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Phytochemical Analysis, Antioxidant and Anti-Acetylcholinesterase Activities of Jordanian *Pistacia palaestina* Bios Leaves Extract

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ABSTRACT

Pistacia palaestina Boiss (*P. palaestina*) is a medicinal plant with multiple dietary and therapeutic applications. The purpose of this work was to investigate the phytochemical content, the antioxidant, and the anti-acetylcholinesterase (AChE) activities of the *P. palaestina* leaves' methanol extract. The antioxidant activity of the extract was tested by 2,2 -azino-bis-3 ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays, while the AChE inhibitory activity of the extract was determined according to Ellman's assay. The composition of secondary phytochemical metabolites was established by HPLC-PDA and GC-MS analysis. *P. palaestina* leaves displayed strong $ABTS^{\bullet+}$ as well as DPPH radical scavenging ability with an IC_{50} value (6.86 µg/mL and 8.31 µg/mL) close to that of ascorbic acid (6.09 µg/mL and 6.96 µg/mL), respectively. For enzyme inhibition, the *P. palaestina* extract showed good activity against acetylcholinesterase in a dose-dependent manner with IC_{50} (57.67 µg/mL) ten folds higher than the standard inhibitor galantamine (IC_{50} = 5.64 µg/mL). The methanol extract displayed high phenolic (242.2 mgGAE/g extract) content. Similarly, the phytochemical profiling of the extract cautiously identified 19 different secondary metabolites, as accessed by HPLC and GC-MS analyses. Benzofenac methyl ester, 3,5-bis(1,1-dimethylethyl)- phenol, N-Dimethylaminomethyl-tertbutyl-isopropylphosphine, 14-methyl-Pentadecanoic acid methyl ester, 13-Tetradece-11-yn-1-ol, and Tridecane were the main compounds detected. Based on the current findings on the antioxidant and anti-AChE activities of *P. palaestina* methanol leaves extract, this plant may be recommended as a source of bioactive plant compounds and presents new alternatives for use in the management of neurological diseases.

***Keywords*:** *Pistacia palaestina*, anticholinesterase, antioxidant, phenolic compounds, HPLC, GC-MS.

Introduction

Human life encounters physiological and environmental stressors that increase the release of reactive oxygen species (ROS) in the cells. ROS induced eustress to maintain the physiological level of oxidant essential for controlling life processes or distress when excessive oxidant challenge causes damage to biomolecules.[1]

Although there are several defense mechanisms employed by human body systems to counteract the drastic effects of ROS, including enzyme and non-enzyme antioxidant molecules, an excess of ROS is uncontrollable leading to damage of biomolecules such as lipids, proteins, and nucleic acids. The failure of human body to scavenge ROS may be connected to cellular aging, cancer, diabetes, and neurological illnesses such as Alzheimer's disease (AD).[2]

AD is an aging neurodegenerative brain disease, characterized by progressive neurodegeneration, cognitive impairment, and memory loss.[3] The incidences of this disease are associated with a decreased level of brain acetylcholine. Therefore, inhibition of acetylcholinesterase (AChE) that hydrolyzes the neurotransmitters is considered a curative strategy in managing and healing AD;[4] therapies of AD is based on improving the levels of acetylcholine in the brain to increase the cholinergic central function, examples of AChE inhibitors used in AD symptom management includes donepezil and galantamine.[5,6] In fact, using AChE inhibitors from natural sources as a safe agents mitigate the side effects associated with using chemotherapeutical agents in treatment of AD patients. Worth noting, the polyphenol content of medicinal plants play an important role in the detoxification of ROS[7] and thereby these plants are utilized for the cure or improvement of diseases.

