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Chemometric discrimination of three *Pistacia* species *via* their metabolic profiling and their possible *in vitro* effects on memory functions

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ABSTRACT

Alzheimer's disease (AD) is the most widespread neurodegenerative disease; there are around ten million new cases of Alzheimer yearly worldwide especially in middle or low-income countries. *Pistacia* is a genus of flowering plants including the well-known, economically important *P. chinensis* Bunge, *P. lentiscus* L. *and P. khinjuk*. In this study, the metabolic profiling of *Pistacia* leaves extracts was achieved *via* UHPLC-ESI-MS analysis and GC–MS analysis employing chemometric analysis for their discrimination. In addition, the methanolic extracts of different *Pistacia* species were assessed for their anti-cholinesterase and anti-inflammatory activities by various *in vitro* assays. 37 and 30 metabolites belonging to different classes were identified by UHPLC-ESI-MS and GC–MS analyses respectively. Chemometric analysis revealed that *P. lentiscus and P. khinjuk* were more closely related chemically to each other. All studied *Pistacia* leaves extracts showed apparent anti-cholinesterase and anti-inflammatory activities, which promotes their use in the prevention and management of AD.

1. Introduction

Alzheimer's disease (AD) is the most widespread neurodegenerative disease; that shows different symptoms including dementia, amnesia, confusion, impaired cognitive and emotional function. According to the World Alzheimer Reports 2015, there are around ten million new cases of Alzheimer yearly worldwide, 66 percent are living in middle or low income countries [1].

The aetiopathology of AD is complex and varied due to multifaceted disease mechanisms, the most common of which is the alteration of the activities of neurotransmitters, as cholinergic dysfunction. At the synaptic cleft, the signalling of the principal neurotransmitter in the autonomic ganglia (acetylcholine), is terminated through the break down by acetylcholinesterase (AChE). Hence, cholinomimetics (drugs that mimic acetylcholine activity) and AChE inhibitors (drugs that limit acetylcholine cleavage) have provided a key role to manage AD [2].

Recently, the anti-inflammatory agents that play a role in the prevention or management of AD received research interest. The cholinergic system and inflammation were linked together as AChE plays a role in cytokine release, in addition, AChE inhibitors play an anti-inflammatory role by indirectly increasing the production of antioxidants in the brain. Hence, AChE inhibitors and anti-inflammatory agents could be employed in prevention and treatment of AD [3].

There are many drugs that are employed for the symptomatic treatment of AD through AChE inhibitors and anti-inflammtory agents but unfortunately these drugs have several drawbacks including side effects, low bioavailability, high cost and requirement of weekly blood monitoring [4]. In view of these limitations, discovering improved therapies for AD becomes a necessity.

Natural products provide a wide array of metabolites that could manage AD through diverse mechanisms such as anti-amyloid production, anti-apoptotic, antioxidant and anti-inflammatory activities, in addition to targeting cholinergic deficits. Thus, systematic *in vitro* and *in vivo* screening of a plethora of plant extracts

Abbreviations: AD, Alzheimer disease; AChE, anti-cholinesterase; DTNB, 5,5′-dithiobis[2-nitrobenzoic-acid]; ESI, electrospray ionization; GC, gas chromatography; HCA, hierarchical cluster analysis; HPLC, high performance liquid chromatography; HRBC, Human Red Blood Cells; MS, mass spectrometry; PC, principal component; PCA, principal component analysis; PDA, photodiode array detection; UHPLC, ultra high performance liquid chromatography.

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can provide novel active material with potential AChE inhibitory activity [2,5,6].

Pistacia is a genus of flowering plants belonging to Anacardiaceae (Cashew family). It contains 10–20 species including the well-known, economically important, *P.vera* (Pistacio nuts), *P. lentiscus* (Mastic) and *P.chinensis* (Ornamental plant). *Pistacia* is found in the flora of many Mediterranean regions [7]. The resins of *P.lentiscus* have high values in Ayurvedic medicine and has its credentials in diverse pharmacopeias.

To our knowledge, extensive researches reported the phytochemical constituents of *P.lentiscus* leaves cultivated in several mediterranean regions as Spain, Morocco, Algeria, Italy, Corsica and Tunisia [8,9]. Fewer researches reported those of *P.chinensis* leaves cultivated in Pakistan and China. In Egypt, there were few studies about the phytochemical composition of the leaves of *P.lentiscus*, *P.chinensis* and *P.khinjuk* [10].

It is well established that *P.lentiscus* has antioxidant and AChE inhibition activities [9], which makes other *Pistacia* species potential targets for evaluation as AChE inhibitors and anti-inflammatory agents.

This study is an attempt to profile the metabolites of the leaves of three *Pistacia* species cultivated in Egypt, namely; *P.lentiscus*, *P.chinensis* and *P.khinjuk via* UHPLC-ESI-MS and GC–MS analysis to find the easiest, most suitable efficient method to explore their compositional heterogeneity. Due to complexity of these hyphenated techniques, chemometric analysis was performed to find simialrities and diffrences among different species to allow their discrimination. In addition the methanolic extracts were assessed for AchE inhibitory and anti-inflammatory activities aiming at discovering newer potent and long lasting treatment for AD with minimal side effects.

2. Materials and methods

2.1. Plant material

Leaves of different *Pistacia* species (*P.lentiscus, P.khinjuk* and *P.chinensis*) were collected from El Zohreya Gardens, Giza, Egypt. Taxonomical identification was kindly carried out by Dr. Mohamed El-Gebaly; National Research Center, Giza, Egypt. Voucher specimens of all collected samples were kept at Pharmacognosy Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA) with codes (MSA-2017-5, MSA-2017-6 and MSA-2017-7 respectively).

