fibroblasts (WI-38) cell line

Human lung fibroblast (WI-38) was seeded in a flat bottom 96-well plate (Corning, USA) at a density of 1×10^4 cell/mL (100 µL) in high glucose DMEM culture medium (Biosera, France) supplemented with 10% fetal bovine serum albumin (Sigma-Aldrich, USA) and 1% Penicillin streptomycin (Sigma Aldrich, USA). Cells were incubated at 37 °C in humidified 5% CO_2 incubator (Eppendorf, Germany) and were allowed to attach for 18 hours before treatment. After complete attachment, the drug was added in triplicates on cells and left for 48 hours under the previously mentioned culture conditions. After 48 hours of incubation, media over the cells were removed and replaced with fresh media (100 µL/well) containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent with a final concentration of (0.5 mg/mL) and cells were replaced in the incubator. After 4 hours of incubation, media over the cells were removed, and DMSO (Merck, Germany) was added (100 µL/well) then plates were placed on a plate shaker for 15 minutes, then the absorbance was measured at 570 nm using a Microplate reader (AccuReader M965+, Metertech, Taiwan) (Mitra et al. 2016).

2.10. Statistical analysis

Data were presented as mean ± SD (standard deviation). Results were analyzed statistically by one-way analysis of variance (ANOVA) and T- test using (IBM SPSS Statistics) software version 26. Significant means were compared by Duncan *post-hoc* multiple comparison test. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. Screening of compounds by thin layer chromatography (TLC)

Thin layer chromatography (TLC) demonstrated the presence of several spots indicating the presence of terpenoids and steroids compounds with different RF values. There was a single spot-on TLC detected when using a mobile phase consisting of (20 % methanol: 80 % chloroform). While there five spots appeared when (100% chloroform) was used as a mobile phase as shown in **Table 1**.

Table 1. RF values of *H. atra* ethyl acetate extract as detected by TLC

	RF (20%methanol:80%chloroform)	***RF (100%chloroform)***
***H. atra* extract**	0.93	0.94
		0.88
		0.56
		0.28
		0.09

3.2. GC-MS analysis of ethyl-acetate crude extract

Ethyl acetate extract was found to contain fatty acids and fatty acid esters, including, Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*, R*)], n-Hexadecanoic acid, oleic acid, and octadecanoic acid as shown in **Table 2** and **Figure 1**.

3.3. AChE enzyme purification:

The purification steps of AChE show that ammonium sulfate precipitation decreased the enzyme yield by 82.53% and increased the purification fold by 5.65. In comparison, the gel filtration step increased the purification fold to 27.6 with an enzyme yield of 81.6%. Fraction (2) contains the lower protein bands number as indicated in **Table 3** and **Figure 2**.

3.4. The in-vitro effect of different concentrations of H. atra ethyl-acetate extract on purified AChE enzyme activity:

The purified AChE enzyme inhibition rate was directly proportional to the concentration of ethyl acetate crude extract up to 0.75 mg/ml and indirectly proportional to concentrations ranging from 1 to 10 mg/ml. In the case of Donepezil hydrochloride, the inhibition rate was directly proportional to drug concentration, with the maximum inhibition rate recorded at a concentration of 5 mg/ml, as indicated in **Table 4**.

3.5. The AChE Kinetic Parameters determination

The tested crude extract acted as an uncompetitive inhibitor as it decreased both Km and Vmax than that of the control purified enzyme. The calculated ki (non-competitive) was 0.0177 µg/ml and ki (competitive) was 0.00529 µg/ml, as shown in **Table 5**.

3.6. Cytotoxicity effect of H. atra ethyl acetate crude extract on (WI-38) cell line

Table 6 shows that the cell viability was indirectly proportional to extract and Donepezil hydrochloride concentrations' gradients. The calculated IC_{50} of the extract was 59.07 mg/mL, while IC_{50} for Donepezil hydrochloride was 56.51 mg/mL indicating their close toxicity profiles.

