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ACETYLCHOLINESTERASE INHIBITORY ACTIVITY OF *DOREMA AMMONIACUM* GUM EXTRACTS AND MOLECULAR DOCKING STUDIES

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ABSTRACT: Ammoniacum gum is traditionally used as an expectorant, stimulant, antispasmodic, catarrh, asthma, chronic bronchitis, and enlargement of liver and spleen in Medicine. The Soxhlet extraction of the oleo gum resin of *D. ammoniacum* was carried out with organic solvents including hexane, dichloromethane, chloroform, ethyl acetate, and methanol. The screening of the anti-alzheimer activity of the extracts was measured by acetylcholinesterase (AChE) inhibitory activity, and the hexane (IC_{50} = 32.34 µg/ml) and chloroform (IC_{50} = 43.5 µg/ml) extract performed highest activity. A profiling experiment of the chloroform extract conducted by LC-MS and literature resources suggested six potential biologically active compounds including doremone A (1), kopetdaghin E (2), dshamirone (3), kopetdaghin D (4), kopetdaghin C (5), and ammodoremine (6). To confirm the responsibility of these compounds for the observed AChE inhibitory activity, the molecular docking studies of these compounds were performed with AChE enzyme which indicated that these compounds show higher AChE inhibitory activity than galantamine as positive control. The molecular docking study was carried out using Glide.

INTRODUCTION: Nature is a rich source of medicinal compounds, some of which are stored in plants and possess numerous biological effects. Medicinal plants are good sources of biologically active compounds showing antioxidant, anti-bacterial and anti-acetylcholinesterase activities [1-4]. One of the most intensively studied subjects in the field of phytochemistry (medicinal plants) is the investigation of plants with an anti-cholinesterase action [5, 6, 7].

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Alzheimer's disease (AD) is the most common cause of senile dementia in later life [8, 9, 10]. A loss of acetylcholine (ACh) is considered to play a vital role in the learning and memory deterioration of AD patients [11, 12]. *Dorema ammoniacum* D. Don commonly known as Ushaq (local Persian names of Kandal, Vasha or Koma-kandal) is a monocarpic plant that is native to the arid and semi-arid regions of Iran such as Yazd, Isfahan and Semnan [13, 14]. In Unani System of Medicine, ammoniacum gum is an antispasmodic which is also used in treating enlargement of the liver, chronic bronchitis, and persistent coughing and asthma [15, 16]. It has been used in treatment of spastic pains, gastric disorders, intestinal parasitic infections, and skin inflammations and as analgesic, stimulant, expectorant and laxative [17, 18, 19].

This gum is exudated from the pores of stems, leaves, and petioles of the flowering and fruiting plants of *Dorema ammoniacum* [20, 21]. The acetylcholinesterase (AChE) inhibitory activity of dichloromethane extract of the gum resin was also reported [22, 23]. In the present study, the anti-Alzheimer activity of methanol, chloroform, hexane and ethyl acetate extracts of oleo gum resin from *Dorema ammoniacum* was investigated using microplate colorimetric assay. Additionally, the major constituents of the chloroform extract were determined by LC-PDA-MS analysis and their activity against the cholinergic enzyme *viz.* AChE was analyzed by molecular docking methods.

MATERIAL AND METHODS:

Chemicals: Acetylcholinesterase (AChE), acetylthiocholine iodide (ATCI), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), Tris-HCl, bovine serum albumin (BSA), and all the chemicals used in the acetylcholinesterase inhibitory assays were purchased from Sigma-Aldrich Chemie GmbH, Germany. Two different buffer systems were used. Buffer A: 50 mm Tris-HCl, pH 7.9 containing 0.1% BSA; Buffer B: 50 mm Tris-HCl, pH 7.9 containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$.

Extraction: Oleo gum resin of the species *Dorema ammoniacum* was collected from the desert of Shahroud (35° 25' 32.01" N, 55° 00' 32.46" E) in Semnan province, Iran. A voucher specimen of the plant has been retained in the herbarium of Science Faculty, Golestan University, Gorgan, Iran (voucher number 6251). Extraction was done using Soxhlet extractor with organic solvents of different polarity including hexane, chloroform, ethyl acetate, and methanol. In each experiment, 250 ml of solvent and 25 g of sample were used. The total extraction process was completed within 4 - 8 h. After filtration and evaporation of the solvents under reduced pressure, 11.5, 9.1, 1.1 and 12.2 g of brown residues were obtained from ethyl acetate, methanol, hexane, and chloroform, respectively.