2.2. Chemicals and reagents

Acetylcholinesterase (EEC 3.1.1.7) from electric eel (*Electrophorus electricus*), acetylthiocholine iodide, 5, 5′-dithiobis[2-nitrobenzoic-acid] (DTNB) and acetyl salicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Voltaren was purchased from (Novartis, Egypt). Buffers and all other chemicals of analytical grade were purchased from Sigma-Aldrich.

2.3. Sample preparation and instrumentation

2.3.1. UHPLC-electro spray ionization-mass spectroscopy (UHPLC-ESI-MS)

The leaves of different *Pistacia* species were air-dried and grinded into coarse powder to give 100 g each. Then, extraction with methanol (1.5 L) by using a Soxhlet apparatus for 8 h was carried out. The obtained extracts were filtered and evaporated under vacuum at low temperature (45 °C) till dryness using a rotary evaporator (Buchi model, Switzerland) to give total methanol extract for each.

All samples (100 μg/mL) solution were extracted utilizing high performance liquid chromatography (HPLC) solvent grade of methanol, filtered using a membrane disc filter (0.2 μm) then subjected to LC-ESI-MS analysis. Samples injection volumes (10 μL) were injected into the UHPLC instrument equipped with reverse phase C-18 column (ACQUITY UHPLC - BEH C18 1.7 μm particle size 2.1 × 50 mm Column). Filter membrane disc 0.2 μm was employed for preparation of sample mobile phase, and then degassed by sonication before injection. Elution was achieved with flow rate of 0.2 ml/min using gradient mobile phase comprising two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. Elution was performed using the above gradient. Analysis parameters were carried out using negative ion mode as follows: source temperature 150 °C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in the ESI negative ion mode between *m/z* 100–1000. The peaks and spectra were processed using the Masslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

2.3.2. Hydro-distillation of the essential oil and GC–MS analysis

Hydro-distillation of the essential oil of different *Pistacia* leaves was achieved utilizing Clevenger-type apparatus for 3 h. Drying of the oils over anhydrous magnesium sulfate was carried out and kept in sealed vials at – 30 °C away from light for the analyses. The yield [expressed in % (v/w)] was calculated based on the initial dried plant weight.

GC–MS analysis was performed on Shimadzu GCMS-QP 2010 (Koyoto, Japan) equipped with Rtx-5MS capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) (Restek, USA). The oven temperature was kept at 45 °C for 2 min (isothermal) and programmed to 300 °C at 5 °C/min, and kept constant at 300 °C for 10 min (isothermal); injector temperature was 250 °C. Helium was used as a carrier gas with constant flow rate set at 1.41 ml/min. Diluted samples (1% v/v) were injected with split ratio 15: 1 and the injected volume was 1 μl. The MS operating parameters were as follows: interface temperature: 280 °C, ion source temperature: 200 °C, EI mode: 70 eV, scan range: 35–500 amu. Each sample was analyzed triplicate.

Different constituents of the essential oils were interpreted with the aid of the NIST 05 database (NIST Mass Spectral Database, PC-Version 5.0, 2005, National Institute of Standardization and Technology, Gaithersburg, MD, USA). The Automated Mass Spectral Deconvolution and Identification System (AMDIS 2.64, NIST Gaithersburg, MD, USA) deconvoluted the measured mass spectra. The spectra of individual components were transferred to the NIST Mass Spectral Search Program MS Search 2.0, where they were matched against reference compounds of the NIST Mass Spectral Library 2005.

2.4. Assessment of in vitro AChE inhibitory activity

Pistacia species leaves extracts were estimated as AchE inhibitors employing the method described by Ellman et al. [11] with slight modifications. The enzyme substrate was acetylthiocholine iodide and DTNB was used for the measurement of thiocholine resulting from cholinesterase activity. Sodium phosphate buffer (100 mM, pH 8.0, 140 μL), DTNB (10 μL, 0.1 M), tested *Pistacia* leaves extracts (20 μL) at an initial concentration of 100 mg/mL, were added to the first well and then serial dilution to ten folds down the plate was carried out. After that, AChE (20 μL) was added and incubated at 25 °C for 15 min. by the addition of acetylthiocholine (10 μL, 21.67 mg/mL), the reaction was initiated. Acetylthiocholine hydrolysis was detected through the development of 5-thio-2-nitrobenzoate anion due to the reaction

between DTNB and thiocholine, because of the enzymatic hydrolysis of acetylthiocholine at a wavelength of 405 nm for 15 min. The tested *Pistacia* leaves extracts were dissolved in DMSO in the assay mixture (which has no effect on AchE activity). All reactions were achieved in triplicate in 96-well microtitre plates and monitored in a SpectraMax 340 spectrometer (Molecular Devices, Sunnyvale, CA, USA). The enzyme solution was replaced by buffer to account for non-enzymatic hydrolysis of the substrate, and the sample background subtracted. The extracts concentrations that caused 50% inhibition of acetylthiocholine hydrolysis (IC_{50} values) were calculated with the software program GraphPad Prism, version 5.01 (San Diego, CA, USA).

2.5. *Assessment of* in vitro *anti-inflammatory activity*

2.5.1. *Effect on albumin denaturation*

The anti-inflammatory activities of *P. chinensis, P. lentiscus* and *P. khinjuk* were evaluated utilizing albumin denaturation inhibition method [12]. The reaction mixture included different concentrations of extracts/standard drug (100 μl) and 1% aqueous solution of bovine albumin (500 μl), the mixture was incubated in the water bath at 37 °C for 20 min, denaturation was inducing by keeping the mixture at 51 °C for 20 min, after cooling the turbidity was measured at 660 nm using UV spectrophotometer (UV-1800 Shimadzu). The experiment was performed in triplicates. inhibition percentage of protein denaturation was measured as follows: Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

2.5.2. *Membrane stabilization method*

2.5.2.1. Preparation of human red blood cells suspension. Collection of 10 ml human blood from healthy human volunteer who is not taking NSAIDS for 2 weeks prior to the experiment into centrifuge tube was carried out. Centrifugation of the tube was at 3000 rpm for 15 min was achieved, the supernatant was discarded and then the pellet was washed with equal volume of saline in triplicate manner. After that the volume was measured and reconstituted as 10% V/V suspension with normal saline [12].

