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In-vitro screening of acetylcholinesterase inhibitory activity of extracts from Palestinian indigenous flora in relation to the treatment of Alzheimer's disease

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ABSTRACT:

Background: Cholinesterase inhibitory therapy serves as a strategy for the treatment of Alzheimer's disease (AD). Several acetylcholinesterase inhibitors (AChEIs) are used for the symptomatic treatment of AD. These compounds have been reported to have adverse effects, including gastrointestinal disturbances.

This study was therefore partly aimed at investigating *in vitro* possible AChEIs in herbal medicines traditionally used in Palestine to treat cognitive disorders, and to point out the role of these plants as potential sources for development of newly potent and safe natural therapeutic agents of AD. Assay of AChE activity plays an important role *in vitro* characterization of drugs including potential treatments for AD. The most widely used method, is based on Ellman's method. The reactant used in this method shows chemical reactivity with oxime antidots and thiol leading to false positive reactions. A new alternative assay could be of high interest.

Methods: The effect on AChE activity of 92 extracts of 47 medicinal plants were evaluated using a new micro-well plate AChE activity (NA-FB) and Ellman's assays. In addition, antioxidant activity using DPPH was determined.

Results: The main advantages of the new method (NA-FB) is that the colorimetric change is better observable visually allowing spectrophotometric as well as colorimetric assay, and does not show any chemical reactivity with thiol. 67.4% and 37% of extracts inhibited AChE by ≥50% using the NA-FB and Ellman's assays, respectively. Using NA-FB assay, 84 extracts interacted reversibly with the enzyme, of which *Mentha spicata* (94.8%), *Foeniculum vulgare* (89.81), and *Oxalis pes-caprae* (89.21) were most potent, and 8 showed irreversible inhibition of

which leaves of *Lupinus pilosus* (92.02%) were most active. Antioxidant activity was demonstrated by 73 extracts *Majorana* syriaca (IC_{50} 0.21mg/ml), and *Rosmarinus officinalis* (0.38) were the most active.

Conclusions: NA-FB assay has shown to be simple, accurate, sensitive, spectrophotometric and colorimetric, and superior to Ellman's, and therefore can be used efficiently for qualitative and quantitative studies of AChEI activities of extracts. Palestinian flora have shown to be a rich source for, new and promising agents (AChEIs) for the treatment of AD Further studies are needed to isolate and identify the active compounds responsible for AChEI activities.

Keywords: Alzheimer's disease, ACh, medicinal plants, *β*-naphthyl acetate, micro-well plate AChE activity Assay (NA-FB)

BACKGROUND:

Numerous medicinal plants have been used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) for the treatment of several diseases, including improvement of memory, Alzheimer's disease (AD) and old age related diseases [1, 2]. However, the use of medicinal plants is mainly based on local tradition and not scientific knowledge.

AD is the most common form of dementia that affects more than 35 million people worldwide and this number is believed to reach 65.7 million by 2030 [3]. It is one of the most widespread neurodegenerative disorders that results in progressive loss of memory and cognition, and deterioration of virtually all intellectual functions [3, 4]. AD has become the fourth leading cause of death in the elderly population (over 65 years of age) as a result of different biochemical pathways [5, 6]. The number of people with AD is expected to increase substantially in the coming years as the proportion of the population aged 65 years or more rises sharply [7].

A loss of acetylcholine (ACh) is considered to play a vital role in the learning and memory deterioration of AD patients. Acetylcholine is an organic molecule released at nerve endings as a neurotransmitter. It is produced by choline acetyltransferase which uses acetyl coenzyme-A and choline as substrates for the formation of acetylcholine in specific cells known as cholinergic neurons. Neurotransmitter disturbances and insufficient cholinergic functions are identified among the pathological features in central nervous system disorders [8].

There are several strategies to improve cholinergic neurotransmission[9], although the one that has been most successful so far is the "cholinergic hypothesis", i.e., stimulation of cholinergic receptors or increasing the availability of ACh released into the neuronal synaptic cleft by inhibiting ACh hydrolysis by acetylcholinesterase (AChE) through the use AChE inhibitors (AChEIs) [10, 11]. AChE is a membrane-bound enzyme found in excitable tissues, such as synaptic junctions. The principle role of AChE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh [12]. Thus, AChEIs (e.g., the drugs used for the AD therapy) promote an increase in the concentration and duration of action of synaptic ACh [13, 14]. The therapy of early and moderate AD is therefore mainly based on AChEIs such as synthetic galanthamine and donepezil isolated from

the bulbs of daffodils [15]. However, these drugs are known to have limitations due to their short-half-lives and/or unfavorable side effects (including gastrointestinal disturbances) and problems associated with bioavailability [16-18], which necessitates the interest in finding better AChEIs from natural resources [19-24].

In traditional practices of medicine, including TAPHM, plants have been used to enhance cognitive function and to reduce other symptoms associated with AD [2, 12]. The search for plant derived AChEI's has accelerated in view of the benefits of these drugs in the treatment of AD and other forms of dementia [25, 26]. Along with the prototype inhibitor of AChE physostigmine, derived from the plant *Phytostigma vevenosum*, other molecules with high anti-cholinesterase activity include galantamine, huperzine-A, alpha-viniferin and ursolic acid obtained from *Galanthus nivalis* and *Narcissus* sp., *Huperzia serrata*, *Caragana chamlague* and *Origanum majorana*, respectively.

Many synthetic anticholinesterase drugs take their origin from plant-derived substances and belong to a diversity of classes of compounds and structures. The majority of these bioactive substances are indole-, steroidal-, piperidine- and Amaryllidaceae alkaloids, glycosides, coumarins, phenylpropanoids and terpenoids [12]. Since AD, the fourth cause of death worldwide, has become a threat to public health, new treatment strategies based on medicinal plants have become focused.

In addition, strong experimental evidences have indicated that reactive oxygen species are associated with the pathogenesis of AD, as some cellular characteristics of this disease are either causes or effects of oxidative stress theory (refers to the physiological condition at which the capacity of the endogenous antioxidant system fails to cope with the damaging effects of free radicals) of AD pathogenesis [27-30]. Generally, the physiological role of antioxidant compounds is to attenuate the oxidation chain reactions by removing free-radical intermediates [28]. Since strong experimental evidences demonstrate that oxidative stress is intimately involved in age-related neurodegenerative diseases, there have been a number of studies which have examined the positive effects of antioxidants in reducing or blocking neuronal death occurring in the pathophysiology of these disorders [31]. Consequently, the use of antioxidants has been explored in an attempt to slow AD progression and neuronal degeneration [11].

Determination of AChE activity has become an important tool in drug design and discovery as well as in medicine and toxicology. A broad variety of methods have been developed over the past decades for AChE inhibitory activity quantification [5, 32, 33]. The most common assay is based on Ellman's method [34] using the substrate acetylthiocholine iodide (ACTI) and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The method is still used, generally with significant modifications [35]. It has some disadvantages, including large interference of some compounds. This method is particularly limited for testing antidots against organophosphorus AChEIs, or for measuring AChE activity in samples of such treated individuals. The antidots contain reactive oxime group splitting DTNB and provide false positive reaction in a process called oximolysis [36].