Acetylcholinesterase Activity: The AChE inhibitory activities of the ammoniacum gum extracts were measured by Ellman's method with the aid of a quantitative colorimetric assay 24, 25. A mixture of ATCI (25 µl, 15 mm), DTNB (125 µl, 3 mm in buffer B), buffer A (50 µl) and sample (25 µl, 10 mg/ml in DMSO diluted with buffer A to a concentration of 1 mg/ml) was placed in a 96-well plate. The optical density was measured at 405 nm, five times every 15 s. Then AChE (25 µl, 0.22 U/ml in buffer A) was added followed by incubation at 25 °C for 10 min. Shortly following this, the absorbance was measured eight times every 15 s again. The hydrolysis of acetylthiocholine iodide was monitored as a result of the reaction of DTNB with thiocholines. Repetition of the assay was done three times for each concentration. The assay was validated by measurement of different concentrations of galantamine as a positive control and the percentage of inhibition was calculated by comparing the absorbance of the sample with a blank. The inhibition rate of the samples on acetylcholinesterase was calculated by the following formula:

% Inhibition = [(Absorbance control – Absorbance sample) / Absorbance control] × 100

The IC_{50} values were calculated from inhibition curves (inhibitor concentration vs. percent of inhibition). The IC_{50} value of galantamine was determined as 2.5 ± 0.20 µl. Tacrine, donepezil, rivastigmine, and galantamine are AChE inhibitors used as anti- Alzheimer's disease (AD) drugs [26, 27].

LC-PDA-MS: LC-PDA-MS analysis was carried out on a Shimadzu Prominence high-performance liquid chromatography (HPLC) system which comprised an LC-20 AD binary pump, CTO-20AC column thermostat, SDP-M20A PDA detector, CBM-20A system controller, and coupled to an LCMS-8030 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). Operating conditions were: capillary voltage, 4.5 V; desolvation line temperature, 250 °C; heat block temperature, 500 °C; drying gas (nitrogen) flow, 15 L min^{-1}; nebulizing gas (nitrogen) flow, 3 L min^{-1}. Full-scan (160 - 1500) was carried out with 6000 u/sec scan speed and 0.150 s per event time for data acquisition in both positive and negative ionization modes. LC separation was performed on a C18 Sun Fire column (3.5 µm, 3 × 150 mm i.d., Waters) equipped with a guard column (3 × 20 mm i.d.). HPLC solvents both contained formic acid (0.1%, v/v), and the flow rate was set to 0.4 ml min^{-1}. The following gradient was used: an isocratic step at 50% A for 5 min, the

concentration of solvent B increased up to 100 % in 25 min. The mobile phase was kept at this concentration for 15 min, then decreased to 40% A for 5 min. Data acquisition was done with lab solutions software (Shimadzu). The mobile phase consisted of 10 µl water (A) and acetonitrile (B).

Molecular Docking: Since the drug discovery process of AD is laborious and expensive [28, 29] thus, structure-based drug discovery has gained importance [30 - 33]. In the present study the molecular docking study was used to investigate the binding affinity of identified compounds to the active site of AChE. For this purpose, docking study was carried out using Glide (Schrodinger package 2016-2). The AChE enzyme protein structure file (PDB ID: 1EVE) was taken from the PDB (Protein Data Bank) and edited using protein preparation on Maestro 10.6, where all cofactors, water molecules and co-crystallized ligands were removed from the protein structure before docking [34, 35].

Partial atomic charges were assigned according to the Optimized Potentials for Liquid Simulations (OPLS3) force field. The Ramachandran diagram was used to clarify the applicability of the prepared protein for further docking analysis. The grid box was centered at particular residues of the protein and was generated with Grid generation application with coordinates x = -2.71, y = 28.02, z = 31.71. The dimensions of the active site box were set at 20 × 20 × 20 Å 36. Compounds were docked inside a sphere with a 15 Å radius centered at the largest cavity detected by the program.