2.5.2.2. Heat induced haemolysis. The reaction mixture was achieved by 1 ml of different concentrations of extracts and 1 ml of 10% RBCs suspension, addition of saline only in the control test tubes instead of extracts was carried out. Aspirin was used as standard drug; all the tubes were incubated in the water bath at 56 °C for 30 min and cooled under running tap water. The reaction mixtures were centrifuged at 2500 rpm for 5 min and the absorbance of supernatants was measured at 560 nm using UV spectrophotometer (UV-1800 Shimadzu) [12]. The experiment was implemented in triplicates. The inhibition percentage of hemolysis was measured as follows:

$$\text{Percentageinhibition} = (\text{Abscontrol} - \text{Abssample})\text{X}100/\text{Abscontrol}$$

2.5.2.3. Hypotonicity induced haemolysis. Different concentrations of extracts, control and standard drug were separately mixed with 1 ml of phosphate buffer, 2 ml hyposaline solution and 0.5 ml of HRBC suspension, Diclofenac sodium was used as reference drug (100 μg/ml), the reaction mixtures were incubated in the water bath at 37 °C for 30 min then centrifuged at 3000 rpm, the supernatant was discarded and the hemoglobin content was measured using UV spectrophotometer (UV-1800 shimadzu) at 560 nm. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100% [12]. Percentage protection = 100-(Abs sample/Abs control) x 100

2.6. *Statistical and chemometric analysis*

The data obtained from UHPLC-ESI-MS and GC—MS were subjected to chemometric analysis. Principal component analysis (PCA) comprises a first step in data analysis in order to provide an overview of all observations and samples to identify and evaluate groupings, trends and strong outliers. Hierarchal Cluster Analysis (HCA) was then applied to allow clustering of different samples. The clustering patterns was constructed by applying the complete linkage method used for group building; this presentation is more efficient when the distance between samples (points) is computed by Euclidean method. Both PCA and HCA were achieved utilizing Unscrambler® X 10.4 from CAMO (Computer Aided Modeling, AS, Norway).

Results of *in vitro* assays were expressed as Mean ± SD. The difference between the groups was compared by One-Way Analysis Of Variance (ANOVA) followed by Bonferroni's Multiple comparison test using the software Graph Pad Prism version 6. All P values reported are two-tailed and $P<0.05$ was considered significant.

3. Results and discussion

3.1. *UHPLC-ESI-MS profiling of different* Pistacia *species*

The analysis of *Pistacia* leaves extracts *via* UHPLC-ESI-MS identified thirty-seven metabolites including flavonoids glycosides, anthocyanins, catechins, different phenolic acids, and their derivatives, which represented about 53.1%, 58.85% and 68.62% with respect to total weight of the total extract in *P. chinensis, P. lentiscus* and *P. khinjuk* respectively. The peak assignments of the metabolites are based on their retention times, molecular mass of parent ion and mass fragmentation behaviors compared to data previously reported in the literature [10]. The identities, observed positive and negative ionization data (molecular and fragment ions) and relative percentages per individual metabolites are given in Table 1. It is noteworthy the predominance of 3,5-*O*- digalloyl quinic acid in the leaves extracts of *P. lentiscus* and *P. khinjuk* (13.48 and 20.6% respectively), while, quercetin-3-*O*-di-hexose-*O*-pentose was the major identified component of the leaves extract of *P. chinensis* (18.1%). Also, several compounds were detected in all studied species such as kaempferol-3-*O*-malonylhexoside (***3***), quercetin-3-*O*-glucoside (isoquercitrin) (***13***), luteolin-3-*O*-rutinoside (***19***), epicatechin-3-gallate (***27***), 3,5-*O*- digalloyl quinic acid (***28***), 3,4,5-*O*-trigalloyl quinic acid (***29***) and quinic acid derivative (***34***) and therefore could not be used to discriminate between the studied species however this favors their employment as landmarks for *Pistacia* species. UHPLC-ESI-MS chromatograms of different *Pistacia* species are shown in Fig. (S1).

3.2. *Chemometric analysis of UHPLC-ESI-MS*

As can be seen from UHPLC-ESI-MS chromatograms Fig. (S1), differentiation between *Pistacia* species cannot be easily observed due to the close peak patterns and diversity of different metabolites. Chemometric analysis acts as a fundamental discriminatory tool *via* applying principal component analysis (PCA) and hierarchal cluster analysis (HCA). PCA score plot of thirty-seven identified metabolites revealed the ability of UHPLC-ESI-MS technique to discriminate between different *Pistacia* species without any overlapping by explaining 100% of the variance in the data (the first PC accounts for 82% of the total variance followed by the second PC 18%) as shown in Fig. 1a). PCA indicated the chemical closeness of *P.lentiscus* and *P.khinjuk* as both of them are located on the negative side of PC1. Additionally, the separation detected in PCA can be elucidated in terms of the inter-

Table 1
Peak assignment of metabolites in *Pistacia* species leaves extracts using LC–MS in the positive and negative modes.