In this work we present experiments to determine AChE activity assay using β-naphthyle acetate as an alternative substrate, and fast blue B salt as the color reagent (absorbance at 600 nm), instead of DTNB. We introduced a new alternative protocol to the Ellman's method, which could be of high interest when DTNB generates unwanted side reactions [37].

Therefore, the aims of this study were (1) to develop an economic, accurate, reproducible, and convenient colorimetric micro-well plate assay for qualitative as well as quantitative spectrophotometric analysis of phytochemical ingredients with activity against AChE; (2) investigate *in vitro* possible AChEIs *in* Palestinian herbal medicines traditionally used in TAPHM, and to point out the role of these plants as potential sources for the development of newly potent and safe natural therapeutic agents of AD. Selection of the plants screened in this study was based on their use as remedies for the central nervous system diseases, as antidotes for human and animal poisoning or to improve memory and cognitive function.

METHODS:

Reagents and Chemicals

Acetylcholinesterase (AChE) type VI-S from an electric eel, Tris-HCl Tris(hydroxymethyl) aminomethane hydrochloride], β-naphtyl acetate, bovine serum albumin (BSA), 3,3'-dimethoxybiphenyl-4,4'-di(diazonium) zinc chloride (fast blue B salt), acetylthiocholine iodide (ATCI), 5,5´-dithiobis [2- nitrobenzoic acid] (DTNB), galanthamine hydrobromide, 2,2-diphenyl-1-picrylhydrazyl (DPPH),Gallic acid, butylated hydroxyanisole (BHA), ascorbic acid were purchased from Sigma-Aldrich.

Plant Materials and Samples Preparation

Forty seven plant species were collected during 2014 from Nablus and Tulkarm districts in the Northern part of Palestine (West Bank), mainly from their natural habitats or rarely from "Attarin" shops. Voucher specimens (Table 1) were deposited at the Herbarium of Biodiversity & Environmental Research Center-BERC, Nablus, Palestine. A total of 92 plant parts were collected and ground to fine pieces using an electric mill (Phillips, France) and plant material was exhaustively extracted with 60% Ethanol (2 ml/g), at room temperature for 24 hours. In all cases, the solutions were filtered and concentrated to dryness under reduced pressure in a rotary evaporator (45 ◦C). Dry extracts were stored at −20 ◦C until used.

Evaluation of AChE Inhibitory Activity Using Ellman's Method

Inhibition of AChE activity was measured using a 96-well microplate reader (Biotek USA) based on Ellman's method [34]. The chemical principle of the reaction is depicted in Figure 1. The enzyme hydrolyzes the substrate ATCI to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity.

In the 96-well plates, a reaction mixture of 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in buffer B and 25 µl of the plant extract were added, and the absorbance was measured at 405 nm. Thereafter, 25 µl of AChE solution (0.22 U/ml) was added to the wells and the microplate was read again at the same wavelength 10 times with 1 min intervals. Galanthamine dissolved in methanol was used as standard drug at 1 mg/ml concentrations; a blank of methanol in 50 mM Tris-HCl, (pH 8) was used. The percentage inhibition for each test solution was then calculated using the following equation:

$$\text{Inhibition (\%)} = 1 - (A_{sample}/A_{control}) \times 100$$

Where *Asample* is the absorbance of the sample extracts and *Acontrol* is the absorbance of the blank.

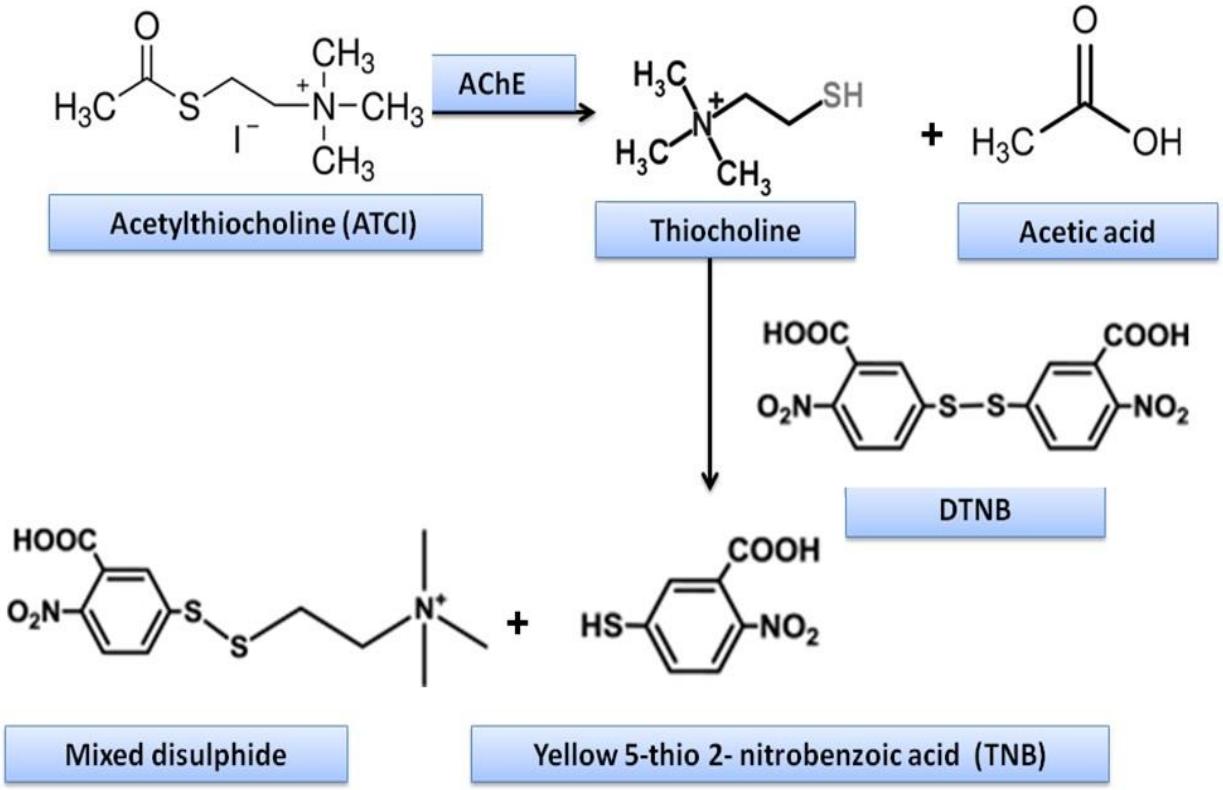

Figure 1. Chemical mechanism of Ellman's method

Evaluation of AChE Inhibitory Activity Using a New Micro-Well Plate AChE Inhibition Assay (NA-FB)

Taking into consideration the relative characteristics of various methods which might be useful in studying the AChE activity in plant extracts, led us to develop a new colorimetric assay to evaluate the activity of AChE, using β-naphthyl acetate as the substrate, and fast blue B salt as the color reagent instead of DTNB. The reaction principle is depicted in Figure 2.The enzyme hydrolyzes the substrate β-naphthyl acetate to naphthol and acetate. Naphthol is allowed to react with fast blue B. This reaction resulted in the development of a stable purple color. The color intensity of the product was estimated either visually, which allowed qualitative detection of the enzyme inhibition, or was measured by UV spectrophotometer, which allowed quantitative detection of the enzyme inhibition. The experiment was organized as a common spectrophotometric test using 96-microwell plats. In each well, 10 ul plant extract, 50 ul (0.25mg/ml) of β-naphthyl acetate dissolved in methanol, and 200 µl of AChE solution (3.33 U/ml) were added, the mixture was incubated at 4^{o}C for 40 min. Later, 10 ul (2.5mg/ml) fast blue b dissolved in water were added to the mixture and the absorbance was measured at 600 nm. To overcome the error in absorbance reading as a result of the plant extract color, the absorbance readings before incubation were subtracted from the absorbance after the addition of the dye. The percentage of inhibition for each test solution was calculated as mentioned above.