RESULTS AND DISCUSSION:

Acetylcholinesterase Inhibitory Assay: In the present study, five extracts of ammoniacum gum were tested for acetylcholinesterase (AChE) inhibitory activity. The results are presented as percentage inhibition values in **Table 1**. As clearly shown the table, the hexane and chloroform extracts with 65.2% and 54.9% inhibition exhibited the highest AChE inhibitory activity while dichloromethane extract with 19.4% inhibition at 50 µg/ml concentration exhibited moderate AChE inhibitory activity in agreement with the previous report [23]. However, ethyl acetate and especially methanol extracts did not show significant activity. The IC_{50} values for AChE inhibition were determined by spectrophotometric measurement of the effect of an increase in the concentrations of test compounds (plant extracts and positive controls) on AChE activity. The IC_{50} values were then calculated from the dose-effect curves using linear regression. As evident in **Table 2**, hexane and chloroform extracts exhibited significantly higher AChE activity than dichloromethane and ethyl acetate extracts.

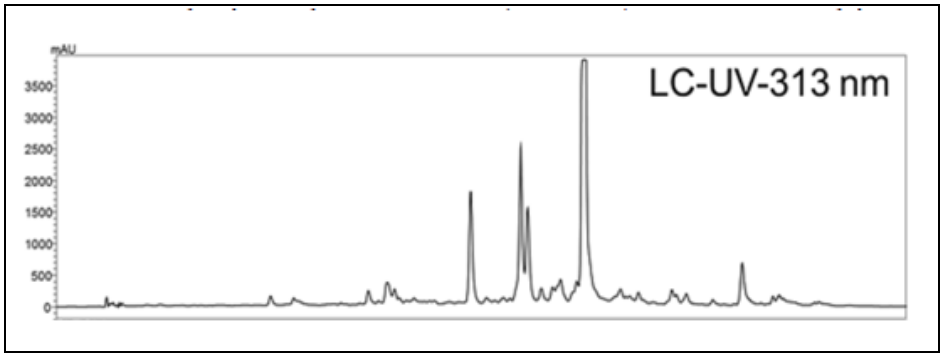
TABLE 1: THE AChE INHIBITORY ACTIVITY OF EXTRACTS (50 µg/ml)

Extracts	Inhibition (%)
Hexane	65.2
Chloroform	54.9
Dichloromethane	19.4
Ethyl acetate	15.7

TABLE 2: AChE INHIBITION OF EXTRACTS OF AMMONIACUM GUM AND THE POSITIVE CONTROL GALANTAMINE (N=3)

Samples	IC_{50}(µg/ml)
Hexane	32.34 ± 1.25
Chloroform	43.50 ± 1.52
Dichloromethane	128.80 ± 1.25
Ethyl acetate	152.40 ± 6.12
Galantamine	2.50 ± 0.20

LC-MS Analysis: The phytochemical profiling of chloroform extract of ammoniacum gum was performed using HPLC-ESI-MS in both positive and negative and PDA analysis. The chromate-grams of LC-MS analysis and HPLC-UV (313 nm) are presented in **Fig. 1**.