Table 2. Compounds identified using GC-MS of the ethyl-acetate extract

No	Compound name	Compound formula	R-time	Molecular weight	Area	Height
1	2-Butanone, 4-hydroxy	$C_4H_8O_2$	3.069	88	4222066	667066
2	Acetic acid	$C_2H_4O_2$	3.219	60	6380480	1322841
3	Butanedioic acid, 2,3-bis(acetyloxy)-, [R-(R*, R*)]-	$C_2H_4O_2$	3.247	60	1974787	1389993
4	1-Chloroheptane	$C_7H_{15}Cl$	6.534	134	251734	43693
5	2H-Pyran-3,4,5-triol, tetrahydro-2-methoxy-6-methyl-	$C_7H_{14}O_5$	11.754	178	129482	33642
6	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	15.507	256	179987	112500
7	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E, E, E)-	$C_{57}H_{104}O_6$	15.666	884	524626	39797
8	9-Octadecenoicacid, methyl ester	$C_{19}H_{36}O_2$	16.600	296	57044	15757
9	Oleic Acid	$C_{18}H_{34}O_2$	16.978	282	621024	209717
10	Octadecanoic acid	$C_{18}H_{36}O_2$	17.145	284	69872	26833

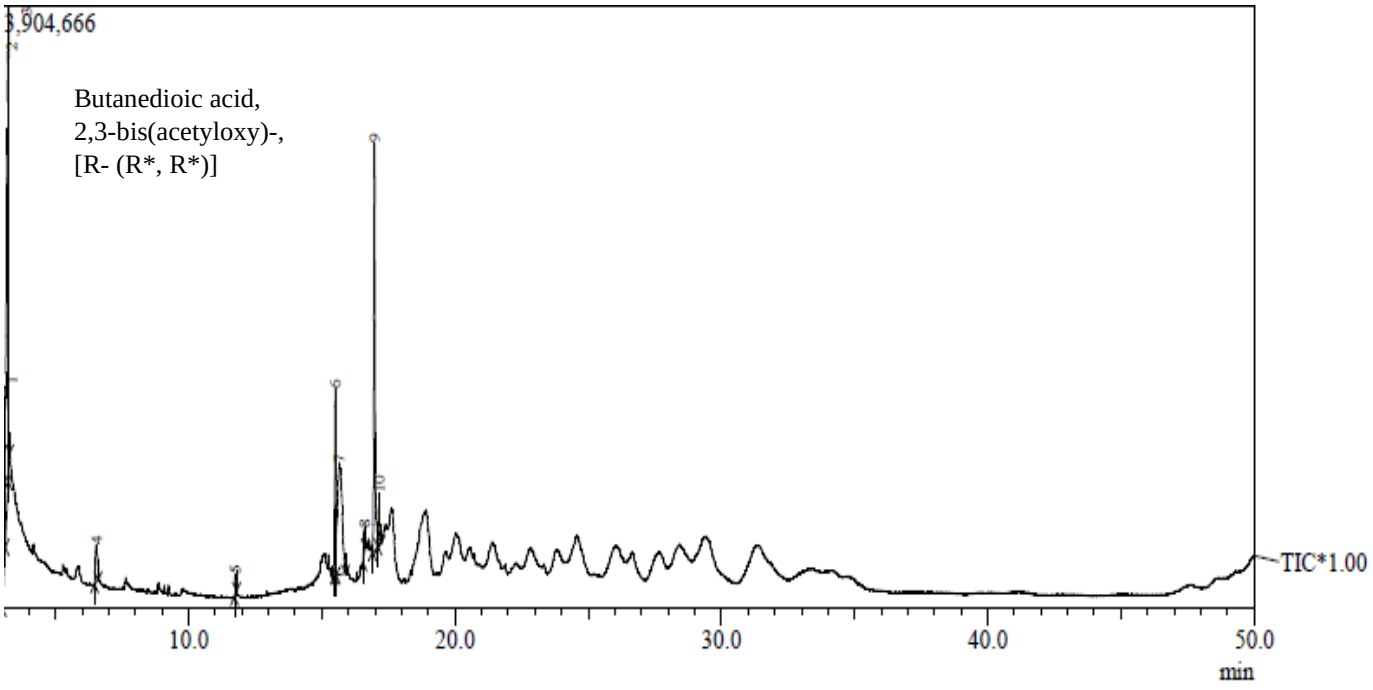

Figure 1. Compounds identified using GC-MS of the ethyl-acetate extract

Table 3. Purification of acetylcholinesterase (AChE)