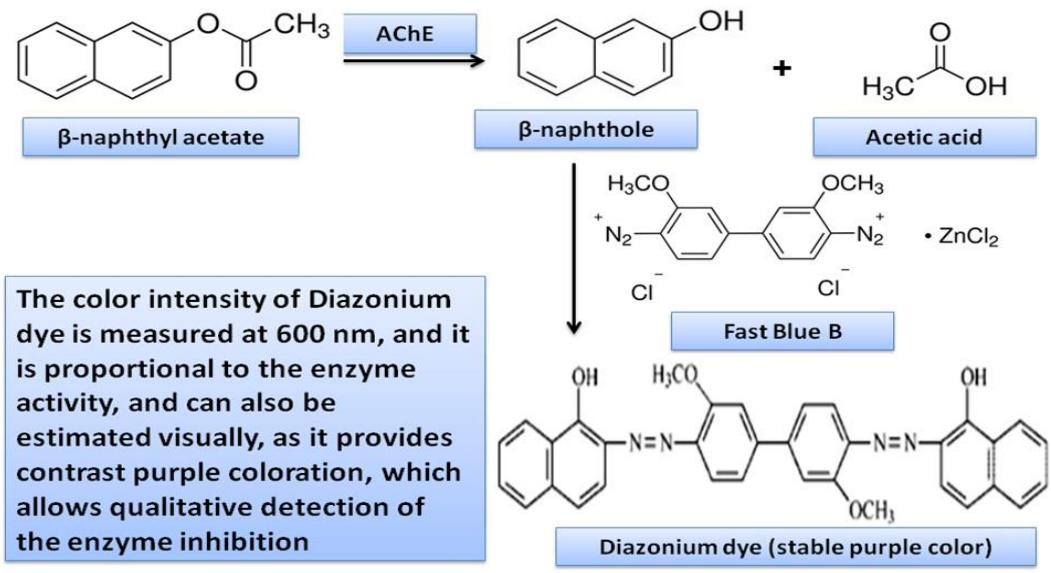

Figure 2. AChE activity Assay using β-naphtyl acetate and fast blue B salt (NA-FB).

Qualitative Determination of AChE Inhibitory Activity

The newly modified method using *β*-naphthyle acetate as a substrate and fast blue B salt as color reagent resulted in the production of a purple color from the reaction between naphthyle and the reagent. However, a scale of 0-3 was used to evaluate qualitatively the inhibition of AChE by plant extracts: (0) No inhibition activity, solution color dark purple (as the negative control); (1) Mild inhibition activity – solution color purple; (2) Moderate inhibition activity –solution color light purple; (3) Strong inhibition activity – no change in solution color (as the positive control).

Estimation of IC_{50} values

The IC_{50} values (concentration of test compounds that inhibits the hydrolysis of substrates by 50 %) were determined by spectrophotometric measurement of the effect of increasing concentrations of test compounds (plant extracts and positive controls) on AChE activity. To calculate the IC_{50} values, each sample was assayed at eight concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml). IC_{50} values were obtained from dose-effect curves by linear regression.

Determination of the Inhibition Type of Plant Extracts on AChE

The type of inhibition of AChE by extracts (reversible or irreversible inhibition) was determined by measuring the restored AChE activity by 10 times dilution of plant extract concentration after mixing and incubation of AChE and plant extract. In reversible inhibition, AChE activity was restored by dilution of plant extract, while there was no change in AChE activity with dilution of plant extract in irreversible inhibition [23].

Determination of Antioxidant Activity Using Scavenging Activity of DPPH Radical

Free radical scavenging activity of the extracts was determined using the free radical 1,1-diphenly-2-picrylhydrazyl-hydrate (DPPH), which is a molecule containing a stable free radical

[38]. In the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical for free DPPH radical decays and the change in absorbance at 517 nm is followed spectrophotometerically. The effect of the plant extracts on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi [39] with minor modification. Twenty five micro liter of plant extract were added to 175µl of 0.004% DPPH methanolic solution, in a 96-well plate. Appropriate blanks were prepared using the solvent only in addition to the same amount of DPPH reagent to overcome any inherent solvent activity. The reaction mixture was shaken well and allowed to stand at room temperature in the dark for 30 min, and then the decrease in absorbance at 517 nm was measured against a control (methanol solution) by using UV-vis spectrophotometer. The radical-scavenging activity of samples, expressed as percentage inhibition of DPPH (I %), and it was calculated according to the formula:

$$\% I = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where *Acontrol* is the absorbance of DPPH radical

IC_{50} (concentration of the extract/compound producing 50% scavenging of DPPH radicals) was determined using non-linear regression analysis of the dose-%I relationship. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml). Antioxidant capacities of the extracts were compared with those of BHA, gallic acid and ascorbic acid. Tests were carried out in triplicates.

Data Analysis

Tests were carried out were possible at least in duplicate on two different occasions. Results are reported as mean ± standard deviation (S.D.). Standard curves were generated and calculation of the 50% inhibitory concentration (IC_{50}) values was done using Excel.

RESULTS:

Forty seven plant species were selected based on their uses as remedies for the central nervous system diseases, as antidotes for human and animal poisoning or to improve memory and cognitive function (Table 1). The inhibition effect of the 92 different extracts on AChE activity was screened using the Ellman's method and the new micro-well plate AChE inhibition assay, NA-FB.

The results obtained by Ellman's method and the NA-FB assay of all plant extracts are shown in Table 1. The screenings were performed at a concentration of 100 mg/ml, and the extracts were considered as active if they only inhibited the enzyme $\geq$50%.

Sixty two (67.4%) and 34 (37%) extracts inhibited AChE by $\geq$ 50% using the NA-FB, and Ellman's assays, respectively. Also, some of the extracts such as *Salvia fruticosa, Galium pisiferum, Anemone coronaria, Juglans regia, Ornithogalum narbonense* and the leaves of *Asphodeline lutea,* which had lower activity against AChE using Ellman's method, exhibited much higher activity using the NA-FB (Figure 3). Therefore, AChEI analysis by Ellman's method was excluded from further discussion.