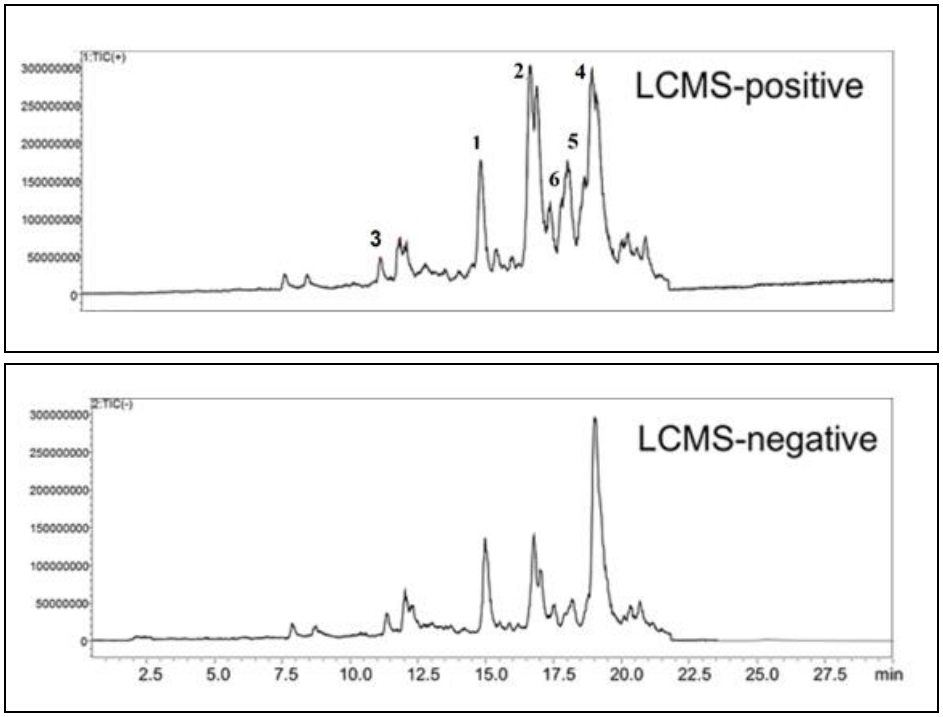
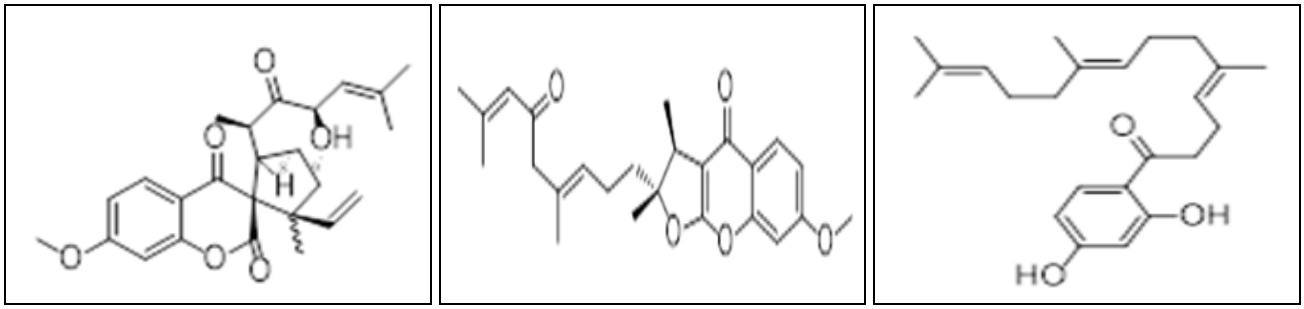
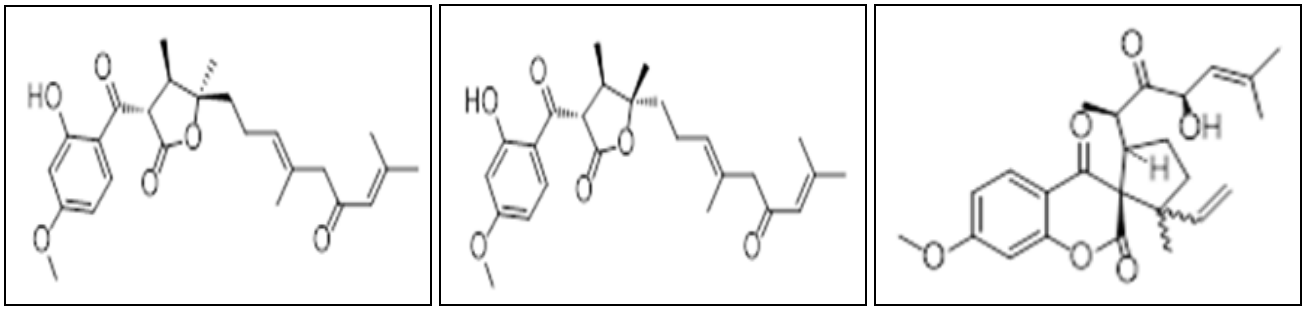


FIG. 1: PHYTOCHEMICAL PROFILING OF A $CHCl_3$ EXTRACT OF AMMONIACUM GUM PERFORMED USING HPLC-PDA-(±)-ESIMS AND UV (313 nm) DETECTION



Doremone A (1) **Kopetdaghin E (2)** **Dshamirone (3)**



Kopetdaghin D (4) **Kopetdaghin C (5)** **Ammodoremin (6)**

FIG. 2: THE CHEMICAL STRUCTURES OF MAJOR COMPOUNDS IN $CHCl_3$ EXTRACT OF AMMONIACUM GUM WHICH ARE IDENTIFIED BASED ON HPLC-PDA-MS ANALYSIS