Peak No.	Positive Ionization		Negative Ionization		Tentative Compound Assignment	Distribution in *Pistacia* species			Reference
	$[M+H]^+$ *(m/z)*	Product ion fragment *(m/z)*	$[M-H]^-$ *(m/z)*	Product ion fragment *(m/z)*		*P.chinensis*	*P.lentiscus*	*P.khinjuk*	
Flavonols									
1	743.1	287.4	741.3	284.5	Kaempferol-3-*O*-di-hexose-*O*-pentose	–	–	**+ (5.5)**	[18]
2	ND	ND	725.1	593.4	Kaempferol-3-*O*-hexose-*O*-deoxy-hexose-*O*-pentoside	–	**+ (1.55)**	–	[18]
3	535.1	287.4	533.0	489.2	Kaempferol-3-*O*-malonylhexoside	**+ (9.57)**	**+ (0.61)**	**+ (0.44)**	[18]
4	479.1	303.1	ND	ND	Quercetin-3-*O*-glucuronide	–	**+ (4.57)**	**+ (0.14)**	[19]
5	631.2	479.3	ND	ND	Quercetin galloyl hexuronide	–	**+ (0.23)**	–	[20]
6	773.2	610.8	ND	ND	Quercetin glucoside-*O*-rutinoside	–	**+ (0.07)**	–	[21]
7	610.8	303.2	608.8	301.2	Quercetin-3-*O*-rutinoside (Rutin)	–	**+ (0.21)**	–	[22]
8	601.3	465.2	ND	ND	Quercetin galloyl deoxyhexose	–	–	**+ (0.31)**	[20]
9	616.6	465.2	615.2	301.3	Quercetin galloyl hexoside	**+ (0.06)**	–	**+ (0.27)**	[20]
10	625.2	303.1	ND	ND	Quercetin-3,4-diglucoside	**+ (0.16)**	–	**+ (0.17)**	[23]
11	551.4	303.2	549.2	505.3	Quercetin-3-*O*-malonyl-hexoside	**+ (0.09)**	–	**+ (0.11)**	[18]
12	ND	ND	595.4	301.1	Quercetin-3-*O*-hexose-*O*-pentoside	**+ (0.34)**	–	–	[18]
13	465.4	303.1	463.0	463.0	Quercetin-3-*O*-glucoside (Isoquercitrin)	**+ (0.37)**	**+ (0.19)**	**+ (0.46)**	[22]
14	758.7	597.4	ND	ND	Quercetin-3-*O*-di-hexose-*O*-pentose	**+ (18.1)**	**+ (7.57)**	–	[18]
15	633.2	481.1	ND	ND	Myricetin galloyl hexoside	–	**+ (0.23)**	–	[20]
16	ND	ND	625.2	284.6	Myricetin rutinoside	–	**+ (0.07)**	–	[21]
17	481.4	319.3	ND	ND	Myricetin 3-*O*-galactoside	**+ (0.18)**	–	–	[20]
Flavones									
18	463	287.2	ND	ND	Luteolin-7-*O*-glycuronide	**+ (0.16)**	–	–	[18]
19	595.2	287.4	593.3	285.2	Luteolin-3-*O*-rutinoside	**+ (0.33)**	**+ (7.84)**	**+ (5.91)**	[23]
20	255	145.1	ND	ND	5,7-dihydroxyflavone (Chrysin)	**+ (4.00)**	**+ (2.17)**	–	[24]
Anthocyanins									
21	579.3	426.8	577.4	425.0	Procyanidin B1	**+ (0.34)**	–	**+ (0.24)**	[25]
22	433.2	271.2	ND	ND	Pelargonidin-3-*O*-hexoside	–	–	**+ (0.51)**	[26]
23	493	331.3	ND	ND	Malvidin-3-*O*-glucoside	–	**+ (0.13)**	–	[27]
24	654.7	492.8	653.3	491.0	Malvidin-3,5-*O*-diglucoside	**+ (0.61)**	–	**+ (0.17)**	[27]
Catechins									
25	469.4	306.8	ND	ND	Epigallocatechin hexoside	–	**+ (0.13)**	**+ (0.13)**	[21]
26	459.3	171.2	456.8	168.9	Epigallocatechin-3-gallate	–	–	**+ (0.17)**	[28]
27	443.1	171.2	441.0	168.9	Epicatechin-3-gallate	**+ (9.03)**	**+ (4.57)**	**+ (18.59)**	[28]
Quinic acid derivatives									
28	ND	ND	495.0	191.3	3,5-*O*- digalloyl quinic acid	**+ (0.84)**	**+ (13.48)**	**+ (20.6)**	[29]
29	649.2	497.3	647.1	495.4	3,4,5-*O*-Trigalloyl quinic acid	**+ (0.11)**	**+ (6.03)**	**+ (7.59)**	[23]
30	679	517.1	ND	ND	3,4,5-*O*-Tricaffeoylquinic acid	**+ (0.32)**	–	–	[30]
31	485.1	165.4	ND	ND	di-*O*-p-coumaroylquinic acid	–	**+ (0.07)**	–	[18]
32	501.3	338.9	499.4	336.7	3,4-*O*-(E)-caffeoyl-p-coumaroylquinic acid	**+ (0.26)**	**+ (0.18)**	–	[18]
33	531.3	368.8	ND	ND	3,4-*O*-(E)-caffeoylferuloylquinic acid	–	–	**+ (0.28)**	[18]
34	525.3	211.2	ND	ND	Quinic acid derivative	**+ (8.00)**	**+ (7.33)**	**+ (7.03)**	[20]
Gluconic and anacardic acids									
35	ND	ND	195.2	177.1	Gluconic acid	–	**+ (0.29)**	–	[21]
36	ND	ND	373.2	328.7	Anacardic acid (17:1)	–	**+ (1.33)**	–	[20]
37	293.2	249.1	ND	ND	Anacardic acid (11:0)	**+ (0.23)**	–	–	[20]