Table 1. Biological activities of Palestinian plants against different bioassays related to Alzheimer disease

No.	Scientific name	Family Name	Voucher No.	Plant part	AChEI on a scale of 0-3	% of Inhibition			IC_{50} (mg/ml)		
						NA-FB	Ellman's	DPPH	NA-FB	Ellman's	DPPH
1.	*Allium neapolitanum* Cirillo	Liliaceae	BERC-414	LE	2	63.4±2.26	6.21± 0.30	42.12±1.24	74.25	NA	NA
2.				FL	2	55.96±1.47	58.04±1.19	53.26±1.78	99.4	44.3	6.16
3.				ST	1	47.72±3.22	28.75±2.33	58.36±2.32	NA	NA	NA
4.				BU	1	33.28±0.40	48.46±3.05	73.4±0.85	NA	NA	NA
5.	*Anemone coronaria* L.	Ranunculaceae	BERC-355	FL	3	83.36±0.91	35.67±2.02	68.94±1.33	71.5	NA	1.7
6.				LE	2	76.04±1.36	33.33±.33	76.73±1.03	71.5	NA	60.17
7.	*Asphodeline lutea* (L.) Rchb.	Liliaceae	BERC-371	LE	2	75±2.83	6.08±0.11	70.16±0.23	17.75	NA	100.21
8.				BU	0	- 25.36±6.56	41.34±3.76	22.1±1.27	NA	NA	NA
9.	*Asphodelus aestivus Brot.* (*Asphodelus microcarpus* Salzm. & Viv)	Liliaceae	BERC-210	BU	1	49.7±0.42	4.64±0.91	53.55±2.05	NA	NA	NA
10.				FL	0	- 6.42±0.82	1.73±0.38	25.3±0.99	NA	NA	NA
11.	*Bellevalia flexuosa* Boiss.	Liliaceae	BERC-374	ST	1	51.27±5.28	2.47±0.75	14±1.41	97.3	NA	NA
12.				LE	2	59.88±1.24	11.38±0.54	52.3±0.99	31.75	NA	12.76
13.				BU	1	49.33±0.95	22.37±0.52	65.34±0.93	NA	NA	NA
14.				FR	2	64.08±1.30	10.96±1.36	82.69±1.85	97.9	NA	2.14
15.				FL	2	62.36±2.32	19.75±1.06	25.3±1.5	73.5	NA	NA
16.	*Chrysanthemum coronarium* L.	Asteraceae	BERC-068	LE	0	20.1±4.10	38.91±1.29	22.4±0.2	NA	NA	NA
17.	*Conyza bonariensis* Cronquist.	Asteraceae	BERC-259	LE	2	70.1±2.69	81.23±2.50	65.25±1.06	2.89	54.8	2.74
18.				FL	3	74.092±2.70	83.12±2.66	83.94±0.08	3.45	0.35	2.74
19.	*Dodonaea viscosa* L.	Sapindaceae	BERC-045	LE	1	31.96±2.77	40.27±0.38	138.83±0.24	NA	NA	NA
20.	*Erodium malacoides* (l.) L'Her.	Geraniaceae	BERC-357	LE	0	- 4.10.57	33.69±0.98	70.5±0.71	NA	NA	NA
21.				FL	0	- 7.51±0.69	1.11±1.26	74.25±1.06	NA	NA	NA
22.	*Eruca sativa* Miller	Brassicaceae	BERC-202	LE	0	17.33±2.18	79.41±1.08	75.8±0.54	NA	36.7	NA
23.	*Euphorbia hierosolymitana*	Euphorbiaceae	BERC-170	LE	0	10.29±1.00	19.75±1.06	93.07±0.10	NA	NA	NA
24.	Boiss.			FL	0	11.38±0.88	29.81±1.15	99.33±1.88	NA	NA	NA
25.	*Foeniculum vulgare* Mill.	Apiaceae	BERC-030	LE	3	89.81±1.03	52.68±0	72.12±0.76	3.5	99.3	27.54
26.	*Fumaria capreolata* L.	Fumariaceae	BERC-367	FR	3	84.36±1.92	97.37±0.52	37.08±1.30	0.025	0.34	NA
27.				LE	3	82.26±2.46	90.27±0.38	69±1.41	0.035	0.55	1.34
28.				FL	3	85.25±1.06	98.14±1.61	76.41±0.83	0.921	2.1	0.514
29.	*Fumaria densiflora* DC.	Fumariaceae	BERC-154	LE	3	79.78±0.31	83.12±0.17	56±1.41	0.62	21	0.51
30.				FL	3	80.81±0.27	82.31±0.44	73.66±1.90	10.5	3.25	0.68
31.	*Fumaria vaillantii* loisel	Fumariaceae	BERC-396	LE	3	81.17±1.17	95.98±1.39	67.25±0.35	10.5	5.75	1.4
32.	*Galium pisiferum* Boiss.	Rubiaceae	BERC-038	FL	2	73.64±1.92	13.38±0.54	60.5±0.71	8.45	NA	24.16
33.				LE	3	77.84±0.23	18.04 ± 0.06	81.58±0.59	10.45	NA	45.53
34.	*Helichrysum sanguineum* (L.) Kostel.	Asteraceae	BERC-091	LE	2	70.84±0.23	28.46 ±0.65	100±1.41	6.35	NA	44.5
35.				FL	2	68.56±0.79	16.5±0.71	100±1.41	35.5	NA	53.5
36.	*Juglans regia* L.	Juglandaceae	BERC-230	FR	3	78.562±2.39	11.36±1.50	78.69±0.20	8.52	NA	43.86
37.	*Lupinus pilosus* L.	Papilionaceae	BERC-019	FL	3	85.24±1.07	53.98±1.39	44.23±1.09	17.75	23.5	NA
38.	(*L. varius* L.)			LE	3	92.02±1.39	70.52±0.74	88.16±1.19	3.49	2.23	1.15
39.				FR	1	23.13±0.034	44.93±1.5	71.26±0.27	NA	NA	NA
40.	*Majorana syriaca* (L.) Rafin.	Lamiaceae	BERC-026	LE	2	70.58±0.59	88.1±1.98	100±1.3	2.9	9.5	0.21
41.	*Mandragora autumnalis* Bertol	Solanaceae	BERC-286	FR	2	71.43±0.81	98.12±0.17	27.21±0.30	10.06	8.5	NA
42.				RT	3	74.21±1.12	97.46±0.65	48.08±1.30	48.1	49	NA
43.				LE	2	64.4±0.85	94.4±0.57	68.91±0.13	5.1	1.48	1.17
44.				FR(ripen)	2	71.78±2.9	72.0±1.94	62.11±1.45	34.85	38.35	5.17
45.	*Mentha spicita* L.	Labiatae	BERC-116	LE	3	94.8±1.93	74.17±0.176	93.52±0.33	6.3	36.35	0.56

Table 1/ Cont.