TABLE 3: THE CHEMICAL COMPOSITION OF MAJOR COMPOUNDS IDENTIFIED IN $CHCl_3$ EXTRACT OF AMMONIACUM GUM

Compound	Rt *	[M+H]+	[M-H]-	UV	Reference
Doremone A (1)	15.03	427	425	205, 285, 312	37
Kopetdaghin E (2)	16.83	-	409	210, 283, 283, 311	38
Dshamirone (3)	11.28	357	355	205, 291, 314	39
Kopetdaghin D (4)	19.22	429	427	206, 280, 302	38
Kopetdaghin C (5)	18.02	429	427	207, 292, 313	40
Ammodoremine (6)	17.55	427	425	205, 277, 306	41

Six major compounds were detected based on data obtained from HPLC-PDA-MS. The compounds were identified by comparing with literature data of fragments of compounds and using Scifinder® and Dictionary of Natural Products 26.2 (Chapman and Hall/CRC). The compounds were identified to be doremone A (1), kopetdaghin E (2), dshamirone (3), kopetdaghin D (4), kopetdaghin C (5) and ammodoremine (6) **Fig. 2**.

Among them, compounds 1, 3, and 6 were reported from the dichloromethane extract of gum-resin of *D. ammoniacum* [23]. The ESI-MS data and UV-VIS peaks are represented in **Table 3**. For doremone A (1) the MS spectra are shown in **Fig. 3**, exploring data showed that m/z 427 corresponding to $[M+H]^+$ in positive mode and m/z 425 correspondings to [M-H]- in negative mode, showing a molecular weight of 426

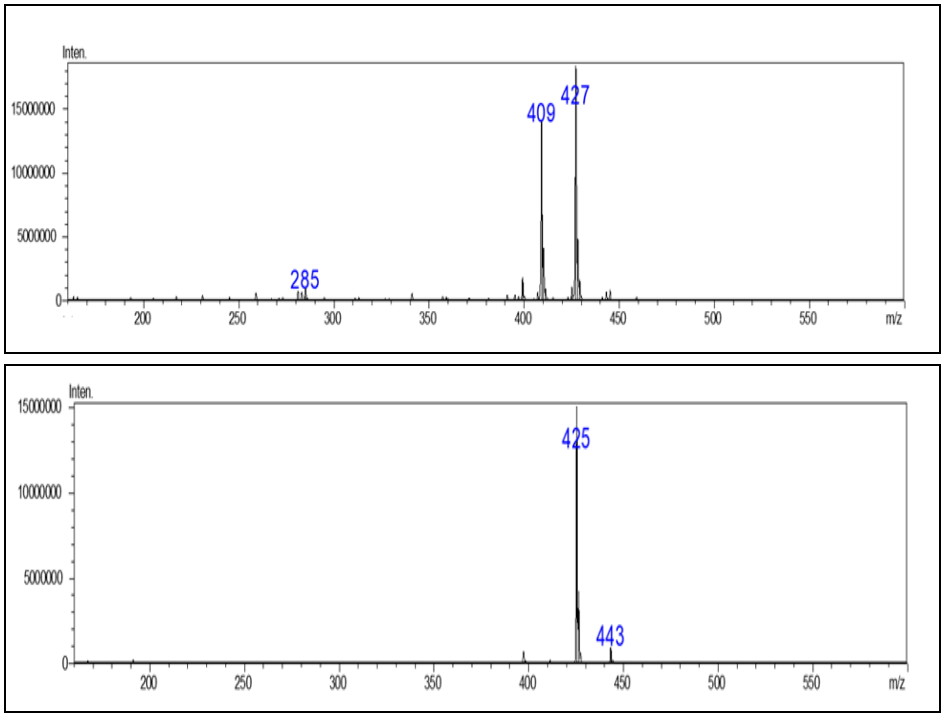


FIG. 3: THE ESI-MS SPECTRUM OF DOREMONE A (1), POSITIVE MODE M/Z 427 [M+H]+ AND M/Z 427 [M-H]- NEGATIVE MODE

Docking Studies: Acetylcholinesterase and butyrylcholinesterase (BChE) are two cholinesterases known to be responsible for the regulation of cholinergic neurotransmission, whose inhibition causes AD. The function of BChE at cholinergic synapses has not been fully recognized while physiological function of AChE is well known 36, so acetylcholinesterase is known to be the main enzyme for AD and its inhibition can have an important role in occurrence of the disease.