Jordan is a rich land of medicinal plants utilized in local traditional medicine.[8-10] *Pistacia palaestina* (in Arabic known as Butom) is a member of the family of Anacardiaceae and widely distributed in Jordan, Palestinian, Lebanon, Syria, and Turkey Hills and mountains.[11] Its extracts are traditionally used as neuro-protectants, treatment of stomach pain, burning skin, asthma, and bronchitis;[12] its leaves are traditionally used as an antiseptic, anti-rheumatic, and in relief of stomach and intestinal pains in Italy.[13] The leaves, fruit, and flowers of Pistacia also demonstrated high antioxidant activity[14-16] with diuretic, laxative, antidiabetic and stimulating properties.[17] It is also used to treat high blood pressure, kidney stones, jaundice, and burns.[7,18,19] *P. palaestina* shoots, leaves, and roots traditionally are also employed as antispasmodic, antiseptic, anti-inflammatory, and for healing of burns and ulcers[20] as well as sources for antimicrobial agents.[20,21]

Herein, the antioxidant activity of *P. palaestina* methanolic leaves extract was evaluated through DPPH and $ABTS^{\bullet+}$ assays. Moreover, the potential neuro-protecting property of the extract was elucidated by examining its ability to inhibit the AChE activity and thus might be involved in the treatment of AD. The phytochemical composition and

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secondary metabolites profiling was established by HPLC and GC-MS.

Materials and Methods

Chemicals

L-Ascorbic acid, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), follin-ciocalteu reagent, sodium carbonate, acetylcholinesterase from *electric eel* (Type VI-S)-C3389-2KU), acetylthiocholine iodide, 5,5-dithio-bis-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich. All reagents and solvents used were of analytical grade.

Plant materials

Leaves of Jordanian *P. palaestina* Boiss were collected in June 2020 from Ajloun mountains, north of Jordan. A voucher specimen of each plant was authenticated botanically by the Biology Department, Faculty of Sciences, Mutah University, Jordan (voucher series: BIO-AA 30). Leaves were dried under shade at 25ºC and kept for further analysis.

Preparation of plant extract

The dry leaves of the *P. palaestina* (50 g) were extracted in methanol (1:10 w/v) under shaking for 3 days, at 120 rpm. The extract was filtered using a Buckner funnel and Whatman No 4-filter paper. The filtrate was concentrated to dryness under vacuum to give the crude extract.

Total polyphenol content

The total polyphenols of methanol extract of *P. palaestina* leaves were determined by the Folin–Ciocalteu method according to Singleton,[22] with slight modification. 0.2 mL of extract (0.5 mg/mL) was mixed with 1 mL of 10% Follin-ciocalteu reagent and was incubated for 5 min and then 0.8 mL of 7.5% sodium carbonate was added. This mixture was then incubated for 30 min at 25 °C. Finally, the absorbance of the sample was measured at 760 nm. Gallic acid was used as a standard in the concentration range of (2.0 - 8.0 µg/mL) ($y = 0.0545x + 0.2318$, $R^2 = 0.9937$). The result was expressed in terms of gallic acid equivalents.

Antioxidant activity

The antioxidant activity of methanol extract of *P. palaestina* was determined using DPPH and $ABTS^{\bullet+}$ free radical scavenging assays.

DPPH assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) was used as a free radical source to determine the free radical scavenging potential of the extracts according to Blois [23] with slight modification. DPPH stock solution (20 mg DPPH /100 mL of methanol) was prepared, and its absorbance was adjusted to 0.7 at 517 nm (control solution). Different concentrations of extracts (2- 100 µg/mL) and Ascorbic acid (2- 10 µg/mL) as a standard were taken and mixed with 1 mL of DPPH solution and incubated for 30 minutes. Finally, the absorbance of extracts and Ascorbic acid were measured at 517 nm. The IC_{50} value was determined for extract and standard which is the concentration at which half of (50%) of DPPH solution has been scavenged. The % free radical inhibition was calculated according to the following formula:

DPPH% inhibition= absorbance of control - absorbance of sample/ absorbance of control x 100.

IC_{50} was calculated from the regression equation (MS Excel-based program) prepared from the concentration of the sample and the percentage of the scavenging activity. Ascorbic acid was used as a positive control. Samples were analyzed in triplicate. The experimental data were expressed as mean ±SD.

$ABTS^{\bullet+}$ Assay

The free radical scavenging activity of the crude extract was determined using $ABTS^{\bullet+}$ assay following the method of Re *et al.*[24] with slight modifications. The $ABTS^{\bullet+}$ was generated by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12h, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.70 at 734 nm with ethanol before it is used.

Different concentrations of the sample solution in ethanol (2- 100 µg/mL) and Ascorbic acid (2- 10 µg/mL) as a standard were taken and mixed with 1 mL of $ABTS^{\bullet+}$ solution. After 10 min, the absorbance was measured at 734 nm by using a spectrophotometer. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of $ABTS^{\bullet+}$ was calculated according to the following equation:

$ABTS^{\bullet+}$ scavenging activity (%) = Abs. control- Abs. sample/ Abs. control ×100.