No.	Scientific name	Family Name	Voucher No.	Plant part	AChEI on a scale of 0-3	% of Inhibition			IC_{50} (mg/ml)		
						NA-FB	Ellman's	DPPH	NA-FB	Ellman's	DPPH
46.	*Myrtus communis* L.	Myrtaceae	BERC-051	LE	3	74.68±0.38	85.59±0.75	94.23±0.35	3.6	34.5	ND
47.	*Narcissus tazetta L.*	Amaryllidaceae	BERC-395	BU	2	47.21±3.95	95.34±0.93	25.21±1.54	NA	NA	NA
48.	*Nigella sativa* L.	Ranunculaceae	BERC-143	SE	2	73.58±0.38	92.49±0.38	88.08±0.35	69.5	7.75	3.62
49.	*Ophrys dinsmore* Schltr	Orchidaceae	BERC-452	BU	1	44.6±1.98	20.96±1.36	17.6±0.57	NA	NA	NA
50.				ST	2	59.12±1.24	32.88±1.24	70.67±0.95	89.5	NA	29.13
51.				FL	2	62.08±1.30	39.79±1.12	85.19±1.15	30.5	NA	35.96
52.				LE	2	73.12±2.66	25.58±0.82	143.33±2.36	12.1	NA	22.22
53.	*Ophrys lutea (*Gouan*)* Cav	Orchidaceae	BERC-432	FL	3	75.8±0.28	51.75±1.06	64.32±0.96	94.21	91.2	11.75
54.				ST	2	53.36±0.51	31.33±0.47	88.3±0.99	26.5	NA	46.4
55.				LE	2	72±1.41	54.5±0.71	94.23±1.09	97.12	95.6	5.08
56.	*Orchis caspia* Trautv	Orchidaceae	BERC-422	ST	2	68.76±1.75	27.75±1.06	79.32±0.96	47.4	NA	57.88
57.				LE	3	88.88±1.58	97.61±0.86	81.75±0.35	5.7	6.75	0.51
58.				FL	2	61.52±2.09	43.79±1.12	87.21±1.12	97.8	NA	2.74
59.	*Orchis collina* Banks &Sol.	Orchidaceae	BERC-448	BU	0	24.48±2.15	26.5±0.71	10.19±1.15	NA	NA	NA
60.				ST	2	57.44±0.79	23.04±0.06	55.61±0.55	98.4	NA	3.2
61.				LE	2	69.08±1.30	38.33±0.95	85.38±2.29	21.85	NA	70.81
62.				FL	2	61.36±0.91	33.25±0.35	91.41±0.83	11.5	NA	19.67
63.	*Ornithogalum narbonense*	Liliaceae	BERC-464	BU	1	38.64±1.92	- 5.67 ± 0 .95	54.71±0.41	NA	NA	NA
64.	L.			FL	2	69.2±0.28	- 0.33 ± 0 .95	46.25±1.06	92.5	NA	NA
65.				ST	2	66.24±1.07	17.21 ± 1.12	67±1.41	78.63	NA	86.32
66.				LE	3	75.88±0.17	-3.71 ± 1.00	80.86±1.22	25.9	NA	21.81
67.	*Oxalis pes-caprae* L.	Oxalidaceae	BERC-265	FL	2	74.33±0.95	100.22±0.31	110.75±1.06	ND	ND	ND
68.				LE	3	89.21±0.35	88.26±0.37	147.43±0.61	6.5	23.3	1.3
69.	*Parietaria judaica* L.	Urticaceae	BERC-063	LE	2	71.19±1.15	31.91±1.29	71.66±0.93	70.3	NA	ND
70.	*Peganum harmala* L.	Zygophyllaceae	BERC-181	SE	2	50.85±0.21	81.06±0.08	80.12±0.15	5.81	ND	3.1
71.	*Pelargonium odoratissimum* (L.) L 'He'r	Geraniaceae	BERC-049	LE	0	- 15.44±0.79	- 1.58±1.24	88.91±0.13	NA	NA	NA
72.	*Phagnalon rupestre* (L.)	Asteraceae	BERC-047	FL	3	80.5±2.12	93.24±0.34	57±0.00	6.25	8.5	0.93
73.	DC.			LE	3	81.1±2.69	95.61±0.86	150.2±.036	10.2	7.1	1.9
74.	*Ranunculus asiaticus* L.	Ranunculaceae	BERC-400	FL	2	71.67±0.47	88.47±0.66	45.75±0.35	14.3	11.54	NA
75.	*Ranunculus millefolius*	Ranunculaceae	BERC-475	LE	1	31.23±2.50	76.75±1.06	57.58±0.82	ND	65.8	2.25
76.	Banks & sol.			FL	0	22.64±1.92	17.33 ± 0 .47	64.61±0.55	ND	ND	ND
77.	*Raphanus rostratus* DC.	Cruciferae	BERC-368	LE	1	28.12±1.24	24.1±1.27	81.75±0.35	ND	ND	ND
78.	*Retama raetam* (Forssk.)	Salvadoraceae	BERC-043	FL	2	69.25±1.77	29.59±0.83	51.5±0.71	85.83	ND	60.2
79.	Webb			LE	0	21.85±1.63	16.62±3.37	59.58±0.59	NA	NA	NA
80.	*Rosmarinus officinalis* L.	Labiatae	BERC-018	LE	3	76.39±0.86	95.32±0.45	99.16±1.19	2.4	14.8	0.38
81.	*Salvia fruticosa* (L.) Mill.	Labiatae	BERC-006	LE	3	85.68±1.26	46.81±2.26	73.1±0.97	4.45	NA	33.08
82.	*Scorzonera papposa* L.	Asteraceae	BERC-401	FL	1	41.08±1.30	17.33 ± 0 .47	44.8±1.13	NA	NA	NA
83.				LE	1	48.24±2.49	15.83 ± 0.24	49.03±1.37	NA	NA	NA
84.	*Smyrnium olusatrum* L.	Apiaceae	BERC-416	LE	0	3.09±8.61	5.01±1.98	57.83±1.17	NA	NA	NA
85.				FL	1	26.21±1.12	3.98±1.39	82.41±0.83	NA	NA	NA
86.	*Tulipa sharonensis* Dinsm.	Liliaceae	BERC-431	LE	1	52.3±2.40	56.5±0.71	38.08±1.30	44.5	26.5	ND
87.				ST	1	41.08±2.72	50.79±0.30	61.9±0.14	ND	48.3	20.38
88.				FL	2	56.88±0.17	37.42±0.82	64.41±0.58	37.5	NA	24.62
89.				BU	1	46.12±1.24	- 18.63±0.89	71.82±0.25	NA	NA	NA
90.	*Urtica pilulifera* L.	Urticaceae	BERC-066	LE	2	58.6±2.4	2.73±0.77	86.66±0.96	10.25	NA	38.32
91.	*Vicia hybrida* L.	Papilionaceae	BERC-420	LE	3	74.27±1.03	49.96±1.36	41.05±1.34	50.23	NA	NA
92.	*Zingiber officinale* Rose.	Zingiberaceae	BERC-501	BU	1	41.25±1.06	48.04±0.06	99±1.2	NA	NA	NA
	Galanthamin			NA	3	88.8±1.13	93.44±2.21	NA	0.015	0.02	NA
	Ascorbic acid										0.07
	BHA										0.009
	Gallic acid										0.033

BU, Bulb, FL, Flower; FR, Fruit; LE, Leaves; SE, Seeds; ST, Stem; RT, Roots; NA, not applicable; ND, not determined