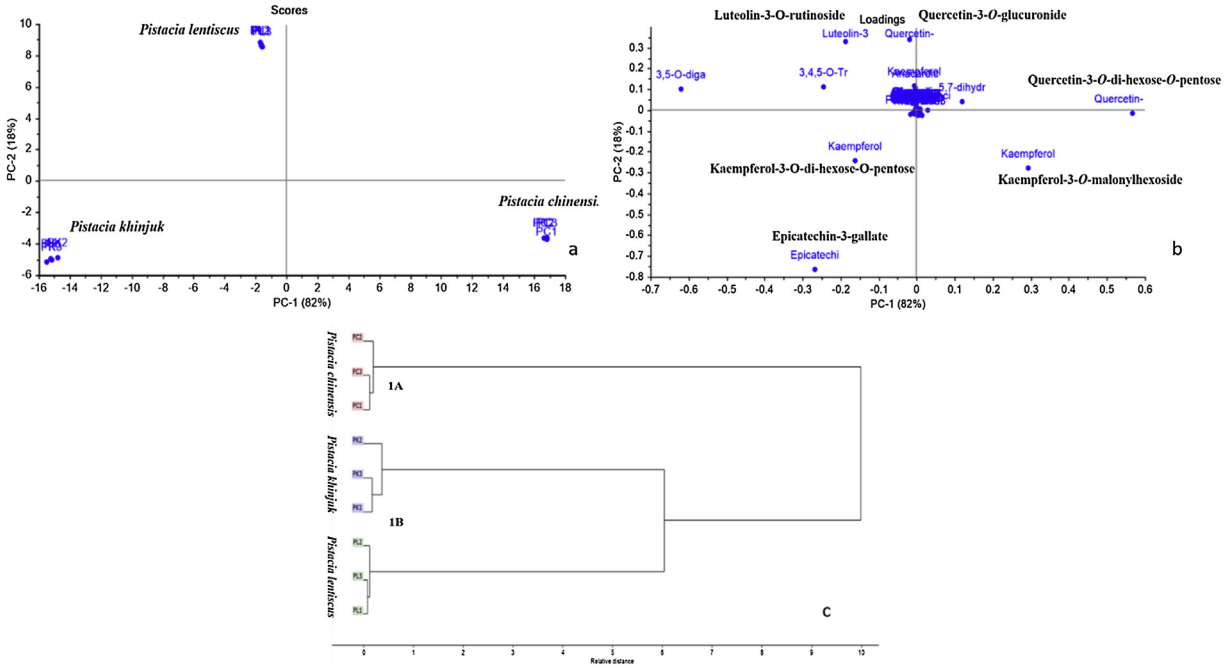

Fig. 1. PCA score plot (a), loading plot (b) and HCA dendogram (c) of different *Pistachia* species based on the analysis of UHPLC-ESI-MS metabolic profile as identified in Table 1,(n = 3).

preted compounds, utilizing the loadings plots for PC components revealing the metabolites responsible for the discrimination as shown in Fig. 1b. Examination of the loadings plot revealed that Kaempferol-3-O-malonylhexoside and Quercetin-3-O-di-hexose-O-pentose accounted for the separation of *P.chinensis* from other species. However, Quercetin-3-O-glucuronide and Luteolin-3-O-rutinoside, contribute most to the discrimination of *P.lentiscus* from *P. khinjuk* that segregated due to prevalence of Epicatechin-3-gallate and Kaempferol-3-O-di-hexose-O-pentose. Moreover, HCA was performed and the obtained dendogram Fig. 1c, displayed two main clusters referred to as groups 1A and 1B, respectively. Inspection of group 1B showed that *P.lentiscus* and *P.khinjuk* were less distant species to each other in comparison to *P.chinensis*, which clustered in one separate group 1A. This reveals that *P.lentiscus* and *P.khinjuk* are more closely related chemically to each other indorsing results obtained from PCA.

Results of UHPLC-ESI-MS in combination with chemometric analysis revealed that complete metabolic profiling was the significant key in the identification and segregation of different *Pistacia* species from each other rather than the major identified compounds in each species.

3.3. *GC–MS analysis of different* Pistacia *species*

The hydro-distillation of the leaves of *P. lentiscus*, *P.khinjuk* and *P. chinensis* gave pale yellow oils with a yield of (0.23% v/w), (0.21% v/w) and (0.18% v/w), on fresh weight basis, after 3 h respectively. The identified components, their percentage, retention indices and retention time are summarized in Table 2. The presented values are the mean of triplicate analyses for the essential oil of different *Pistacia* species. Representative chromatograms of *P.lentiscus*, *P.khinjuk* and *P. chinensis* were displayed in Figs. (S2–S4) of Supplementary Material respectively.

Results from GC–MS revealed the identification of thirty components in the oils of *P. lentiscus, P.khinjuk* and *P.chinensis*, which represented about 96.03%, 98.62% and 93.71% of the total detected components respectively. Major annotated metabolites were monoterpenes, which represent the major class of the oil composition in the three species (76.73%) for *P.lentiscus,* (78.98%) for *P.chinensis* and (81.65%) for *P.khinjuk*, whereas sesquiterpenes and other classes are present in a lower quantity.

The qualitative composition of both *P.lentiscus* and *P.khinjuk* essential oils was comparatively the same, where α-Pinene was recognized as the major constituent in *P.lentiscus* (38.14%) and *P.khinjuk* (50.71%). Regarding *P.lentiscus,* these results were in accordance to that reported in literature [13]. However, *P.chinensis*, showed different quantitative composition when being compared to other *Pistacia* species as 3-carene was the main identified component (72.46%), which is completely absent in other *Pistacia* species.

3.4. *Chemometric analysis of GC–MS*

As obvious from Table 2, there are many qualitative and quantitative similarities and differences between the essential oils of different *Pistacia* species. The relative peak areas of all the detected components (30 component) of different *Pistacia* species (three replicates for each species) were subjected to both PCA and HCA.