	Crude Homogenate	Fraction (1)	Fraction (2) (purified enzyme)
Volume(ml)	846	225	135
AChE enzyme activity (U/ml)	0.00062	0.00191	0.00315
Total enzyme activity (U)	0.52	0.429	0.4245
Protein(mg/ml)	205	112.5	37.9
Total protein (mg)	173430	24187.5	5116.5
Specific activity (U/mg)	0.3×10^{-5}	1.69×10^{-5}	8.30×10^{-5}
Yield (%)	100	82.53	81.64836
Purification fold	1	5.65	27.68

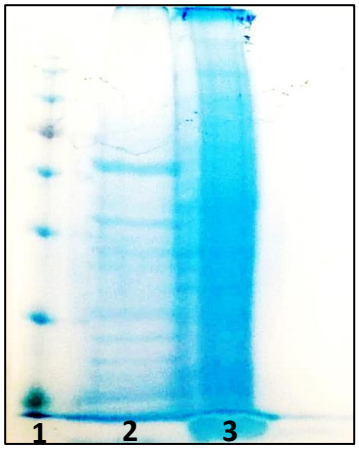

Figure 2. Electrophoretic behavior of acetylcholinesterase enzyme during different purification steps using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) where lane 1: protein ladder; lane 2: fraction 2; lane 3: fraction 1.

Table 4. The *in-vitro* effect of different concentrations of *H. atra* ethyl-acetate crude extract on purified AChE enzyme activity

Concentrations (mg/ml)	Ethyl acetate crude extract	Donepezil hydrochloride
	The rate of inhibition%	
0.1	71.94±0.03[a]	55±0.02[b]
0.5	74.30±0.04[a]	67.5±0.06[b]
0.75	102.08±0.05[a]	68.89±0.05[b]
1	70.83±0.03[a]	70.97±0.01[a]
1.5	68.47±0.05[b]	74.03±0.04[a]
2.5	66.25±0.01[b]	77.92±0.03[a]
5	62.78±0.06[b]	81.11±0.04[a]
10	53.75±0.23[c]	

The mean and SD of three replicates make up the reported data. Means in the same columns followed by different lower-case letters are significantly different ($p<0.05$). Means with similar letters are not significantly different at ($p<0.05$), letter a represents the highest value**.**

Table 5. AChE kinetic parameters in the presence of ethyl acetate crude extract and Donepezil hydrochloride

	Control	Donepezil hydrochloride	Ethyl-acetate crude extract
Type of inhibition	-	Uncompetitive	Uncompetitive
Km	164.7±15.46[a]	5.04±3.49[b]	1.77±0.49[c]
Vmax	3.2±0.29[a]	0.07±0.028[c]	0.15±0.080[b]
Ki_{comp} (µg/ml)	-	0.018371489	0.005297508
$Ki_{non\text{-}comp}$(µg/ml)	-	0.008011562	0.017726357

The mean and SD of three replicates make up the reported data. Means in the same column followed by different lowercase letters are significantly different (p <0.05). Means with similar letters are not significantly different at (p < 0.05). The mean with letter (a) was the highest value.

Table 6. The WI-38 viability (%) at different concentrations of extract and reference drug

Concentration (mg/ml)	Cell viability %	
	Ethyl-acetate extract	Donepezil hydrochloride
1000	5±0.47	5±0.47
500	6±0.13	6±0.13
250	9±0.86	9±0.86
125	14±0.22	53±0.49
63	48±0.73	63±0.52
31	79±0.51	80±0.78
IC_{50} value (µg/ml)	**59.07±0.38[a]**	**56.51±0.08[b]**

The mean and SD of three replicates make up the reported data. Means in the same row followed by different lowercase letters are significantly different at p <0.05. the mean with litter a was the highest value.

4. Discussion

Acetylcholine (ACh), the major neurotransmitter responsible for learning abilities and memory, was decreased due to AChE enzyme hyperactivation in the brain leading to memory deficits. Therefore, the inhibition of AChE is a critical strategy used to increase the brain's ACh level, thus improving cholinergic functions in AD patients (Murray et al. 2013). The currently available AChE inhibitors (tacrine, donepezil, rivastigmine, and galantamine) can only relieve the AD symptoms but not the progression of the disease. These drugs have limited effects and several harmful side effects (Chopra et al. 2011).