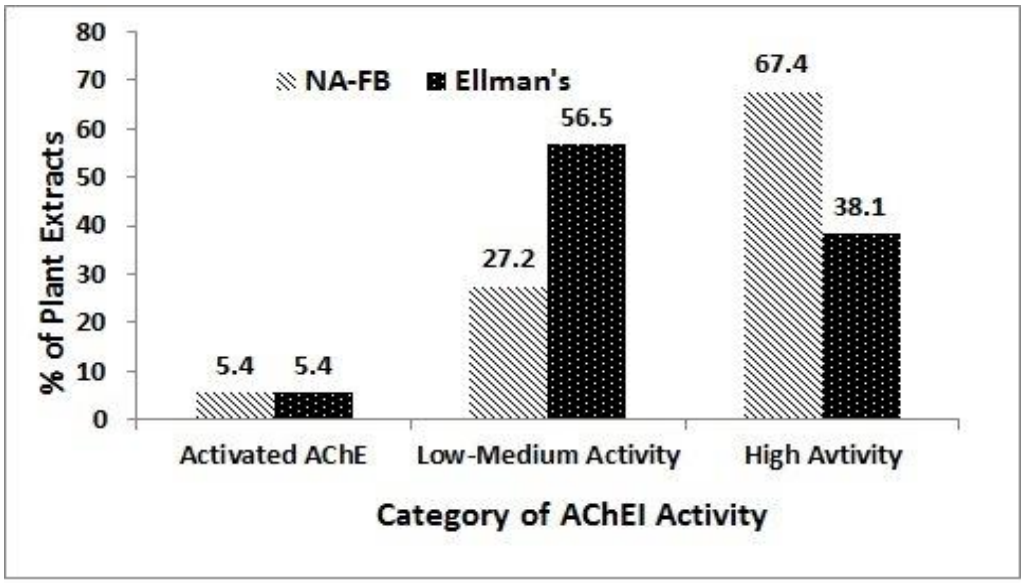

Figure 3. Categories of AChE inhibitory activities based on Ellman's and NA-FB AChE inhibition assays

The new method produced colorimetric change better observable visually over the Ellman's, allowing quantitative as well as qualitative assay, and did not show any chemical reactivity with thiol used for the Ellman's method, therefore avoid false positive reaction of enzyme activity provided by samples containing thiol-bearing molecules (e.g., *Bellevalia flexuosa*). Qualitative determination of AChE inhibitory activity using the NA-FB method revealed that 66.3 % (61 extracts) of the plant extracts showed moderate to high AChE activity, 19.6 % (18) showed low activity, and 14.1% (13) were not active (Figure 4).

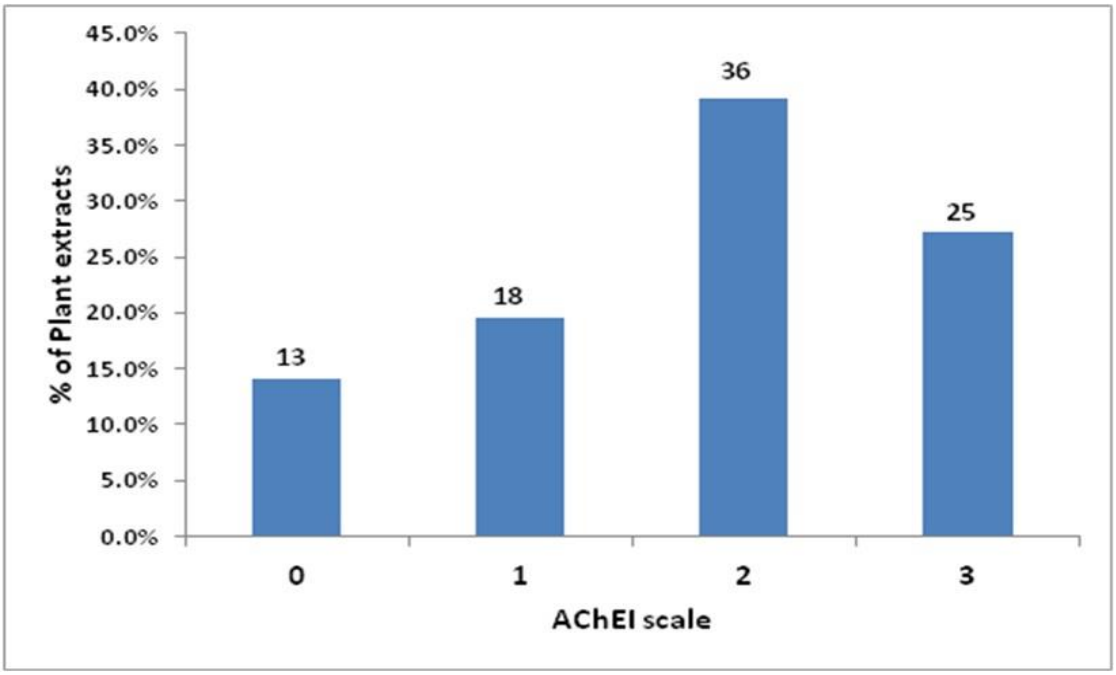

Figure 4. Qualitative determination of AChE inhibitory activities based on the NA-FB AChE inhibition assay

Extracts from the leaves of *Pelargonium odoratissimum*, and bulb of *Asphodeline lutea.* activated AChE more than 15% and 25 %, respectively (Figure 5). The most potent extracts using the modified method were *Mentha spicata* (94.8 %), *Lupinus pilosus* (92.02), *Foeniculum vulgare* (89.81), *Oxalis pes-caprae* (89.21), and *Orchis caspia* (88.88) (Figure 5).

The dose-dependent AChE inhibitory activity of the active herbs was further studied, and the IC_{50} values of inhibition are presented in Table 1. Ethanol extracts of the tested plants were found to have high AChE inhibitory activities in a dose-dependent manner. Further testing and analyses of the inhibition of AChE by leaves of *Fumaria capreolata*, *Fumaria densiflora*, *Rosmarinus officinalis*, *Conyza bonariensis*, and *Majorana syriaca* revealed IC_{50} values of 0.035, 0.62, 2.4, 2.9, and 2.9 mg/ml, respectively.