Where Abs. control is the absorbance of the initial concentration of the $ABTS^{\bullet+}$ and Abs. sample is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of the sample. The extract concentration providing 50% radical scavenging activity (IC_{50}) was calculated from the curve of $ABTS^{\bullet+}$ scavenging effect percentage against extract concentration (MS Excel-based program). Ascorbic acid was used as antioxidant standards for comparison of the activity. Samples were analyzed in triplicate. The experimental data were expressed as mean ±SD.

Anti-Acetylcholinesterase inhibitory activity

Acetylcholinesterase (AChE) inhibitory activity was measured spectrophotometrically following the method described by Ellman *et al.* [25] with slight modifications. Briefly, 300 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution at different concentrations (final concentration 10-100 µg/mL), and 5 µL AChE (5 U) solution were mixed and incubated for 20 min at 25°C, and then 10 µL of 0.01 mM DTNB was added. The reaction was then initiated by the addition of 2 µL of acetylthiocholine iodide (0.71 mM). The reaction of thiocholine with DTNB was monitored at 412 nm, utilizing a 96-well microplate reader (Bio-Tek ELx800UV, USA). The results were recorded as IC_{50}, which indicates that 50% of enzyme activity was decreased. Different concentration from galanthamine stock solution (1 mg/mL) were prepared and used as a positive control.

The percentage of inhibition of the AChE enzyme was determined by using the formula [(y-x)/y] *100, where Y is the activity of enzyme without test sample, and x is the activity of the enzyme with the test sample.

Chemical characterization

HPLC chromatography analysis

The HPLC analysis of the crude extract was performed using ODS column (4.6 ID x 150 mm, 5 µm), at 25ºC, 20 µL injection volume, and PDA detector at wavelength range 210-500 nm. The column temperature was set at 25ºC. The mobile phase is a mixture of phosphoric acid in water (0.1%) (solvent A) and acetonitrile (solvent B); the run was done in a linear gradient mode. 100% (solvent A) descended to 70% (solvent A) in 40 min, then to 40% (solvent A) in 20 min and finally to 10% (solvent A) in 2 min; it was held at that composition for 6 min with 1 mL/min flow rate, and back to the initial conditions in 2 min. The HPLC system was equilibrated for 5 min with the initial acidic water mobile phase (solvent A) before injecting the next sample. All the samples were filtered with a 0.45 µm filter.

GC-MS chromatographic analysis

The GC-MS analysis was done using GC type 6890 (Agilent technology, USA) equipped with split-splitless injector and HP-5MS capillary column coated with a film of 5% phenylmethylpolysiloxane (30 m x 0.25 mm, 0.25 µm film thickness). The instrument was equipped with an inert performance turbo MSD mass spectrometer type 5973C. The column oven temperature was programmed as follows: start temperature at 60°C, increased to 300°C with a ramp of 15ºC/min, the temperature was held at 300°C for 7 min until elution was complete. After 15 seconds the split valves were opened for 3 min to purge the injector. All injections (1µL) were made with a 10 µL syringe. Helium gas (purity of 99.999%) was used as the carrier gas at a flow rate of 1.0 mL/min.[26]

Statistical analysis

All the assays were carried out in triplicate and values were expressed as means ± SD. Significance between antioxidant activity of extract

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and control was analyzed using Student's t-test. The P values less than 0.05 were considered statistically significant.

Results and Discussion

Yield and total phenolic content

The total yield of the methanol extract was determined after drying via a rotatory evaporator. The yield of *P. palaestina* leaves methanol extract was 28.2% (w/w). The total phenolic content of the extract of *P. palaestina* leaves was 242.2 mg Gallic acid equivalent (GAE)/g extract. This result was consistent with previous studies showing that methanol is an excellent solvent for the extraction of Pistacia phenolic components.[27-29] It was also in accordance with the previously reported high phenolic content (124.1 mg GAE/g DW) in Pistacia leaves.[7]