PCA score and loading plots are presented in Fig. 2a and (b) respectively. By examination of the score plot, the contribution rates of the first, second principal component were 97% and 3%, respectively explaining 100% of the variance in the data. Different *Pistacia* species could be discriminated from each other, where they are clustered into three main groups. Nevertheless, the plot showed the closeness of *P.khinjuk* and *P.lentiscus* as both species were grouped on the right side of the plot although they are completely segregated, very far away from *P.chinensis* that located solely on the left side. The loading plot showed that main discriminating makers were α-Pinene, 3-Carene and α-Phellandrene for *P.khinjuk, P.chinensis* and *P.lentiscus* respectively. HCA dendogram as shown in Fig. 2c absolutely discriminated the three species establishing three main clusters. The dendogram confirmed results obtained from PCA.

By comparison of different applied techniques, the discrimination power was compared from their corresponding PCA and HCA results. The PCA score plot derived from UHPLC-ESI-MS and GC—MS displayed the same segregation pattern, where all *Pistacia* species

Table 2
Metabolic profiling of *Pistacia* species leaves essential oils by.GC–MS

No.	R_t	Compound	RI^a exp	RI^b Rep	Content %			Molecular Formula	Identification [f]
					PL[c]	PC[d]	PK[e]		
1	7.276	Tricyclene	912	918	0.89	0.09	0.47	$C_{10}H_{16}$	MS, RI
2	7.648	α-Pinene	927	927	38.14	0.69	50.71	$C_{10}H_{16}$	MS, RI
3	8.072	Camphene	941	943	3.75	0.25	2.56	$C_{10}H_{16}$	MS, RI
4	8.846	Sabinene	967	964	0.14	0.17	0.21	$C_{10}H_{16}$	MS, RI
5	8.935	β-Pinene	972	972	9.533	0.20	9.98	$C_{10}H_{16}$	MS, RI
6	9.388	β-Myrcene	989	989	0.80	0.62	0.68	$C_{10}H_{16}$	MS, RI
7	9.793	α-Phellandrene	1003	1003	10.05	0.22	1.71	$C_{10}H_{16}$	MS, RI
8	9.995	3-Carene	1009	1009	–	72.46	–	$C_{10}H_{16}$	MS, RI
9	10.428	p-Cymene	1022	1023	1.08	0.27	3.74	$C_{10}H_{14}$	MS, RI
10	10.563	D-Limonene	1029	1031	11.91	2.41	10.86	$C_{10}H_{16}$	MS, RI
11	11.24	Cis-β-Ocimene	1049	1047	–	0.21	–	$C_{10}H_{16}$	MS, RI
12	12.44	α-Terpinolene	1097	1099	0.14	0.81	0.1	$C_{10}H_{16}$	MS, RI
13	12.926	Nonanal	1115	1112	–	0.28	–	$C_9H_{18}O$	MS, RI
14	15.245	4-Terpinenol	1178	1180	0.23	0.30	0.19	$C_{10}H_{18}O$	MS, RI
15	15.656	α-Terpineol	1191	1191	0.92	0.31	0.86	$C_{10}H_{18}O$	MS, RI
16	20.285	α- Cubebene	1353	1352	0.41	–	0.65	$C_{15}H_{24}$	MS, RI
17	21.308	β- Bourbonene	1386	1386	0.11	0.35	0.71	$C_{15}H_{24}$	MS, RI
18	21.428	β -Cubebene	1393	1392	0.22	–	0.44	$C_{15}H_{24}$	MS, RI
19	22.271	*β*-Caryophyllene	1420	1420	1.89	0.27	1.77	$C_{15}H_{24}$	MS, RI
20	23.182	α-Caryophyllene	1452	1452	0.42	–	0.36	$C_{15}H_{24}$	MS, RI
21	23.916	ɣ- Muurolene	1486	1489	7.05	4.01	1.29	$C_{15}H_{24}$	MS, RI
22	24.365	α- Muurolene	1504	1505	0.2	0.14	–	$C_{15}H_{24}$	MS, RI
23	24.458	α- Farnesene	1508	1508	0.15	–	–	$C_{15}H_{24}$	MS, RI
24	24.958	δ-Cadinene	1527	1529	1.7	0.58	1.98	$C_{15}H_{24}$	MS, RI
25	25.862	Germacrene B	1562	1561	–	0.10	–	$C_{15}H_{24}$	MS, RI
26	26.115	Z-3-Hexenyl benzoate	1573	1573	0.43	1.21	0.98	$C_{13}H_{16}O_2$	MS, RI
27	26.544	Caryophyllene oxide	1585	1583	0.12	0.35	1.47	$C_{15}H_{24}O$	MS, RI
28	28.229	α-Cadinol	1655	1660	1.37	2.02	2.4	$C_{15}H_{26}O$	MS, RI
29	30.743	Benzyl Benzoate	1766	1773	0.63	2.37	–	$C_{14}H_{12}O_2$	MS, RI
30	37.682	Phytol	2099	2096	–	0.26	–	$C_{20}H_{40}O$	MS, RI
Monoterpene HC					75.57	78.35	80.59		
Oxygenated Monotrpenes					1.16	0.62	1.06		
Sesquiterpenes					12.17	5.48	7.22		
Oxygenated Sesquiterpenes					1.5	2.376	3.87		
Others					1.96	4.22	1.45		
Total identified					96.03	93.71	98.62		

Compounds listed in order of their elution on Rtx-5MS capillary column, [a] Kovats index determined experimentally on Rtx-5MS capillary column relative to C8–C28 n-alkanes, [b] Published Kovats retention indices, [c] *P. lentiscus* essential oil, [d] *P. chinensis* essential oil, [e] *P. khinjuk* essential oil, [f] Identification, was based on comparison of the compounds mass spectral data (MS) and Kovats retention indices (RI) with those of NIST Mass Spectral Library (2011), Wiley Registry of Mass Spectral Data 8th edition and literature. Data presented are the mean values of three replicates.