Therefore the experimental and theoretical studies of the ammoniacum gum extracts were evaluated for anti-AD activity by this enzyme. Acetylcholinesterase inhibitory activities of the extracts of ammoniacum gum were investigated using Ellman's method and the results are shown in **Table 1**. The major compounds of $CHCl_3$ extract of ammoniacum gum were identified based on data obtained from HPLC-PDA-MS **Table 3** and comparing these data with those published in literature such as SciFinder and dictionary of natural products. The activity of compounds in Dorema gum investigated against the cholinergic enzyme *viz.* AChE using the molecular docking methods.

The compounds showed good affinity with the enzyme with docking score ranged from -10.89 to -8.62 kcal/mol and Gibbs Free Energy (ΔG) ranged from -49.06 to 90.53 kcal/mol **Table 4**. The dshamirone (3) and kopetdaghin C (5) were found to possess the highest binding scores with corresponding binding energy values of -10.89 and -10.27 kcal/mol, respectively **Table 4** and **Fig. 4**, where galantamine as positive control showed binding energy of -5.92 kcal/mol. The top-ranked active compound 3 interacted through the

formation of two backbone hydrogen bonds with the active site residues (Glh199 and Tyr130 with a hydroxyl group) and showed a hydrophobic interaction between phenol ring and Tyr116, Ile444 and Leu127 **Fig. 5**.

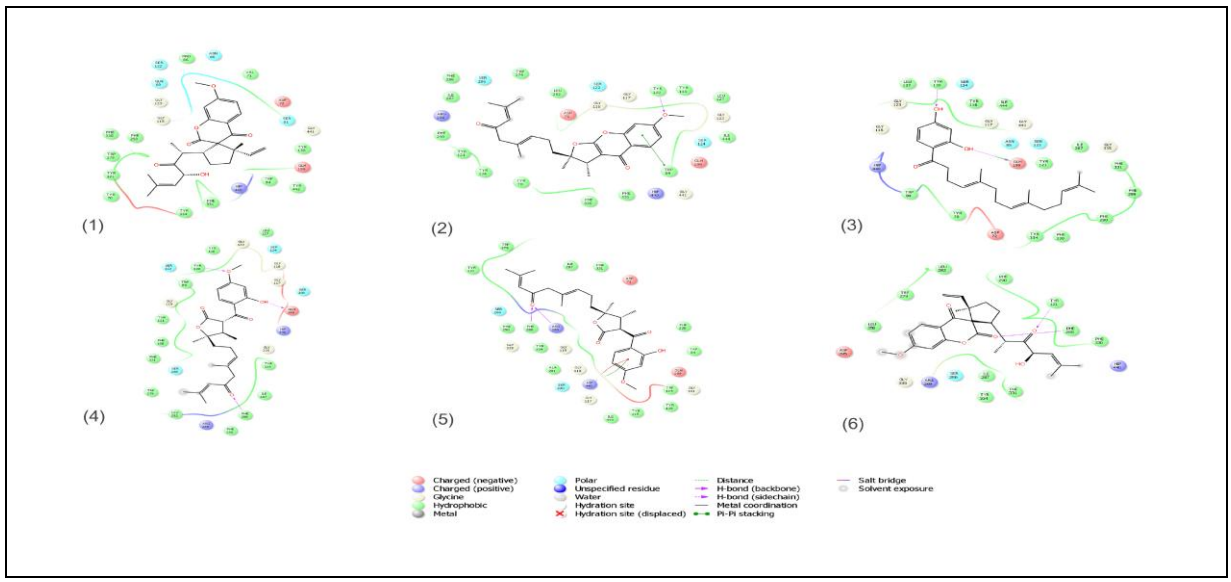


FIG. 4: DOCKING STUDY OF COMPOUNDS (1-6) IN AChE ASSAY

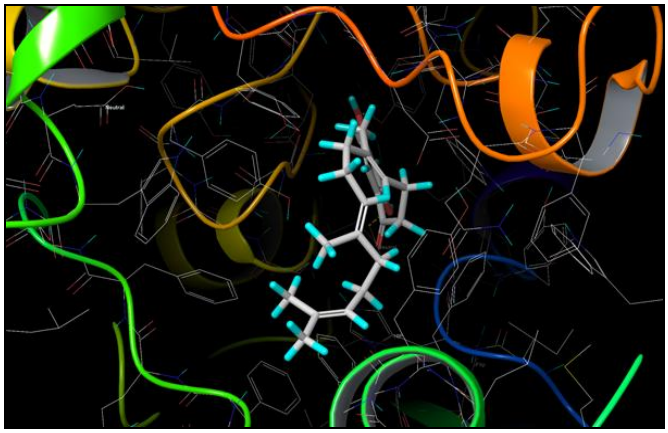


FIG. 5: THE 3D REPRESENTATION OF INTERACTION BETWEEN THE AChE ENZYME AND DSHAMIRONE (3)