HPLC-PDA Profiles and GC–MS analysis of P. palaestina extract

HPLC-PDA and GC- MS analysis indicated presence of 19 compounds in the methanolic extract of *P. palaestina* leaves (Figure 1). Their chromatographic runs on both systems are presented in Figure 2a and 2b. The eluted compounds were detected in the range of 50-64 min (Figure 2b); indicating nonpolar constituents. The extracted constituents of *P. palaestina* were investigated using GC–MS (Table 1). Each constituent in the methanolic extract was quantified and identified by comparing mass fragmentation patterns with those listed in Wiley 9 library spectral data and NIST. The dominant constituents in *P. palaestina* extract were 14-methyl-pentadecanoic acid methyl ester (4.99%), 13-Tetradece-11-yn-1-ol (3.423%), benzofenac methyl ester (2.569%), 3,5-bis(1,1-dimethylethyl)-phenol (2.52%), and N-Dimethylaminomethyl-tert.-butyl-isopropylphosphine (1.845%). Moreover, other phenolic derivatives such as, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-Benzenepropanoic acid methyl ester**,** 4-Hydroxyphenyllactic acid ethyl ester di-TMS derivative, Salicylic acid di-TMS derivative and 4-Hydroxyphenyllactic acid ethyl ester were also identified.

The results of the HPLC confirmed that *P. palaestina* extracts have polyphenol components; The UV-VIS of such molecules was between 210-350 nm, which indicated an abundance of flavonoids.[26] The previous reports on the phytochemical examination of this plant has confirmed the presence of flavonoids and flavonols in its methanol extracts, in addition to bioactive metabolites such as gallic acid, digalloylquinic acid, procyanidine, epicatechin, epigallocatechin gallate, trigalloyquinic acid, myricettin, myricetine, and epicatechin gallic acid.[28,30,31] GC–MS chromatographs have shown that the *P. palaestina* methanol extract comprises 19 maximum retention times from 2.009 to 39.33 minutes (Table 1). The study found that the extract is made up of oxygenated hydrocarbons, carboxylic acids, amines, and aromatic compounds.

Table 1: Main chemical components of *P. palaestina* leaves extract analyzed by GC–MS

Pk #	Rt (min)	Compound Name	Peak Area (%)
1	2.009	Benzofenac methyl ester	2.569
2	2.194	3,3'-Oxybis-1-Propene	0.554
3	2.219	methylnitroso-carbamic acid ethyl ester	0.378
4	11.002	6-methyl-1-Heptene	0.633
5	16.199	6-methyl-1-Octene	1.275
6	19.105	3,5-bis(1,1-dimethylethyl)-Phenol	2.520
7	20.996	6-methyl-1-Octene	1.344
8	25.338	1-Tridecyn-4-ol	0.552
9	26.223	16-Heptadecenal	0.475
10	27.729	N-Dimethylaminomethyl-tert.-butyl-isopropylphosphine	1.845
11	27.989	14-methyl-pentadecanoic acid methyl ester	4.990
12	28.094	3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid methyl ester	0.946
13	31.100	Tridecane	1.318
14	31.215	13-Tetradece-11-yn-1-ol	3.423
15	31.415	trans-2-Undecen-1-ol	0.537
16	31.700	2-Methylheptanoic acid	0.673
17	38.248	Salicylic acid, 2TMS derivative	0.741
18	38.938	4-Hydroxyphenyllactic acid ethyl ester, di-TMS	0.438
19	39.33	tripropyl-Bismuthine	0.405

DPPH and ABTS$^{\bullet+}$ radical scavenging activity

The DPPH and ABTS$^{\bullet+}$ assays were used to assess the antioxidant activity of the extract. DPPH scavenging activities of *P. palaestina* leaves extract and the standard (vitamin C) are given in Table 2. The crude methanol extract from leaves was able to scavenge 50% of generated DPPH radicals at IC_{50} (8.31 ± 0.91 µg/mL) compared to Vitamin C with IC_{50} (6.96 ± 0.35 µg/mL); there was a non-significant difference between the activity of the tested *P. palaestina* leaves extract and the standard ($p > 0.05$) (Table 2). However, the methanol crude extract from leaves of *P. palaestina* exhibited antioxidant activity in ABTS$^{\bullet+}$ assay with an IC_{50} (6.86 ± 0.52 µg/mL); a concentration comparable with that of the standard (Vitamin C with IC_{50} (6.09 ± 0.73 µg/mL) (Table 3). Although there were no significant differences between antioxidant activity of *P. palaestina* leaves extract and ascorbic acid IC_{50} in both assays, a significant difference between extract and ascorbic acid activities at 2 and 10 µg/mL ($p<0.05$) was noticed in scavenging the DPPH and ABTS$^{\bullet+}$ radicals.