Natural products derived AChEIs have taken scientific attention due to their diversity, efficiency, safety, and acting as a multi-targeting candidate (Ayaz et al. 2018, Taqui et al. 2022). Despite this, sea cucumber has several biological activities due to the presence of different bioactive compounds (Nursid et al. 2019). There is a lack of studies on sea 'cucumbers' anti-Alzheimer's effect (Xu et al. 2018).

In the current investigation, after extracting the powdered *H. atra* crude extract with ethyl acetate, TLC was used to screen the existence of steroid and terpenoid compounds in *H. atra* ethyl-acetate crude extract, to be used in the biological activity explanation. The RF values obtained showed that this extract contains extremely nonpolar compounds that were soluble and fractionated by a nonpolar mobile phase, chloroform, This result was similar to those (Nishikawa et al. 2015).

To examine a substance's effect on enzyme kinetics, the enzyme must be investigated in its purest form; because of lowering the environmental impurities and high desired quantity and quality (Khattab et al. 1994). AChE was purified from the brain tissue in our study using the technique described by (Ghareeb et al. 2010). The final stage of purification increased the specific activity of brain AChE. approximately 27-fold. This outcome resembled those in some ways (Ghareeb et al. 2010)

The GC-MS analysis of the sea cucumber ethyl acetate extract revealed the presence of ten natural compounds, mainly fatty acids and fatty acid esters, including oleic acid, octadecanoic acid, n-Hexadecanoic acid, and 9-octadecanoic acid methyl ester. The findings of this study were in agreement with those of (Muflihunna et al. 2021) and (Dhinakaran and Lipton 2014), who reported the presence of esters such as 9-Octadecenoic acid (Z)-, methyl ester; Hexadecanoic acid, methyl ester; Octadecanoic acid, methyl ester; and 9,12-Octadecadienoic acid (Z, Z)-, methyl ester in *H. atra* ethanolic extract.

The essential oils contained in *Salvia potentillifolia*'s ethanolic extract demonstrate remarkable *invitro* cholinesterase inhibitory action (Syad et al. 2012). Fatty acids and oils of marine origin improve the memory of AD patients by reducing oxidative stress and increasing neurogenesis (Khunt et al. 2020).

Most research has found that oleic acid and the most abundant dietary fatty acids are deemed protective against AD (Kao et al. 2020) and (Hafez Ghoran and Kijjoa 2021). Therefore, the presence of fatty acids and esters in this extract could be attributed to the AChE inhibitory activity.

Our study demonstrated that the ethyl acetate crude extract exhibited an uncompetitive inhibition type where it decreased Km and Vmax compared with control purified enzyme values. These findings agreed with the results of Syad et al (2012), who presented the benzene extract of *Gelidiella acerosa* and found that the type of inhibition involved was uncompetitive toward butyrylcholinesterase.

The *in-vitro* cytotoxicity results of the extract against the WI-38 cell line revealed that the ethyl-acetate extract is safer, with an IC_{50} value of 59.07 µg/ml. In comparison with the reference drug (donepezil), its IC_{50} at the same concentrations was 56.51 µg/ml, indicating similar toxicity. These results are similar to those of Nursid et al (2019) on different cell lines as anticancer agents where, the IC50 values for the ethanol extract of H. atra against T47D, MCF7, WiDr, and HeLa cells were 9.6, 14.3, 11.4, and 10.4 g/ml, respectively.

5. Conclusion

In the current study, *H. atra* ethyl acetate extract demonstrated an uncompetitive profile like donepezil hydrochloride but, its Ki $_{Competitive}$ was lower than donepezil hydrochloride by 3.4-fold. At the same time, its Ki as a non-competitive inhibitor was higher than the donepezil hydrochloride by 2.1-fold, which indicated that this crude extract was more potent than the reference drug (donepezil hydrochloride). Both extract and donepezil hydrochloride showed the same safety pattern on WI-38 (normal cell line).

Author contributions

All authors contributed to the conception and realization of the work. All the authors have contributed to the paper redaction and approved the final version of the manuscript.

Conflict of interests: There are no conflicts of interest stated by the authors.

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