The compound 1 showed an interaction through hydrophobic binding with amino acid residues at the catalytic site (Tyr121, Tyr334, Tyr70, Tyr130, Pro86, Trp279, Phe288, Phe290, Phe330, and Val71) and furthermore a hydrophobic interaction between phenol ring and Tyr116, Tyr84, Tyr121, Phe330, Phe331, and Leu127. The Compound 5 had a hydrophobic interaction with important amino acid residues at the catalytic residues (Trp114, Trp84, Tyr130, Tyr116, and Phe330) and $\pi - \pi$ interaction of phenolic group with Hip440 in addition to backbone hydrogen bonding between carbonyl group of kopetdaghin C with Phe288 and Arg289. Compound 2 had two hydrogen-bond interaction between Trp84 with phenyl group and Tyr130 with methoxy group of kopetdaghin E, furthermore to hydrophobic interaction with residues of the protein (Tyr121, Tyr334, Tyr70, Trp279, Phe288, Phe290 and Ile287), hydrophobic interaction between aromatic ring with Tyr116, Ile444, Phe330, Phe331 and Leu127 and $\pi - \pi$ interaction of aryl group with Trp84. Compound 4 depicted three hydrogen-bond interactions between Phe330 with carbonyl group, Glh199 with hydroxyl of phenolic group and Tyr130 with methoxy group of kopetdaghin D, in addition to hydrophobic interaction between side-chain substituent with the protein residues (Tyr334, Ile287, Leu282 and Phe290) and hydrophobic interaction between phenol ring and Tyr116, Tyr84, Tyr121, Phe330, Phe331 and Leu127. Compound 6 showed two backbone hydrogen-bond interactions between Tyr121 with carbonyl group and one side-chain hydrogen bond with Phe288, a hydrophobic interaction between side-chain with important amino acid residues at the catalytic residues (Ile287, Tyr334, Phe331, and Phe330) and hydrophobic interaction between aryl ring with Trp279, Leu358, Phe290, and Leu282 **Fig. 4**.

The results of molecular docking were in good agreement with *in-vitro* results confirming the inhibitory activity of six major compounds and the overall activity of chloroform extract of ammoniacum gum in inhibition of AChE enzyme.

TABLE 4: DOCKING SCORES AND H-BOND INTERACTIONS OF THE ACETYLCHOLINESTERASE ACTIVE SITE WITH COMPOUNDS (1-6) AND GALANTAMINE AS POSITIVE CONTROL

Compound	Binding Energy (kcal/mol)	ΔG (kcal/mol)	H-bond formation
doremone A (1)	-8.66	-58.97	-
kopetdaghin E (2)	-7.85	-72.84	Tyr130, Trp84
dshamirone (3)	-10.89	-68.20	Glh199, Tyr130
kopetdaghin D (4)	-9.97	-90.53	Phe288, Glh199, Tyr130
kopetdaghin C (5)	-10.27	-87.16	Phe288, Arg289
ammodoremine (6)	-8.62	-49.06	Phe288, Tyr121
galantamine	-5.92	-43.049	-

According to the results in **Table 4**, dshamirone (3) exhibited high AChE inhibitory activity, which is probably responsible for the observed activity of the chloroform extract in the present study. The presence of phenolic compounds such as sesquiterpene coumarins/phenols could be associated with the appearance of noticeable antioxidant activity of the chloroform extract following the other reported data for different dorema species [42].

CONCLUSION: In conclusion, we have shown that the ammoniacum gum (collected from Shahroud, Iran) extracts exhibited significant acetylcholine esterase inhibitory activity (with the IC_{50} (μg/ml) of 32.34 and 43.50 for hexane and chloroform extracts, respectively).

Six major compounds from this gum-resin were identified based on HPLC-PDA-MS analysis including doremone A (1), kopetdaghin E (2), dshamirone (3), kopetdaghin D (4), kopetdaghin C (5), and ammodoremine (6). The experimental results were also confirmed by docking analysis. These results may suggest that the ammoniacum gum extracts could be applied for medicinal purposes.

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CONFLICTS OF INTEREST: Authors declare no conflicts of interest.

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