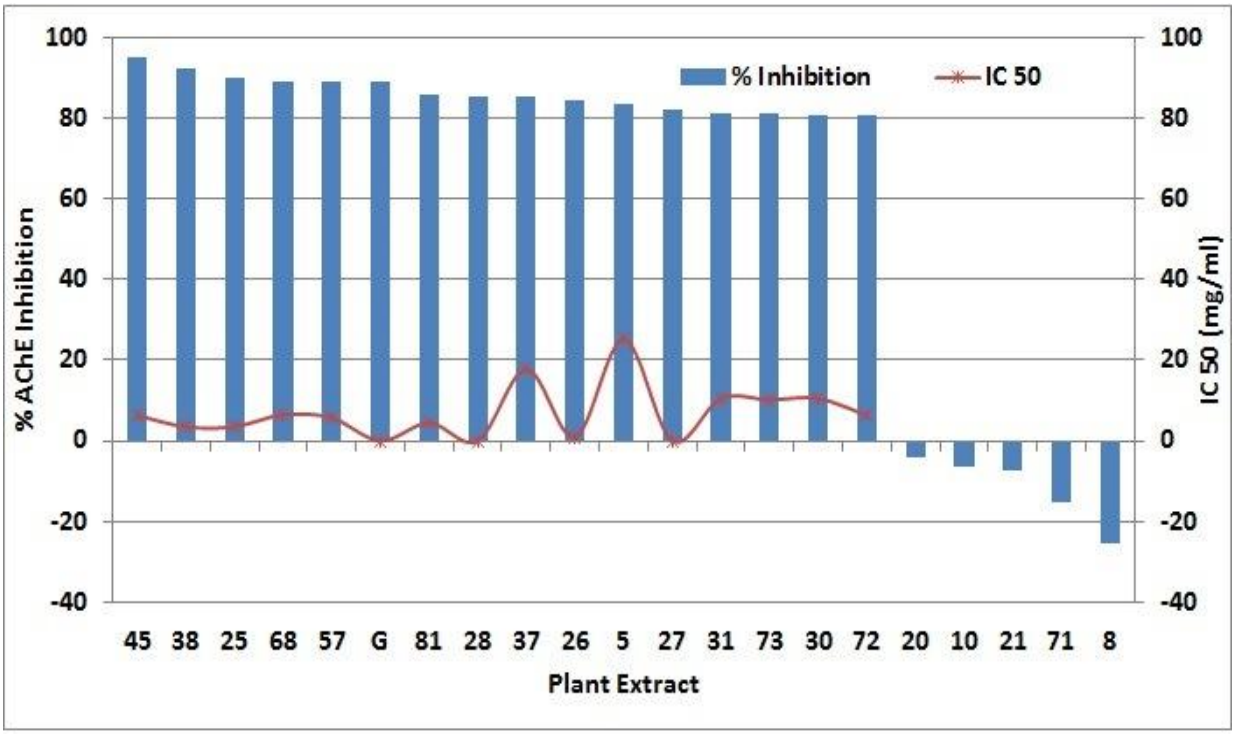

Figure 5. % inhibition of AChE by different plant extracts at 100 mg/ml. G, Galanthamine. Plant extract numbers on X axis as in Table 1.

The inhibition type of plant extracts which showed ≥ 50% inhibition activity was determined by assaying the change in the remaining AChE activity of the mixture of AChE and the plant extract before and after the dilution of the plant extract in the same mixture. While, AChE activity was restored more than 5 fold by 10 times dilution of *Juglans regia, Oxalis pes-carpae*, and *Foeniculum vulagare*, the same dilution of *Fumaria capreolata, F. densiflora, Lupinus pilosus, Peganum harmala* and *Mandragora autumnalis* did not show any effect on the remaining activity of AChE after dilution. This result indicates that AChE is inhibited reversibly by *Juglan regia*, *Oxalis pes-carpae*, and *Foeniculum vulagare* and irreversibly by the leaves, flowers, and fruits of *F. capreolata*, the leaves and flowers of *F. densiflora*, the seeds of *Peganum harmala*, and the leaves of *Lupinus pilosus* and *Mandragora autumnalis.*

Table 1 shows the antioxidant results of the tested plant extracts. Seventy-three extracts showed ≥ 50% antioxidant activity, of these *Phagnalon rupestre, Oxalis pes-caprae, Ophrys dinsmor, Dodonaea viscosa, Helichrysum sanguineum,* and *Majorana syriaca* were the most active. The IC_{50} of the antioxidant activity for the plants extracts, which showed ≥ 50 % AChE inhibition activity using the NA-FB was determined (Table 1). Of these eight extracts; leaves of *M. syriaca* (IC_{50} 0.212mg/ml), leaves of *Rosmarinus officinalis* (0.377 mg/ml), leaves of *Fumaria densiflora* (0.514 mg/ml), leaves of O*rchis caspia* (0.514 mg/ml), leaves of *Mentha apicata* (0.56 mg/ml) flowers of *Fumaria densiflora* (0.678 mg/ml), flowers of *Fumaria capreolata* (0.69 mg/ml), and flowers of *Phagnalon rupestre* (0.928 mg/ml) were particularly strong antioxidants when compared to the reference radical scavengers (BHA, gallic acid, and ascorbic acid) recording IC_{50}'s < 1 mg/ml.

DISCUSSION:

Palestine is enriched with great plant diversity, and 368 of these plants have been reported to be used in TAPHM for the treatment of several diseases [40]. However, the use of medicinal plants is mainly based on local tradition and not scientific knowledge [41-43]. The chemical constituents of most of these plants are unknown and may have dangerous effects on human health. On the other hand, some plants, which are not reported to be used in herbal medicine, might also possess potential activity.

The deficiency of ACh is one of characteristics of AD and responsible for most of its symptoms, such as a decline in memory and cognition. AChE inhibitors such as tacrine, donepezil, rivastigmine, and galantamine are currently used as anti-AD drugs [44]. The side effects of these anti-AChE drugs, such as toxicity, tolerability, and loss of efficiency, have interested the researchers to consider alternative natural anti-AD substances in place of current synthetic medications [45].

In the present work, the selected extracts were screened for AChE inhibition using the Ellman's method and the NA-FB assay at 100 mg/ml dose. The Ellman's method is the most widely used AChE inhibitory assay [32]. This method has some advantages and disadvantages. Its main advantages are simplicity, rapid processing of large numbers of samples, fast conversion of ACTI comparing to other artificial substrates such as naphthyle acetate and relatively low cost [46, 47]. On the other hand, Ellman's method has some disadvantages, including the interference of some compounds. The -SH groups in the plant extract may react with DTNB and ATCh, thus the natural substrates are not identical from a kinetic point of view. False positive reaction of enzyme activity can be provided by samples containing a lot of thiol-bearing molecules.

To overcome this problem an alternative method using *β*-naphthyl acetate as the substrate and fast blue B as the color reagent (absorbance at 600 nm) instead of DTNB was developed in this study. The main advantages of this method is that the colorimetric change is better observable visually, allowing spectrophotometric as well as colorimetric assay, and does not show any chemical reactivity with thiol, therefore avoid false positive reaction of enzyme activity provided by samples containing thiol-bearing molecules. The NA-FB method can be considered superior and more sensitive than the Ellman's assay. In the present study, 67.4% of plant extracts inhibited AChE by ≥ 50% using the NA-FB method, while only 37% extracts inhibited AChE by ≥ 50% using Ellman's assay. This result can be attributed to the accuracy and

stability of the reaction using naphthyle acetate. Naphthyle acetate splits with lower turnover rate, and does not show any chemical reactivity with thiol used for the Ellman's method [37], thus NA-FB can be advantageously used for accurate measurements of AChE activity.

Sixty two extracts belonging to 34 plant species (Table 1) have been identified to effectively inhibit AChE enzymes, which is considered to be related to the mechanism of memory dysfunction in this study. In the light of these findings, we can conclude that most of the plant extracts screened showed inhibitory activity against AChE and could be considered worthwhile in future studies in the treatment of AD. In particular, the species belonging to Apiaceae, Papilionaceae, Oxalidaceae, Orchidaceae, Lamiaceae, Fumariaceae families had the highest activity ranging between 94.8 and 85.25% at 100 mg/ml concentration against AChE. Since most of the AChE inhibitors are known to contain nitrogen, the higher activity of these extracts may be due to their rich alkaloidal content [9].