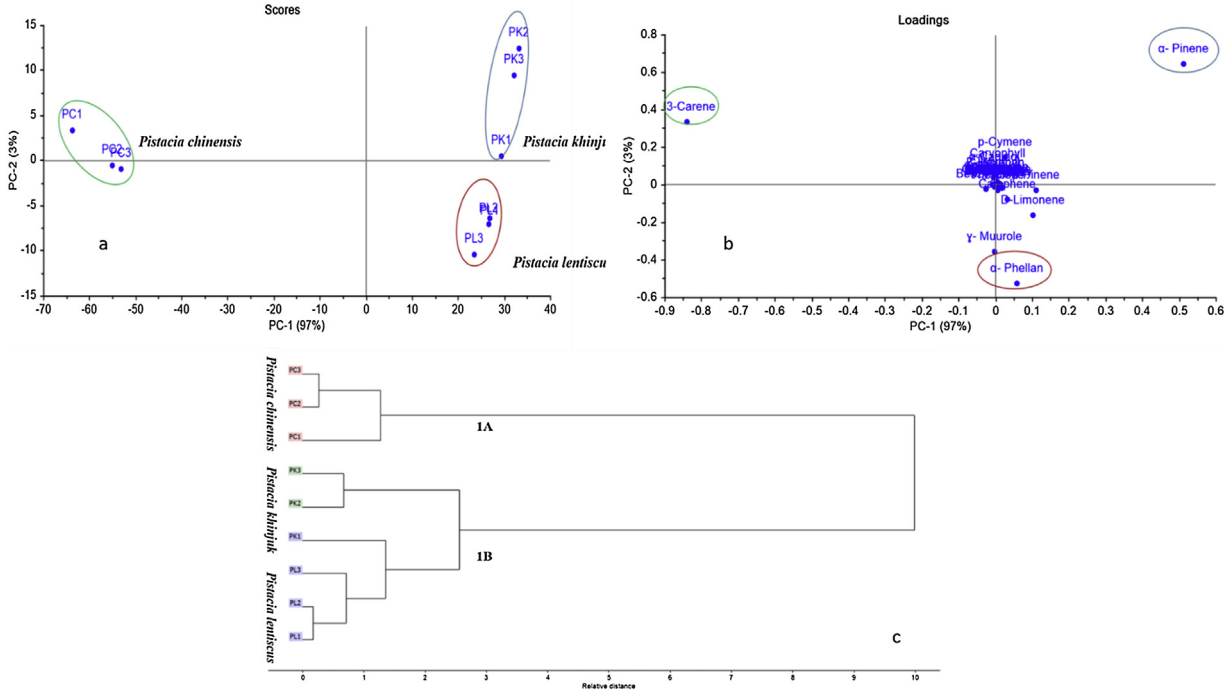

Fig. 2. PCA score plot (a), loading plot (b) and HCA dendogram (c) of GC/MS analysis of essential oils of different *Pistacia* species based on the identification of 30 compounds as shown in Table 2, (n = 3).

Table 3
Effect of *Pistacia* species leaves extracts on heat induced protein denaturation, heat induced hemolysis and hypotonicity induced hemolysis of erythrocyte.

Treatment (s)	Concentrations	Heat induced protein denaturation		Heat induced hemolysis		Hypotonicity induced hemolysis of erythrocyte	
		Mean ± SD	% inhibition	Mean ± SD	% inhibition	Mean ± SD	% inhibition
Control	–	0.923 ± 0.01		0.942 ± 0.035		0.11 ± 0.002	
P. chinensis	100 μg/ml	0.759 ± 0.016*	17.7	0.681 ± 0.015*	27.7	0.087 ± 0.004*#	20.9
P. chinensis	200 μg/ml	0.611 ± 0.005*	33.8	0.615 ± 0.004*	34.7	0.05 ± 0.001*	54.5
P. chinensis	300 μg/ml	0.529 ± 0.026*	42.6	0.579 ± 0.005*	38.5	0.044 ± 0.001*	60
P. chinensis	400 μg/ml	0.439 ± 0.017*	52.4	0.511 ± 0.01*	45.7	0.044 ± 0.002*	60
P. chinensis	500 μg/ml	0.386 ± 0.008*	58.1	0.367 ± 0.001*	61	0.036 ± 0.002*#	67.2
P. chinensis	1 mg/ml	0.228 ± 0.005*#	75.2	0.23 ± 0.002*#	75.5	0.03 ± 0.002*#	72.7
P. lentiscus	100 μg/ml	0.672 ± 0.02*	26.3	0.757 ± 0.007*	19.6	0.091 ± 0.003*#	17.2
P. lentiscus	200 μg/ml	0.393 ± 0.004*	57	0.702 ± 0.004*	25.4	0.063 ± 0.003*#	42.7
P. lentiscus	300 μg/ml	0.248 ± 0.005*	72.8	0.586 ± 0.003*	37.7	0.052 ± 0.002*	52.7
P. lentiscus	400 μg/ml	0.234 ± 0.004*	74.3	0.579 ± 0.003*	38.5	0.048 ± 0.002*	56.3
P. lentiscus	500 μg/ml	0.209 ± 0.007*	77.1	0.478 ± 0.008*	49.2	0.035 ± 0.003*#	68.1
P. lentiscus	1 mg/ml	0.146 ± 0.005*#	**84**	0.139 ± 0.007*#	**85.2**	0.028 ± 0.001*#	74.5
P. khinjuk	100 μg/ml	0.495 ± 0.004*	45.7	0.406 ± 0.005*	57	0.081 ± 0.001*#	26.3
P. khinjuk	200 μg/ml	0.453 ± 0.002*	50.3	0.389 ± 0.007*	58.7	0.052 ± 0.001*	52.7
P. khinjuk	300 μg/ml	0.423 ± 0.003*	53.6	0.329 ± 0.022*	65	0.047 ± 0.002*	57.2
P. khinjuk	400 μg/ml	0.384 ± 0.003*	58	0.301 ± 0.005*	68	0.042 ± 0.001*#	61.8
P. khinjuk	500 μg/ml	0.371 ± 0.001*	59.4	0.274 ± 0.004*	71	0.034 ± 0.003*#	69
P. khinjuk	1 mg/ml	0.305 ± 0.004*#	66.5	0.191 ± 0.003*	79.7	0.027 ± 0.001*#	**75.4**
Aspirin	100 μg/ml	0.304 ± 0.004	67	0.250 ± 0.007*	73.4	–	–
Diclofenac	100 μg/ml	–	–	–	–	0.050 ± 0.001*	54.5