These results are in line with those detected in Algerian *P. lentiscus*.[32] Achili *et al*.[33] reported that the Algerian *P. atlantica* leaf extracts were able to neutralize DPPH and ABTS$^{\bullet+}$ radicals at IC_{50} of 2.87 ± 0.16 and 2.76 ± 0.17, respectively. Mohammadi *et al*.[34] also documented Pistacia's nutritional and antioxidant capabilities.

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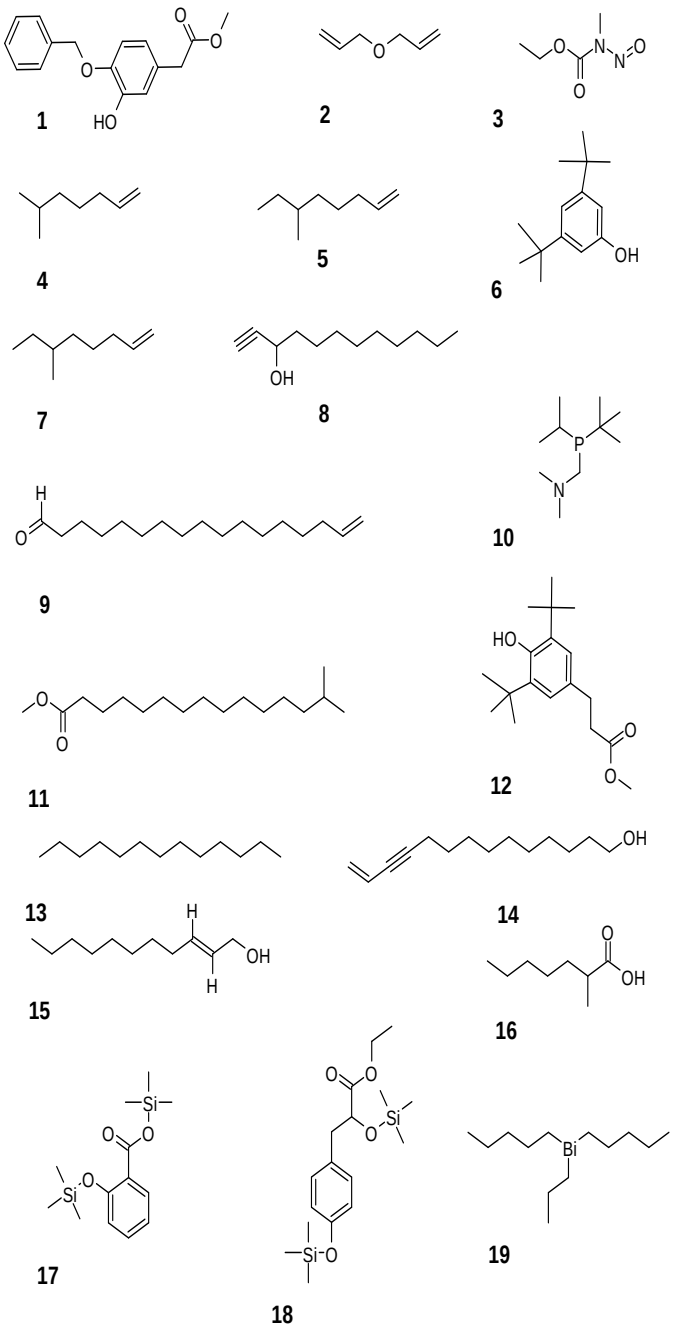

Figure 1: List of secondary metabolites of *P. palaestina* leaves methanolic extract identified by the GC-MS analysis