The most potent extracts were the leaves of *Fumaria capreolata*, *Fumaria densiflora*, *Rosmarinus officinalis*, *Conyza bonariensis*, and *Majorana syriaca* with IC_{50} values of 0.035, 0.62, 2.4, 2.9, and 2.9 mg/ml respectively. At a concentration of 100 mg mL–1, they reduced the enzymatic activity of AChE to 17.74%, 20.22, 23.61, 29.9 and 29.42 respectively. The inhibition type of AChE varied among plant extracts, while 84 extracts showed reversible inhibition, 8 showed irreversible inhibitions. Although IC_{50} values of *Fumaria capreolata*, *Fumaria densiflora*, are higher than that of *Rosmarinus officinalis* and *Majorana syriaca*, the inhibition type in this study showed that *Rosmarinus officinalis* and *Majorana syriaca* reversibly inhibits AChE and can be used for AD's medication rather than *Fumaria capreolata*, *F. densiflora* which inhibits irreversibly AChE. This recommendation was supported by the toxicity reports in literature which indicated the higher safety margin of *R. officinalis* and *M. syriaca* as compared to *Fumaria* species.

Rosmarinus officinalis (rosemary) contains the natural COX‑2 inhibitors (e.g. Apigenin, carvacrol, eugenol, oleanolic acid, thymol, and ursolic acid, which can prevent Alzheimer's disease [48] . In addition, rosemary contains antioxidants and anti‑inflammatory compounds. Some of the strongest antioxidant substances in the plant are carnosic acid and ferulic acid, which have been reported to posses antioxidant activity much higher than the widely common synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [3].

Conyza bonariensis is a medicinal plant, which has been reported to be used for constipation and diarrhea, has been attributed to the spasmogenic and spasmolytic constitute of the plant [49]. The leaves and flowers of the plants have shown to possess high AChEI activity > 70% with IC_{50} value ranging between 2.89-3.45 mg/ml.

Majorana syriaca possesses an important food-flavouring ingredient in the Middle Eastern culture, known commonly as Za'atar. The plant is used traditionally for the treatment of several ailments and is associated as memory enhancer [2]. The main components of the plant extract according to GS-MS analysis were thymol, and carvacrol. The remaining compounds comprise flavonoids and phenolic acids that provide the antiradical and antioxidant activity [50]. The plant has shown to have strong activity against AChE, ursolic acid which has been isolated from the plant, has shown to be a potent AChE inhibitor in Alzheimer's Disease [51].

Fumaria species have been used in traditional medicine as antihypertensives, diuretics, hepatoprotectants and laxatives (to treat gastrointestinal disorders), as well as in the treatment of rachis and conjunctivitis [52]. The plant has also been evaluated pharmacologically and shown to possess antihelmintic, antipyretic and hypoglycemic properties [53-55]. The biological activities of *Fumaria species* have been mainly associated with the presence of isoquinoline alkaloids [56]. The toxicity of the species have been evaluated, *Fumaria capreolata* has shown to be non-toxic [56], while *F. densiflora* was reported to be toxic [57]. The AChEI activity of *Fumaria* species has been reported by several researchers; the plant was reported to have strong AChEI activity [9]. In this study, *Fumaria* were among the most active plant extracts against AChE activity, however, the reaction was shown to be irreversible, thus the plant cannot be used for the treatment of AD as the activity of the enzyme cannot be restored.

AChEI activity of the methanolic extract of *Peganum harmala,* has previously been reported by Ali et al. [26]. The plant contains β-carboline alkaloids, which demonstrated potent activity against AChE [58]. Harmaline, the major active constituent of *P. harmala,* is a common dihydro β-carboline type; it possess interesting pharmacological activities and can interact with several enzymes and neurotransmitters including topoisomerase I, and monoamine oxidase-A [59, 60]. Although, *P. harmala* has been used in traditional medicine, there are reports of severe intoxication in cattle, donkeys, sheep and horses [61]. Digestive and nervous syndromes have been reported in animals that consume a sub-lethal amount of the plant. Harmaline and harmine are toxic alkaloids characterized in the seeds of *P. harmala*. Harmaline is almost twice as toxic as harmine and in moderate doses cause tremors and clonic convulsions, but with no increase in spinal reflex excitability [62]. The seeds of *P. harmala* were among the potent plant extracts against AChE activity, however, the reaction have been shown to be irreversible, thus the plant cannot be used for the treatment of AD.

Some insecticides including organophosphate and carbamates cause AChEI which lead to the accumulation of ACh at neuromuscular junctions causing rapid twitching of voluntary muscles and eventually paralysis of the insects. However, in this study, leaves and flowers of *Fumaria* species, seeds of *P. harmala,* and the leaves of *Lupinus pilosus* and *Mandragora autumnalis,* which have shown high irreversible AChEI activity, can be considered potent natural insecticides.

Alzheimer's appears to be caused to a large degree by oxidative damage [63]. Therefore, antioxidants, in general, should have positive effects in both the prevention and treatment of Alzheimer's. A study found that antioxidants such as vitamin A, vitamin D, lycopene, and beta carotene were all significantly lower in Alzheimer's disease patients compared to controls [64]. Another study of 633 patients aged $\geq$ 65 years found that high dose supplementation with vitamin C decreased the risk of developing AD [65]. Therefore, the plant extracts which demonstrated potent free radical scavenging properties are expected to play a vital role in reducing the oxidative stress and this may explain their use in traditional medicine for improvement of AD and/or ageing related diseases. It's worth mentioning that some of the plant extracts which have high antioxidant activity including *M. spicata* (93.52), *Z. officinale* (99), *R. officinalis* (99.16), *M. syriaca* (100), and the leaves of *O. pes-caprae* (147.43), are wild edible plants widely consumed among the Palestinian population [66]. Some of these plants have been reported to be used traditionally for memory enhancement [2, 3, 23, 66].

CONCLUSION:

The new micro-well plate AChE activity assay (NA-FB) has shown to be simple, accurate, sensitive, spectrophotometric and colorimetric, and superior to the Ellman's method, and therefore can be used efficiently for qualitative and quantitative studies of AChE inhibitory activities of plant extracts of a wide range of diverse plant species and to give high detection rates from a range of plant parts. The extracts of *R. officinalis*, *M. spicata*, *M. syriaca*, and *N. sativa* were proved to have a great potential and should be considered for further studies to identify the constituents responsible for the AChE inhibitory activity, which can be eventually utilized in the prevention and treatment of AD.

The pathophysiological process of AD is thought to begin many years before the diagnosis of AD dementia. This long "preclinical" phase of AD would provide an important opportunity for therapeutic intervention. It is hoped that plants with strong reversible AChEI and strong antioxidant activities will aid in earlier intervention at a stage of AD when some disease-modifying therapies may be most efficacious.

List of abbreviations:

TAPHM	Traditional Arabic Palestinian Herbal Medicine
AD	Alzheimer's disease
ACh	acetylcholine
AChE	acetylcholinesterase
AChEIs	AChE inhibitors
ACTI	acetylthiocholine iodide
DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
BSA	bovine serum albumin
DPPH	diphenyl-1-picrylhydrazyl
BHA	Butylated hydroxyanisole
NA-FB	New Micro-Well Plate AChE Inhibition Assay

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