Results were expressed as mean ± SD, N = 3 and analyzed using one-way ANOVA followed by Bonferroni's post hoc test; * Significant from control at P < 0.0001, # Significant from Aspirin/Diclofenac at P < 0.0001.

were completely separated from each other. However, it showed that *P.khinjuk,* and *P.lentiscus* were more closely related to each other. HCA dendograms confirmed these results as it showed the close distance of *P.khinjuk,* and *P.lentiscus* to each other as they were grouped in one cluster with regard to that of *P.chinensis*.

3.5. *Assessment of* in vitro *AChE inhibitory activity*

A wide range of plant metabolites was found to possess AChE inhibitory activity, mainly alkaloids, xanthones and flavonols, among others. Hence, the intake of polyphenols through diets was stated to reduce incidence of certain age related neurological disorders including AD [14]. It is well established that quercetin, myrecetin, kaempferol and their glycosides possess AChE inhibitory activities [15]. However, anthocyanins as pelargonidin, delphinidin and cyanidin were found to be the most efficient AChE inhibitors [16]. Interestingly, the UHPLC-ESI-MS analyses of *Pistacia* leaves extracts revealed the presence of relatively high concentrations of polyphenolic compounds, which could synergistically contribute to the AChE inhibitory activities of the studied extracts.

Although the *in vitro* AChE inhibitory activity of *P. lentiscus* was previously reported [9] we hereby extend the research to other *Pistacia* species. Our results, Table (S1), showed that the leaves extracts of *P. lentiscus* showed the least IC_{50} (8.46 mg/ml) compared to the other two studied species (*P. chinensis and P. khinjuk*) extracts with IC_{50} values of (13.83 mg/ml and 9.23 mg/ml) respectively. Overall, all studied extracts exhibited a significant dose-related AChE inhibitory activity.

3.6. *Assessment of* in-vitro *anti-inflammatory activity*

To combat the multifactorial nature of AD, additional subsidiary activities such as anti-inflammatory activities evaluation is considered an add-on. The anti-inflammatory activity of the extracts was evaluated *in vitro* as the implementation of *in vivo* studies in the experimental pharmacological research had some drawbacks such as ethical issues, lack of rational of their use when some other methods could be used. Most of the inflammatory diseases are characterized mainly by the presence of inflammation where the proteins are denatured so the agents that could prevent protein denaturation would be used as ant-inflammatory drug development [17]

Human Red Blood Cells (HRBC) membrane stabilization has be used as a method to evaluate the anti-inflammatory effect of the extracts knowing that the erythrocyte membrane is analogous to lysosomal membrane. Stabilization of lysosomal is crucial point in limiting the inflammatory response *via* inhibiting the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. When theses enzymes are released during inflammation lead to various disorders. The extra cellular activity of these enzymes are related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [12]

3.6.1. *Inhibition of protein denaturation*

The ability of the plant extract to inhibit protein denaturation is considered to be an anti-inflammatory effect, maximum inhibition of 84% was observed in *P. lentiscus* at dose 1 mg/ml, while Aspirin at dose 100 μg/ml as a standard drug; its inhibition was 67%

3.6.2. *Heat induced hemolysis of erythrocyte*

The different extracts showed a significant protection for human erythrocyte membrane against lysis induced by heating at different concentrations at $P < 0.0001$; *P. lentiscus* at dose 1 mg/ml showed the best protection of RBCs membrane against hemolysis at 85.2% while Aspirin used as the standard drug showed protection at 73.4% as shown in Table 3

3.6.3. *Hypotonicity induced hemolysis of erythrocyte*

Concentrations at (100 μg/ml - 1 mg/ml) of the extracts showed a significant protection of erythrocyte membrane against lysis induced by hypotonic solution, at dose 1 mg/ml, *P. khinjuk* showed a maximum protection of 75.4% while Diclofenac sodium (100 μg/ml) showed 54.5% inhibition of hemolysis of erythrocyte when compared to control at $P < 0.0001$. Non-significant differ-

ence was found between *P. khinjuk* and *P. lentiscus* against the lysis induced by hypotonic solution at high dose.

4. Conclusion

In this research, the metabolic profiling of the leaves extracts of three *Pistacia* species cultivated in Egypt were assessed *via* UHPLC-ESI-MS and GC–MS in an attempt to find the most suitable method for their identification and discrimination. To the best of our knowledge, this study provides the first challenge for the analyses of different *Pistacia* species in a comparative manner utilizing various applied techniques. UHPLC-ESI-MS revealed the presence of diversity of metabolites including flavonoids, anthocyanins and phenolic acids. Regarding GC–MS thirty compounds were detected including various monoterpenes and sesquiterpenes. The discrimination among different species based on metabolomics was achieved utilizing PCA and HCA. The present study showed that the complete metabolic profiling obtained by different applied techniques represent a fingerprint for each *Pistacia* species that could be utilized for complete identification and segregation from each other. The methanolic extracts of different species exhibited promising anti-cholinesterase and anti-inflammatory activities as evaluated *via in vitro* assays which are the impetus for *in vivo* studies and we hereby definitely recommend that the studied *Pistacia* species extracts are potential dual-targeted candidates for *in vivo* studies.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.112840.

Declaration of Competing Interest

The authors declare no conflict of interest.

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