1: Benzofenac methyl ester; **2**: 3,3'-Oxybis-1-Propene; **3**: methylnitroso-carbamic acid ethyl ester; **4**:, 6-methyl-1-Heptene; **5**: 6-methyl-1-Octene; **6**: 3,5-bis(1,1-dimethylethyl)- Phenol; **7**: 6-methyl-1-Octene; **8**: 1-Tridecyn-4-ol; **9**: 16-Heptadecenal; **10**: N-Dimethylaminomethyl-tert.-butyl-isopropylphosphine; **11**: 14-methyl-pentadecanoic acid methyl ester; **12**: 3,5-bis(1,1-dimethylethyl)-4-hydroxy-benzenepropanoic acid methyl ester; **13**: Tridecane; **14**: 13-Tetradece-11-yn-1-ol; **15**: trans-2-Undecen-1-ol; **16**: 2-Methylheptanoic acid; **17**: Salicylic acid, 2TMS derivative; **18**: 4-Hydroxyphenyllactic acid, ethyl ester, di-TMS; **19**: tripropyl-bismuthine.

Table 2: Antioxidant activity of *P. palaestina* leaves methanol extract in DPPH free radical scavenging assay.

Concentrations (µg/mL)	Percent inhibition (mean ±SD)[a]	IC_{50} (µg/mL) (mean ±SD)[a]
Pistacia		
2	29.27 ± 0.98*	8.31± 0.91
5	44.52 ± 0.96	
10	57.93 ± 0.83*	
20	68.55 ± 2.30	
50	82.66 ± 1.30	
100	92.45 ± 0.82	
Ascorbic acid[b]		
2	13.25 ± 0.57*	6.96 ± 0.35
4	26.54 ± 1.56	
6	38.42 ± 0.80	
8	61.88 ± 0.63	
10	73.83 ± 1.19*	

[a]Results expressed as %inhibition (mean ± SD of n = 3) and IC_{50} values. *Values significantly different as compared to control at $P < 0.05$. [b] Standard antioxidant.

The antioxidant effect of phenolic compounds is mostly attributable to their redox property.[35] As a result, the high phenolic and flavonoid content of Pistacia species may account for its free radical scavenging activity. Furthermore, the substantial antioxidant activity of *P. palaestina* is consistent with the findings of Hacbekirolu *et al*.[28] who found that ethanol and ethanol-water extracts of *Pistacia terebinthus* demonstrated strong antioxidant capacity in $ABTS^{\bullet+}$, DPPH, and CUPRAC tests.

Acetylcholinesterase (AChE) inhibition

The methanolic extract of *P. palaestina* revealed a dose dependent inhibition in activity of AChE enzyme (Table 4). The inhibition in enzyme activity reached to 80.2% at the highest tested concentration (100 µg /mL) and with IC_{50} of 57.67 µg /mL; a concentration significantly 10 times higher than that required by the standard galantamine (IC_{50} = 5.64 µg /mL) positive control.

The fact that excess formation of ROS might causes DNA, lipids, and protein damage as well as the advancement of numerous diseases such as Alzheimer's, the antioxidant potential of plant extract is corporate with its anti-AChE activity in treatment of stress-related disorders such as anxiety and Alzheimer's disease.[4,15,33,36,37] AChE is required for the breakdown of nerve impulses during transmission at the cholinergic synapse, where acetylcholine hydrolysis occurs.[38] In a previous study, *P. terebinthus* ethanol and ethanol-water extracts have proved a stronger anticholinesterase action than galantamine.[28]

Intriguingly, some studies have shown that acetylcholinesterase enzyme inhibitors are generally nitrogenous substances, such as alkaloids.[28,40-44] AChE inhibition by *Abutilon indicum* methanol extract has also been reported[45,46] and it caused improvement cognitive impairment and memory in rats with Alzheimer's disease caused by aluminum chloride.[47] Furthermore, some report highlighted the role of *Pistacia* species in improving memory processes.[48,49]

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A

B

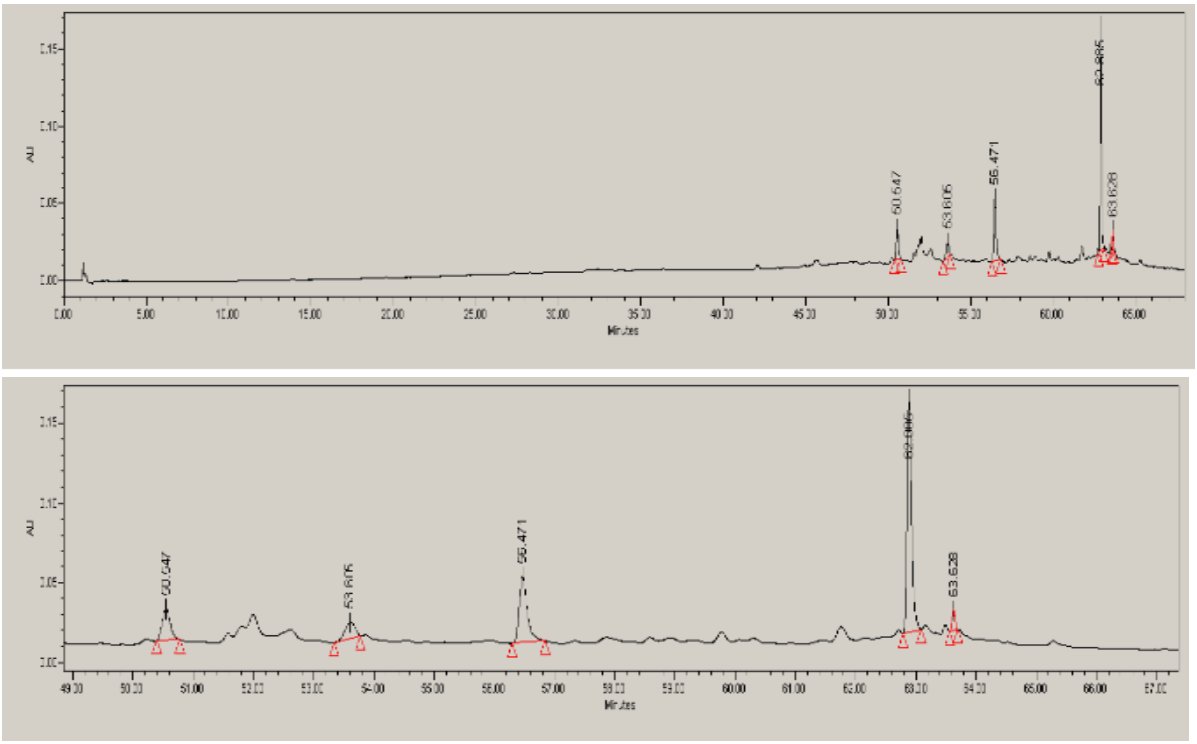

Figure 2: A: HPLC-PDA chromatogram of *P. palaestina* leaves methanolic crude extract at 340 nm. B: eluted compounds were detected in the range of 50-64 min

Table 3: Antioxidant activity of *P. palaestina* leaves methanolic extract in $ABTS^{\bullet+}$ free radical scavenging assay.

Concentrations (µg/mL)	Percent inhibition (mean ±SD)[a]	IC_{50} (µg/mL) (mean ±SD)[a]
Pistacia		
2	32.71 ± 1.57*	6.86 ± 0.52
5	49.42 ± 0.56	
10	60.21± 1.45*	
20	72.11± 0.47	
50	85.75± 1.30	
100	99.49± 0.37	
Ascorbic acid[b]		
2	13.48± 0.60*	6.09± 0.73
4	25.95± 0.89	
6	51.05± 0.94	
8	70.00± 0.58	
10	85.29± 0.71*	

[a] Results expressed as % inhibition (mean ± SD of n = 3) and IC_{50} values. *Values significantly different as compared to control at $P < 0.05$. [b] Standard antioxidant.

Table 4: Anti-acetylcholinesterase activity of *P. palaestina* leaves methanolic extract.

Concentrations (µg/mL)	AChE Percent inhibition (mean ±SD)[a]	IC_{50} (µg/mL) (mean ±SD)[a]
Pistacia		
10	9.90±3.67	57.67±6.9*
25	26.05±4.25	
50	48.84±2.46	
100	80.17±2.76	
Galantamine[b]	-	5.64± 0.89*

[a] Values expressed as means ± SD of three measurements; Inhibitions were determined between 10 µg/mL and 100 µg/mL. IC_{50} values significantly different as compared to control, $*P < 0.05$. [b] Standard and reference drug Galantamine.

Conclusion

P. palsteina possessed antioxidant and anticholinesterase properties. These activities might be attributed to presence of phenolic derivative in its methanol leave extract detected by HPLC and CG-MS.

Conflict of Interest

The author declares no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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